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**THE EXPRESSION IN SOIL BACTERIA
OF SYMBIOTIC GENES FROM
RHIZOBIUM LEGUMINOSARUM BIOVAR TRIFOLII**

A THESIS PRESENTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE IN MICROBIOLOGY
AT MASSEY UNIVERSITY

Michael Fenton

1994

*This Thesis is dedicated to
my family;*

*Annette and Frank Fenton,
my wife Christine
and my daughter
Jamie Jessica.*

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To my wife Christine I owe the biggest debt; for her constant support, our many brain-storming sessions, and her good humour. Without my best friend I would not have been able to complete this project.

ABSTRACT

Rhizobium leguminosarum biovar *trifolii* strain ICMP2163::Tn5 was able to spontaneously transfer its pSym to the non-nodulating *Rhizobium loti* soil isolate NR40 in sterile soil microcosms containing Ramiha hill soil or Ashurst silt loam soil at pH 6.0 or higher. In sterile soil microcosms at pH 6.0 containing sterile ryegrass or white clover plants the frequency of NR40 transconjugants was higher than in microcosms containing soil alone. The survival of the parent strains decreased in soil with a pH of 5.5 or less, and no transconjugant NR40 bacteria were detectable. Southern blots of the genomic digests probed with *nodA* DNA confirmed that transconjugant NR40 contained symbiotic genes.

On artificial media strain ICMP2163::Tn5 transferred its symbiotic plasmid, by conjugation, to *Sphingobacterium multivorum*, an organism that can be found in soil. The transconjugant bacteria were able to nodulate white clover seedlings but were unable to fix nitrogen. Microscopic examination revealed that the root nodule structure, and bacteroid formation, were abnormal. The bacteria occupying the nodules were isolated and the total DNA extracted. The partial 16S RNA gene sequence from a transconjugant derived from a nodule was shown to be identical with that of the recipient *S. multivorum*. Southern blots of the genomic digests probed with *nodA* DNA confirmed that the transconjugant contained symbiotic genes.

A *Caulobacter crescentus* sewage isolate was also able to induce a tumour-like growth on white clover seedlings after receiving the pPN1 co-integrate plasmid from *E.coli* strain PN200. Eckhardt gel analysis confirmed that the transconjugant *Caulobacter* carried the R68.45:pSym co-integrate plasmid.

Bacteroids were absent but *Caulobacter* cells were found in the outer two or three layers of the growth and the plant cells in this region had degenerated.

Sequence data was obtained for a 260 bp fragment of the 16S rRNA gene from *Sphingobacterium multivorum* and *Caulobacter crescentus* corresponding to positions 44 to 360 on the *Escherichia coli* genome. A distance matrix was constructed showing the relationship between *S. multivorum*, *C. crescentus*, *Rhizobium*, and related bacteria and neighbor-joining was used to construct a tree. From the tree given it is concluded that the ability to carry or express symbiotic genes is not dependant on having a phylogenetic relationship with *Rhizobium*.

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INTRODUCTION

1. The significance of the genus *Rhizobium*.

Pasture growth is limited by the quantity of fixed nitrogen available when other soil nutrient deficiencies have been corrected by top-dressing. Nitrogen fixation is the process by which atmospheric nitrogen gas is made available for incorporation into organic compounds. Only certain bacteria are capable of carrying out this process, the genus *Rhizobium* being the most common (Raven *et al.*, 1981). Members of this genus are Gram negative aerobic rods that occur free-living in soil or as micro-symbionts in root nodules of leguminous plants (Jordan, 1984).

Rhizobia in root nodules are estimated to carry out 50-70% of the world biological nitrogen fixation (Quispel, 1974), reducing approximately 20×10^6 tonnes of atmospheric nitrogen to ammonia (Beringer *et al.*, 1980). Biological nitrogen fixation is of particular importance to New Zealand agriculture, providing 1 million tonnes of nitrogen annually (Ball and Field, 1985). Compared to the 26,373 tonnes (Douglas and Cochrane, 1989) of nitrogenous fertiliser used by New Zealand farmers this is more than 97% our annual requirements. Although this process is free, self-sustaining and non-polluting, it does not necessarily operate with optimum efficiency.

Although New Zealand pasture soils contain high numbers (e.g. 10^4 - \rightarrow 10^6 /g soil) of indigenous clover rhizobia (Bonash and MacFarlane, 1987) the introduction of superior nitrogen fixing strains is still considered an important management practice. However, the inoculant strains may be prone to loss of

symbiotic traits such as infectiveness and effectiveness (O'Hara, 1985), and may not be competitive with the indigenous strains already present in the soil (Rhys and Bonish, 1984). The recommended inoculum for white clover consists of a mixture of three strains of *Rhizobium leguminosarum* biovar trifolii which includes strain ICMP2163, ICMP2666, and ICMP2668. Stock cultures are maintained by the Plant Diseases Division of the Crown Research Institute, Auckland (Bianchin, 1989).

2. The Biology of Nitrogen Fixation.

Rhizobium bacteria are able to invade the root hairs of leguminous plants via an infection thread formed by the plant cells. The plant cells then respond by undergoing rapid cortical division to form either a tumour or a refined structure called a nodule.

The genetic requirements for nodulation are divided between the *Rhizobium* bacteria and the host plant. Both contain genes that are only expressed in the presence of the other. The process is reviewed in Djordjevic *et al.* (1987). Flavanoids, excreted by the plant, activate nodulation (*nod*) genes carried by the bacteria. The *nodAB* genes on the *Rhizobium* symbiotic plasmid may produce a low molecular weight substance that induces plant cell division (John *et al.*, 1988). Attachment of the bacterial cell to the root hair is proposed to be mediated by binding to lectin. Rhizobia appear to attach in an end-on fashion followed by involution of the plant cell wall to form an infection thread. As the infection thread grows through 3 to 6 layers of root outer cortex cells, meristematic activity is initiated in a small group of root cortical cells directly in front of the tip of the infection thread. Growth of the infection thread continues into this meristematic region where rhizobia are released into the inner most

cells, where the bacteria continue to divide until the cytoplasm is filled with bacteroids (Robertson and Farnden, 1980). The nodules formed on clover are called indeterminate nodules. The infection threads continue to penetrate the plant cortical cells in the nodule meristem, providing a continuous release of rhizobia into the plant cells as the nodule increases in size (Beringer *et al.*, 1979).

In the process of nodule development, the bacteria undergo morphological and physiological changes that lead to the formation of bacteroids (Irigoyen *et al.*, 1990). Free living rhizobia are not capable of fixing atmospheric nitrogen as oxygen inactivates the nitrogenase enzyme that converts nitrogen to ammonia and blocks the transcription of nitrogenase genes. The atmosphere in the nodule environment is micro-aerophilic due to high concentrations of the plant protein leghaemoglobin. This protein plays a role in the transport of oxygen by maintaining a sufficiently high pO_2 in the plant cytoplasm for oxidative phosphorylation, while providing a sustained low level of oxygen to the bacteroids (Verna and Long, 1983). In this environment, bacteroids are able to supply the plant with ammonia which is assimilated into glutamate, glutamine and other translocatable products. In return, the bacteria is supplied with an abundance of carbon compounds such as sugars, and is provided with a protected environment from the outside world. An ineffective nodule which is not able to fix nitrogen may be formed if the plant is infected by a *Rhizobium* strain with a mutation in the nitrogen fixing (*fix*) genes.

3. Taxonomy of *Rhizobium*.

Until recently, the rhizobia that infect beans, peas, and clovers were clustered in a single species, *Rhizobium leguminosarum* (Jordan, 1984), which had three biovars; *Rhizobium leguminosarum* bv phaseoli, *Rhizobium leguminosarum* bv viceae, and *Rhizobium leguminosarum* bv trifolii. The artificial nature of this simplistic classification scheme is becoming more evident as knowledge is acquired and new species discovered. Currently three species, *Rhizobium leguminosarum* bv phaseoli, *R. etli* bv phaseoli, and *R. tropici*, two new *Rhizobium* genomic species, and other unclassified genotypes have been isolated from nodules of *Phaseolus vulgaris* (Laguerre *et al.*, 1994). Figure 1 indicates that there may be a greater diversity of bacteria capable of nodulating legumes than was previously recognised (Laguerre *et al.*, 1994).

Within *R. leguminosarum* biovar trifolii there is considerable phenotypic variability (Dughri and Bottomley, 1984; Harrison *et al.*, 1987), reflected by the genetic diversity observed (Jarvis *et al.*, 1980; Crow *et al.*, 1981). Jarvis *et al.* (1980) compared reference DNA from clover inoculant strains NZP561 and TAI with DNAs from 18 other *R. leguminosarum* bv trifolii strains. The range of DNA-relatedness and $\Delta T_{m(e)}$ values with strains NZP561 and TAI was 61 - 91% and 0 - 8.2°C and 49 - 94% and 1.3 - 7.0°C respectively. $\Delta T_{m(e)}$ is a statistic which expresses the base sequence homology in the fraction of DNA which hybridises. Each 1°C represents a 1% miss-match in the hybridising sequences (Jarvis *et al.*, 1991). The values quoted extend well beyond the phylogenetic limits for a bacterial species as proposed by Wayne *et al.*, (1987). It is concluded that, *Rhizobium leguminosarum* bv trifolii may

not be a single species but a group of inter-related species capable of expressing the appropriate symbiotic genes.

Normally the primary isolation of *Rhizobium* strains is from nodulated legumes (Schofield et al., 1987; Vincent, 1970; Young, 1985) and this has made it difficult to define phylogenetic relationships with other bacteria in the soil. However, the ability to nodulate leguminous plants is regarded as the characteristic function of the genus *Rhizobium* with nitrogen fixation a normal but not essential consequence of nodulation (Jordan, 1984). The nodulation and nitrogen fixation genes are usually located on a symbiotic plasmid (pSym), that encodes distinct nodulation specificities (Johnston et al., 1978; Hirsch et al., 1980). The plasmid may be lost under certain environmental conditions, so that soil bacteria lacking this plasmid cannot be classified as rhizobia although they may be able to express the symbiotic genes. Strains of bacteria exist that fail to satisfy Jordan's definition but are clearly rhizobia lacking the symbiotic plasmid (Scott and Ronson, 1982; Soberon-Chavez and Najera, 1988; Segovia et al., 1991). Another difficulty arises from the ability of the symbiotic plasmid to be transferred from one strain of *Rhizobium* to another. This may change the strains host specificity or lead to the loss of the ability to nodulate. It has been shown that pSym genes can be expressed to a limited degree in *Agrobacterium* species (Hooykass et al. 1981; Kondorosi et al., 1982; O'Connell et al., 1987), *Pseudomonas aeruginosa* and *Lignobacter* species (Plazinski and Rolfe, 1985).

Jarvis et al. (1989) suggested that *Rhizobium* classification should be defined in terms of DNA-DNA or rRNA-DNA homology to accepted reference bacteria. In addition, it may be useful to use the 16S ribosomal DNA sequence to determine what is a 'true' rhizobia. PCR-RFLP analysis has been described as a rapid method for the identification of nodule isolates and new taxa (Laguette *et al.*, 1994). The use the fatty acid composition profiles has also been described as another reliable means of rapid identification (Jarvis and Tighe, 1994).

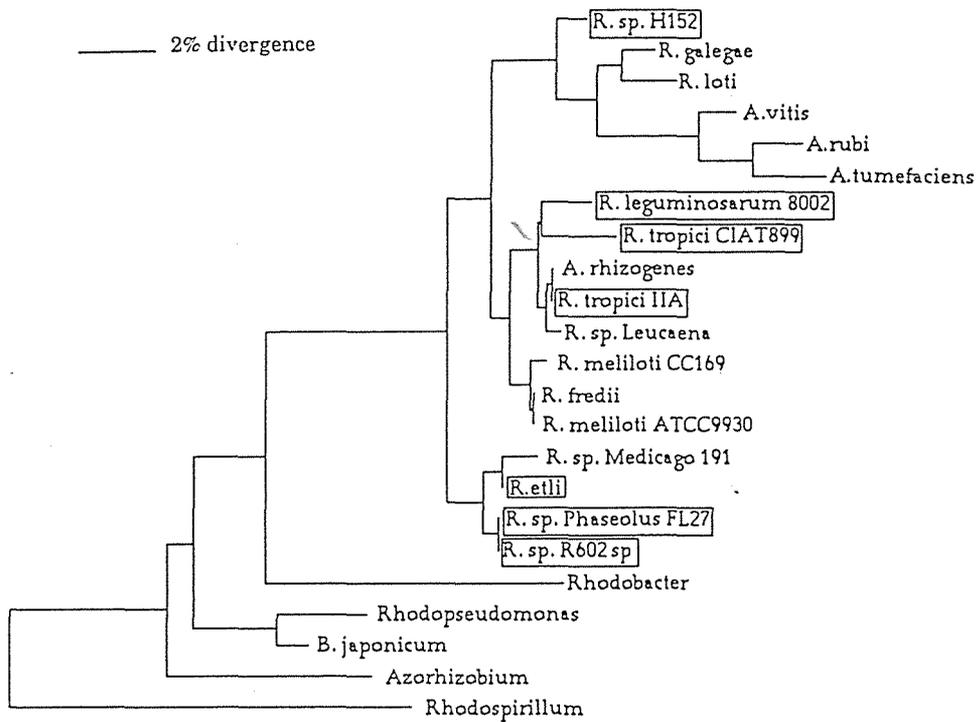
Figure 1. **PHYLOGENETIC RELATIONSHIPS AMONG BEAN RHIZOBIA
AND OTHER STRAINS OF *RHIZOBIUM* AND RELATED
BACTERIA BASED ON PARTIAL 16S rDNA SEQUENCES**

(LAGUERRE *ET AL.*, 1994).

The taxa in boxes are the taxa that infect *Phaseolus vulgaris*.

Genus abbreviations: *R*, Rhizobium; *A*, Agrobacterium;

B, Bradyrhizobium.



4. The Symbiotic Plasmid.

The nodulation and nitrogen fixation genes are usually located on large (>100 kb) symbiotic plasmids (pSym or Sym plasmid), some of which can be transferred to other bacteria via conjugation (Djordjevic *et al.*, 1983; Johnston *et al.*, 1978).

There is evidence that pSym transfer occurs in natural field populations., Schofield *et al.*, (1987) studied 16 soil isolates of *Rhizobium leguminosarum* and observed similar Sym plasmids in different host chromosomal backgrounds and different Sym plasmids in similar host chromosomal backgrounds, as well as the presence of a putative recombinant Sym plasmid. Jarvis *et al.*, (1985) reported the isolation of soil bacteria that showed DNA homology to *Rhizobium leguminosarum* but were unable to nodulate white clover. Transconjugation experiments with the co-integrate plasmid pPN1 (Scott and Ronson, 1982) showed that these bacteria could express symbiotic genes from clover rhizobia. Plasmid transfer in non-sterile soil has been demonstrated between *Rhizobium fredii* and a pSym cured *Rhizobium leguminosarum* (Kinkle and Schmidt, 1991) and between *Rhizobium leguminosarum* and *Enterobacter* (Dohler and Klingmuller, 1988).

Indigenous soil bacteria, including native rhizobia, are well adapted to survive in the absence of a host plant. Potential competitors may not initially be able to nodulate crop plants but may be enabled to by obtaining the appropriate symbiotic plasmid (Dowling and Broughton, 1986). If complemented by a Sym plasmid from an introduced *Rhizobium* strain, the indigenous soil bacteria will compete for nodulation sites and may form the majority of nodules on the host plant (Meade *et al.*, 1985; Weaver & Frederick, 1974a, 1974b). The

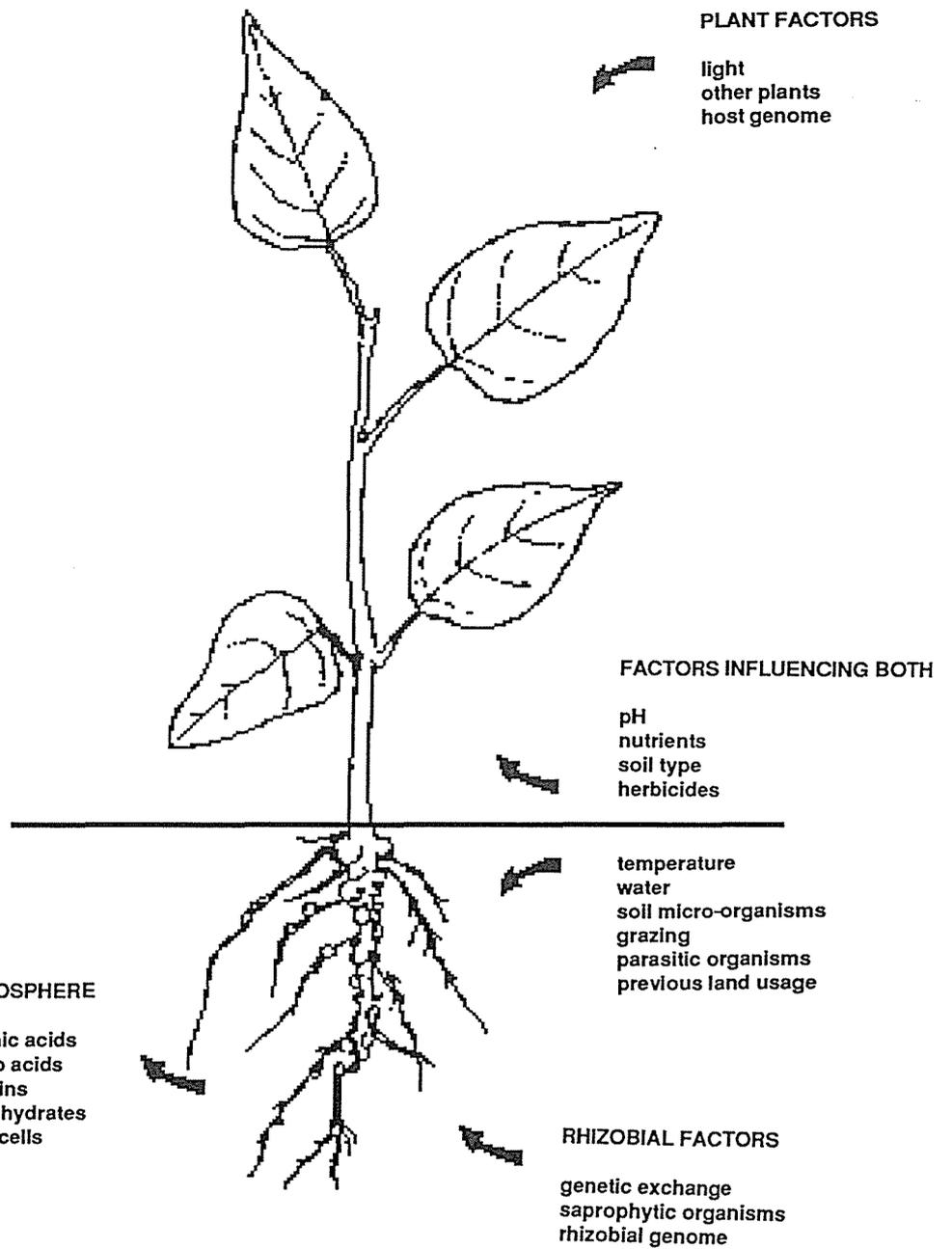
inoculant strain may need to be supplied at 1000X the level of the indigenous *Rhizobium* population in order to form 50% of the nodules. For the inoculation industry this may yield unexpected benefits if it were possible to isolate indigenous soil bacteria able to nodulate and fix nitrogen better than the commercial *Rhizobium* inoculant. However, it becomes a problem when the indigenous soil bacteria form ineffective nodules incapable of nitrogen fixation. In this instance, increasing the inoculum added to the soil is simply adding more DNA for the competitors to pick up. There may also be important consequences for the release of genetically engineered micro-organism.

However, many factors influence the competitive ability of a *Rhizobium* strain, and any factor which adversely effects plant growth will also profoundly effect competition for nodulation (see Fig. 2). Phosphorous limitation has been shown to be exacerbated by low pH and the combination of low pH and phosphorous levels can have a strong influence on competition for nodulation (Dowling and Broughton, 1986). Most soils in New Zealand are moderately acidic, having a pH between 5.0 and 6.5. It appears that an acidity of pH 5.8-6.0 is considered ideal for the legume to prevent aluminium and manganese toxicity, but the other partner in the symbiotic relationship appears to have been overlooked. Other environmental factors such as soil type, temperature, and moisture also affect the outcome of competition. Biological factors, such as bacteriophage effects, epiphytic bacteria, mycorrhizal effects predation by protozoa should all be considered when applying laboratory results outside.

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.

Figure 2. FACTORS THAT MAY INFLUENCE THE OUTCOME OF
COMPETITION AMONG RHIZOBIUM STRAINS FOR
NODULATION OF LEGUMES.

(Dowling, D.N. and W.J. Broughton, 1986).



5. Aims of this investigation.

- 1) To examine the transfer of symbiotic genes from *Rhizobium leguminosarum* biovar trifolii to a soil bacterium in sterile soil microcosms and observe the effect of:
 - a) soil type
 - b) soil pH
 - c) the presence of plants

- 2) To examine the expression of symbiotic genes from *Rhizobium leguminosarum* biovar trifolii in soil bacteria.

- 3) To examine the expression of the co-integrate plasmid pPN1 in soil bacteria.

- 4) To determine whether the ability to carry or express symbiotic genes is dependant phylogenetic relationship with *Rhizobium*.

MATERIALS AND METHODS

1. MICROBIOLOGICAL METHODS.

1.1 Bacterial Strains and Maintenance.

The bacterial strains used in this study are listed in Table 1. Bacteria were grown at 28°C on tryptone yeast extract (TY) agar (Beringer, 1974) or Luria broth (LB) agar (Maniatis, 1972) supplemented, where necessary, with rifampicin (Rif), 50 µg/ml; neomycin (Neo), 200 µg/ml; or spectinomycin (Spc), 200 µg/ml. *Rhizobium* strains ICMP2163::Tn5 and NR40 did not grow on Luria agar and required incubation for 3 to 4 days for isolated colonies to arise on TY streak plates. *Sphingobacterium* strain NZRM1228 needed only 24 hour incubation and grew on LB agar. All strains were maintained on slopes at 4°C and sub-cultured at intervals of 3 - 4 months.

1.2 Media Used.

1.2.1 Tryptone Yeast Extract (TY) (Beringer, 1974) contains (g/l):

Bacto Tryptone (Difco), 5; Yeast Extract (Difco), 3; in distilled water. Autoclave then add 5ml 30% CaCl₂.6H₂O. TY agar was obtained by adding 15 g/l agar (Davis).

1.2.2 Luria Broth (LB) (Miller, 1972) contains (g/l):

NaCl, 5; Bacto Tryptone (Difco), 10; Yeast Extract (Difco), 5; in distilled water. The pH was adjusted to 7.0 with 5M NaOH and the media sterilised by autoclaving. LB agar was obtained by adding 15 g/l agar (Davis).

TABLE 1. Bacterial strains used in this study.

Bacterial strains	Characteristics	Source
<i>Rhizobium leguminosarum</i> biovar trifolii		
ICMP2163::Tn5	Nod ⁺ Fix ⁺ Rif ^S Spc ^R Neo ^R Growth on TY No growth on L	J. R. Rao, Massey University, Palmerston North, New Zealand.
<i>Rhizobium loti</i>		
NR40	Nod ⁻ Fix ⁻ Rif ^R Spc ^S Neo ^S Growth on TY No growth on L	B.D.W. Jarvis, Massey University, Palmerston North, New Zealand.
MFNR series	Nod ⁺ Fix ⁻ Rif ^R Spc ^S Neo ^R Growth on TY No growth on L	This study. (ICPM2163::Tn5 X NR40)
<i>Sphingobacterium multivorum</i>		
NZRM1228 (NHI75/1026)	Nod ⁻ Fix ⁻ Rif ^R Spc ^S Neo ^R Growth on TY Growth on L	NZCDC.
MF100, MF200	Nod ⁺ Fix ⁻ Rif ^R Spc ^S Neo ^R Growth on TY Growth on L	This study. (ICMP2163::Tn5 X NZRM1228)

TABLE 1. continued.

Bacterial strains	Characteristics	Source
<i>Escherichia coli</i>		
PN200	Nod ⁻ Fix ⁻ Rif ^S Spc ^S Neo ^R Growth on TY & L pPN1 (R68.45;pSym)	Scott and Ronson, 1982.
<i>Caulobacter</i>		
MCDF23	Nod ⁻ Fix ⁻ Rif ^R Neo ^S Growth on TY No growth on L	C.D. Fenton, Massey University, Palmerston North, New Zealand.
MCDF100	Nod ⁺ Fix ⁻ Rif ^R Neo ^R Growth on TY No growth on L	This study. (MCDF23 X PN200)

^a Nod⁺, nodulates white clover seedlings; Fix⁺, fixes atmospheric nitrogen.

^b Rif^S, rifampicin sensitive; Rif^R, rifampicin resistance; Spc^R spectinomycin resistance;
Neo^R, neomycin resistance.

^c TY, Tryptone Yeast extract agar; L, Luria agar.

^d NZCDC, New Zealand Centre for Disease Control, Porirua, New Zealand.

1.2.4 Hoaglands Trace Elements Solution (Hoagland and Arnon, 1938)

contained (g/l100ml): H_3BO_3 , 2.86; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1.81; $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 0.22; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.095; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.080; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.054; in de-ionised water. The solution was autoclaved.

1.2.5 Thornton's Seedling Agar (Thornton, 1930) contained (g/l):

$\text{Ca}_3(\text{PO}_4)_2$, 2.0; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl , 0.1; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.017; Fe_3PO_4 , 1.0; Agar (Davis), 15.0; Hoaglands Solution, 1 ml; in distilled water. The pH was adjusted to 7.0, the agar dissolved, dispensed (10 ml) in 20 X 150 mm tubes, autoclaved and the tubes sloped to set.

1.3 Culture Growth and Purity.

All stock cultures were streaked on LB (section 1.2.2) and TY (section 1.2.1) plates and checked for purity. Only well isolated single colonies were used to inoculate liquid media which was incubated at 28°C on a gyratory shaker (200 rpm). *Rhizobium* strains ICMP2163::Tn5 and NR40 required 2 days incubation while over-night incubation was sufficient for the other strains.

1.4 Plant Inoculation Tests.

1.4.1 Materials:

- (1) HgCl_2 solution, 0.2%
- (2) Methanol (AR grade)
- (3) Sterile universal
- (4) Sterile water
- (5) Water agar plates (section 1.2.3)
- (6) Thornton's agar slopes (section 1.2.5)

1.4.2 Method:

White clover seedlings (*Trifolium repens* cv Grasslands Huia) in a sterile universal bottle were surface sterilised in methanol (10s), 0.2% mercuric chloride (2 min), and washed free of the sterilising agents using several rinses of sterile water. A sample (0.1 ml) of water used in the last rinse was spread on LB agar (section 1.2.2) and incubated at 30°C as a sterility check. Sterilised seeds were germinated on water agar plates (section 1.2.3) incubated in darkness at 22°C. One healthy seedling with a straight radical was aseptically transferred to a Thornton agar slope (section 1.2.5). The plants were grown under controlled environment conditions at 22°C in artificial light with an intensity of 550 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Einsteins) and a 16 hour photoperiod. After 3 days, seedlings were inoculated with 1 ml of a washed cell suspension (10^3 cfu ml⁻¹) of a log phase culture of the test bacteria. Ten seedlings were inoculated with each culture. The number of nodules and shoot length were recorded at weekly intervals. Plant size was compared to the negative controls and served as a visual indicator of the ability to fix atmospheric nitrogen. Strain ICMP2163::Tn5 served as a positive control and plants inoculated with *E. coli* (PN200) or water were used as negative controls. All other parent strains used in this investigation were tested on 10 plants to confirm that they could not incite nodule formation.

1.5 Isolation of Bacteria From Nodules. (Vincent, 1970).

1.5.1 Materials:

- (1) HgCl₂ solution, 0.2%
- (2) Methanol (AR grade)
- (3) Sterile water
- (4) TY agar plates (section 1.2.1)
- (5) Sterile petri dishes
- (6) Sterile forceps
- (7) Sterile scalpel blade

1.5.2 Method:

The nodules were cut from the plant root using a sterile scalpel blade leaving a short section of root attached to the nodule to aid handling. Sterile petri dishes (3) were set up in the following order: 15 ml of methanol; 15 ml of mercuric chloride; aliquots of sterile water (8 x 200 µl). Sterile forceps were used throughout the procedure. Nodules were surface sterilised by immersion in methanol for a few seconds, followed by washing in mercuric chloride solution for a maximum of 90 seconds. After rinsing the nodules in at least 8 changes of sterile water, the nodules were crushed in 100 µl of TY broth (section 1.2.1) with the end of a sterile glass rod. The contents were drawn into a 100 µl autopipette tip and spread on TY (section 1.2.1) plates. After incubation at 28°C for 2-3 days, single well isolated colonies were streaked on media suitable for the selection of putative transconjugants (Table 1 and section 1.1). The ability to form nodules was confirmed by a further round of streak plating, a nodulation test and re-isolation from nodules.

2. CONJUGATIVE PLASMID TRANSFER.

Liquid cultures (10 ml, section 1.3) of approximately equal concentrations were used. The concentration of cells (10^8 CFU/ml) in each culture was estimated by using a Petroff-Hausser counting chamber, unless stated otherwise, as the reliability of optical density readings can be influenced by the amount of exopolysaccharide present in the culture. The number of viable cells present was checked by plate counting.

2.1 **Membrane Filter Method.** (Buchanan-Wollaston *et al.*, 1980)

2.1.1 Materials:

- (1) Cellulose nitrate filters (0.2 μm pore size) sterilised by autoclaving
- (2) TY agar plates (section 1.2.1)
- (3) Petroff-Hauser counting chamber (1/400 mm^2 , 1/50 mm deep)

2.1.2 Method:

A sterile cellulose nitrate filter on the surface of a TY plate (section 1.2.1) was spotted with 50 μl of donor cells, and after absorption into the filter, 25 μl of the recipient cells were added. The plate was incubated overnight at 28°C, and then growth from the membranes was resuspended in 2.0 ml of sterile water. Duplicate 0.1 ml samples of cell suspension were spread on suitable antibiotic media (table 1, section 1.1) to select for putative transconjugants and incubated for 24 - 48 hours. When possible, isolated colonies were streak plated for purification. The mix of putative transconjugants and residual recipients was resuspended in 50 ml sterile water and diluted to 10^3 cfu ml^{-1} in sterile water for the plant inoculation tests.

2.2 Conjugation in Sterile Soil Microcosms.

2.2.1 Preparation of Soil Microcosms.

Ramiha hill soil, taken from the hill country of the Tararua Ranges, and Ashurst silt loam soil, taken from flat terrace land, were used in this study. The top 3 cm of soil, taken from ryegrass/white clover pastures, was allowed to air dry to aid in the removal of plant material. After passing through a 2 mm sieve, the water holding capacity of the air dried soil was determined by the Department of Soil Science, Massey University. Sieved soil was dispensed (5g) into screw-capped universal bottles and autoclaved on two consecutive days. Random bottles were chosen to check the weight before and after autoclaving to determine that the soil had not absorbed moisture. Adding the calcium hydroxide and washed cell suspensions to the bottles adjusted the soil water holding capacity to 50% by the adding a total volume of 2.1 ml. The acidity of sieved and autoclaved soil was determined by the method of Metson (1961). Triplicate 1:2.5 soil suspensions (10 g of soil added to 25 ml of distilled water) were left overnight at room temperature and the pH measured. Sterile 0.1M or 1M calcium hydroxide was used to change the soil pH from a minimum of 5.0 to a maximum of 8.0.

For experiments involving plants, sodium dihydrogen phosphate (4 mg) was added to each bottle and six sterile seedlings were allowed to grow for seven days in the controlled environment used for plant inoculation tests (section 1.4).

2.2.2 Inoculation and Sampling Procedure.

Log phase cultures were washed twice in sterile distilled water, resuspended to an optical density at 600 nm (OD_{600}) of approximately 0.60 (absorbance) and plate counted to estimate the actual viable cell numbers in each suspension. Four sets, of 10 universal bottles each, were inoculated with each of the following: sterile water, strain ICPM2163::Tn5, NR40 or a 1:1 mixture of ICPM2163::Tn5 and NR40, respectively. All treatments were held at 22°C in the controlled environment used for plant inoculation tests (section 1.4) until they were examined. Pairs of bottles from each treatment were examined after 1, 3, 7, 10 and 18 days incubation. Sterile water (20 ml) was added to each bottle, it was briefly vortexed, then shaken for 5 minutes on a gyratory shaker. After a short settling period, the supernatant was diluted 10 fold from undiluted to 10^6 . Samples were plated in duplicate on TY (section 1.2.1) agar; TY containing Spc and Neo; TY containing Rif, and TY containing Rif and Neo (section 1.1). All plates were incubated at 28°C for 6 days.

2.2.3 Statistical Analysis of Data.

The Student's-t test (Bailey, 1981) was used to compare the significance of apparent differences due to the different treatments. The means of two treatments were considered significantly different at $P < 0.001$ if the calculated t-value was greater than the P-value obtained from the Student's-t distribution.

The data was subjected to analysis of variance and treatment means were compared at the 95% probability level ($P < 0.05$). For graphed data the bars at each point indicate 95% confidence limits (Results, section 1).

2.2.3 Frequency of Plasmid Transfer

The frequency of NR40 transconjugants formed was calculated as follows:

$$\frac{\text{Transconjugant NR40 CFU/g of soil}}{\text{Parent NR40 CFU/g of soil}}$$

3. LIGHT AND ELECTRON MICROSCOPY.

Nodules from test plants 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.

4. IDENTIFICATION BY FATTY ACID PROFILE.

High resolution gas chromatography analysis of total fatty acids from whole bacterial cells was used to generate a profile that could be compared with a database of approximately 600 bacterial strains, including *Rhizobium* and *Sphingobacterium*. This work was done by Analytical Services Inc., PO Box 626, Essex Junction, Vermont 05453.

5. GENOMIC DNA EXTRACTION. (Jarvis *et al.*, 1992).

5.1 **Materials.**

(1) Tris electrophoresis (TE) buffer contained (mM): Trizma base (Sigma), 10; Ethylenediaminetetra-acetic acid:disodium salt (EDTA), 1. The pH was adjusted to 8.0 by adding concentrated hydrochloric acid.

(2) TEL buffer contained (mM): Trizma base (Sigma), 50; EDTA , 20.

(3) Tris buffer contained (M): Trizma base (Sigma), 0.1. The pH was adjusted to 8.0 by adding concentrated hydrochloric acid.

(4) Protease contained: Protease type XIV, 5 mg/ml in TEL buffer. This was prepared fresh and incubated for an hour at 37°C to digest impurities.

(5) 10% SDS contained: Sodium-dodecyl-sulphate, 1g; TEL buffer, 10ml.

(6) Phenol contained: Phenol, 1 kg; deionised water, 110 ml; m-Cresol, 140 ml; 8-hydroxyquinoline, 1 g. These components were mixed overnight on a magnetic stirrer, saturated with 0.1 M Tris buffer (pH 8) and stored in a dark bottle at 4°C.

(7) Sodium acetate, 3M, pH 4.85.

(8) Diethyl ether, (Analar)

(9) 100% ethanol, (Analar)

(10) 70% ethanol

5.2 Method

Bacterial cells grown on TY slopes were washed in 3 ml of TEL buffer and collected in a sterile Falcon tube. Protease (375 µl) and 10% SDS (375 µl) were added and the suspension was left to lyse for 30 minutes on ice, and then 30 minutes at room temperature. Phenol was added (100 to 15 ml), and the tube capped tightly and inverted 25 times. The lysate/phenol solution was centrifuged at 4500 x g to separate the two phases. The aqueous phase (top) was transferred to a clean, sterile glass bottle, washed twice with 3 ml of diethyl ether and then allowed to stand. The aqueous phase (lower) was removed and 0.2 ml of sodium acetate was added and gently mixed. DNA was precipitated by adding cold (4°C) 100% ethanol and collected on a hooked glass rod. The DNA threads were washed in 70% ethanol, air dried, dissolved in 0.2 ml of TE buffer and stored at -70°C, or for short term, 4°C.

6. DETERMINATION OF DNA PURITY AND CONCENTRATION.

The purity of the DNA was determined by measuring the optical density at 230 nm, 258 nm and 280 nm using a PYE-Unicam SP1800 UV spectrophotometer. The following spectral ratio values indicated that the purity of the sample was satisfactory: absorbance (A) at A_{258}/A_{230} = between 1.8 and 2.3, (no significant protein contamination); A_{258}/A_{230} = between 1.8 and 2.0 (no significant phenol contamination) (Jarvis *et al.*, 1980). DNA concentration of 1 mg/ml have extinction coefficients of 20 for unsheared DNA and 24 for sheared DNA. The final concentration was calculated using the following formula: DNA concentration mg/ml = $(A_{258} - A_{300})/20 \times$ dilution factor (Brenner and Falkow, 1971).

7. HORIZONTAL AGAROSE GEL ELECTROPHORESIS.

(Sambrook et al, 1989).

Agarose gels (1%) were used for restriction endonuclease digests (section 8) and 0.7% agarose gels were used for Eckhardt gels (section 10). Agarose gels (2%) were used to check DNA products amplified by the Polymerase Chain Reaction (section 11).

7.1 **Materials.**

(1) Tris borate electrophoresis buffer (TBE) x 10 contained (g/l):

Trizma base (Sigma), 108.0; sodium EDTA, 9.3; boric acid, 55.0; in Milli-Q water. After diluting to 1 x TBE with milli-Q water the pH was adjusted to 8.2 with dilute hydrochloric acid. The buffer was stored at room temperature.

(2) Ethidium bromide contained: Ethidium bromide, 100 mg; in de-ionised water, 10 ml.

(3) Magnesium sulphate stock solution contained: Magnesium sulphate, 2.5 g; in de-ionised water, 10 ml.

7.2 Method.

The agarose (Sea Kem agarose FMC) was melted in 1 X TBE buffer and cooled to 55°C. The gel platform was put into the electrophoresis system (Horizon 11.14 BRL) and the end dams and comb were inserted. Agarose was poured onto the gel platform and allowed to set for at least 2 hours. The comb was carefully removed, 1 X TBE added to cover the gel, and the wells loaded. Electrodes from the power supply (BioRad Model 500/200) were inserted with the black negative electrode nearest to the wells and the power turned on. After an appropriate time (section 11, section 10, or section 8) the gel was removed from the gel platform, stained in 0.1% ethidium bromide for 20 minutes, and destained in 1% magnesium sulphate for 10 minutes. The gel was viewed on a UV transilluminator (Chromato-Vue^R, Watson Victor, model TS-15) and photographed (aperture, f11; shutter speed, 1 minute) with a MP-4 land camera using polaroid 667 or 665 or Kodak Tri-X pan professional film through a Wratten 23A (red) filter.

8. RESTRICTION ENDONUCLEASE DIGESTS. (Maniatis *et al.*, 1982).

8.1 Materials

(1) Restriction enzyme buffer (Promega) contained (mM): Tris-HCl (pH 8), 50 ; MgCl, 10 ; NaCl, 100. Purchased in the 10 X concentrated form.

(2) EcoRI restriction endonuclease (Promega). Purchased as 10 units/μl. One unit is the amount of enzyme required to cleave 1 μg of lambda DNA for one hour at 37°C in the appropriate buffer.

(3) Tris-acetate electrophoresis (TAE) buffer contained (mM): Trizma base, 40; sodium acetate, 5. The pH was adjusted to 7.8 using glacial acetic acid.

(4) Loading buffer x 10 contained: Ficoll 400,000 (Sigma), 20 %; EDTA, 0.1 M; sodium dodecyl sulphate, 1 %; bromophenol blue, 0.2 %.

(5) Agarose gel: 1 % agarose (Sea Kem Agarose FMC).

(6) Ethidium bromide: Stock solution of 10 mg/ml.

(7) Destaining solution: 1M Magnesium sulphate.

8.2 Method

Restriction enzyme buffer (2 μ l) was added to 15 μ l of genomic DNA, 2 μ l of sterile Milli-Q water and 1 μ l of *Eco*RI restriction endonuclease. The tubes were pulsed for 2 - 3 seconds in a microcentrifuge and incubated for 2 to 5 hours at 37°C. A 1% agarose gel was poured as outlined in section 7. Loading buffer (2 μ l) was added to the DNA digest, and 10 to 15 μ l of the mixture was loaded into a well on the gel. The gel was run for an hour at 1.5 volts/cm and then for 6 hours at 6.5 volts/cm. After viewing, as described in section 7, gels could be used for Southern blotting (section 9).

9. SOUTHERN BLOT TECHNIQUE (Southern, 1975).

9.1 Materials

(1) Depurinating solution (0.25M HCl) contained: Concentrated HCl, 20 ml in Milli-Q water, 980 ml.

(2) Denaturing solution contained (g/l): NaOH, 20; NaCl, 29; in Milli-Q water.

(3) Neutralising solution contained (g/l): Trizma base, 60.55; NaCl, 116; in Milli-Q water 800 ml. The pH was adjusted to 7.2 with concentrated hydrochloric acid and the volume made up with Milli-Q water.

(4) Standard saline citrate solution (20 x SSC) contained (g/l): NaCl, 175.3; sodium citrate, 88.2; in Milli-Q water, 800 ml. The pH was adjusted to 7.0 with 10 M NaOH and the volume made up to 1 litre with Milli-Q water.

(5) Nylon Membrane Hybond - N (Amersham)

9.2 Method

Gloves were worn throughout the procedure. The DNA to be transferred had been separated by gel electrophoresis (section 7). It was soaked in depurinating solution until the blue dye front turned yellow. After rinsing in de-ionised water, the gel was soaked in denaturing solution until the dye front returned to a blue colour, and then in neutralising solution for 1 hour.

Four layers of 3MM Whatman filter paper were laid in the bottom of a Pyrex dish. A piece of Gladwrap was placed over the whole dish, smoothed out, and a hole slightly smaller than the gel was made. The Whatman 3MM paper was wetted with 20 x SSC. The gel, with the end wells cut off, was placed over the hole in the gladwrap so that all four edges overlapped the hole. A piece of nylon membrane, slightly larger than the gel and wet with de-ionised water, was placed on the gel and all air bubbles between the layers were excluded. Layered on top of the membrane were a sheet of Whatman 3MM paper wet with 20 x SSC, two sheets of dry Whatman 3MM paper, and finally, a stack of paper towels 5 - 8 cm deep. A glass plate was placed on the paper towels and weight of about 300 g was placed on top. The whole apparatus was left overnight after adding enough 20 x SSC underneath the Gladwrap to keep the

3MM paper at the base moist for a few hours. The following day the membrane was washed briefly with 2 x SSC, placed between two sheets of Whatman 3MM paper and baked for 2 hours at 80°C under vacuum. The gel was checked to ensure that the DNA had transferred to the membrane by staining with ethidium bromide and viewing as outlined in section 7. Successful blots were probed as described in section 15.

10. THE ECKHARDT GEL PROCEDURE.

A modified version of the method proposed by Eckhardt (1978) was used in this study.

10.1 Materials.

All solutions are prepared from analytical grade reagents in deionised (Milli-Q) water.

(1) TE sarcosyl buffer contained (g/l):

Trizma base, 1.21; sodium EDTA, 0.372g; N-Laurylsarcosine, 1.0. The pH was adjusted to 8.0 and the buffer stored at 4°C until required.

(2) TBE x 1: section 7.1

(3) Lysozyme solution contained: Lysozyme, 4 mg; TBE x1, 2 ml. The lysozyme was dispensed into 0.1 ml aliquots and stored at -20°C until required.

(4) RNAse contained: RNAse, 20 mg; in sodium acetate (0.4 M, pH 4), 2 ml. After boiling in a water bath for 2 minutes, the RNAse was stored at -20°C until required.

(5) Solution 1 contained: Ficoll 400,000, 2.0 g; bromophenol blue, 0.01 g; lysozyme powder, 0.004 g; RNase, 0.02 ml; in TBE x 1 to 20 ml. The solution was stored at 4°C until required.

(6) Solution 2 contained: SDS, 0.02 g; Ficoll 400,000, 1 g; Proteinase K, 0.04 mg; in TBE x 1 to 10 ml. The solution was dispensed into 0.5 ml aliquots and stored at -20°C until required.

(7) Solution 3 contained: SDS, 0.04 g; Ficoll 400,000, 1.0 g; TBE x 1 to 20ml. The solution was stored at 4°C until required.

(8) Agarose gel: 0.8% w/v agarose gel (Sea Kem or Sea Plaque Agarose FMC) in TBE x 1 buffer.

10.2 Method.

A single colony was inoculated in 5 ml of TY broth (section 1.2.1) and incubated at 30°C with shaking (200 rpm) until the cells were in log phase growth ($OD_{600} = 0.4$) which was usually after 18 to 24 hours. Aliquots of the broths (0.1 ml) were dispensed into Eppendorf tubes and cells harvested by centrifugation (15600 x g for 5 minutes in a 5414 S Eppendorf centrifuge) and washed once in TE sarcosyl buffer. The pellet was resuspended into 1 ml of TE buffer and cells recovered by centrifugation. Lysozyme (50 µl) was added to 500 µl of solution 1 and mixed well. The pellet was resuspended in 20 µl of this solution and immediately loaded into a well on an agarose gel (section 7). After incubating for 15 minutes at room temperature, 20 µl of solution 2 was added to the well, and gently mixed with a fine blunt glass rod by stirring once. The mixture was left for 15 minutes, then the wells were overlaid with 20 µl of solution 3. The gel was then flooded with cold 1 x TBE buffer and run at 4°C for 1 hour at 1.5 volts/cm and then for 5 hours at 6 volts/cm. When examining for the presence of pPN1 the gel was run at 1.5 volts/cm for 1 hour and 6

volts/cm for 16 hours. After electrophoresis the gel was examined as outlined in section 7.

11. POLYMERASE CHAIN REACTION (PCR)

A method of thermal cycling (Ausabel *et al.*, 1991) was used to amplify a 260 bp 16S rRNA gene fragment corresponding to positions 44 to 337 in the *E.coli* 16S rRNA sequence (Young *et al.*, 1991) from the isolates used in this investigation. The primers for the rRNA amplification was chosen by careful inspection of the published bacterial 16S rRNA sequences available in the EMBL/Genbank data library. The primers themselves are complementary to conserved sequences and have amplified the correct fragment from all the alpha proteobacteria that were tested (Young *et al.*, 1991). The primers required for rRNA amplification was synthesised by Separation Science Unit - Molecular Genetics Unit, Massey University, Palmerston North, New Zealand or Oligos Etc. Inc. (USA).

11.1 Materials

(1) Deoxynucleotide triphosphates (dNTPs) (Pharmacia).

2 mM of each of the four bases; dATP, dCTP, dGTP, and dTTP.

(2) Primers for 16S rRNA Gene (Young *et al.*, 1991).

Y1 5' - TGG CTC AGA ACG AAC GCT GGC GGC - 3'

Y2 5' - CCC ACT GCT GCC TCC CGT AGG AGT - 3'

Y1 corresponds to positions 20 to 43 in the *Escherichia coli* 16S RNA sequence (Brosius *et al.*). Y2 corresponds to positions *E.coli* positions 361 to 338. Before use, the primers were diluted 1:50 with sterile Milli-Q water.

(3) Taq Polymerase Buffer x 10 (Life Technologies) contained: KCl, 500 mM; Tris-HCl (pH 8.8); MgCl₂, 15 mM; and 1% Triton-X - 100 .

(4) Taq Polymerase: (Life Technologies) isolated from *Thermus aquaticus* YT1. The storage buffer contained: Tris-HCl (pH 8), 20 mM; EDTA, 0.1 mM; dithiothreitol, 1 mM; 50 % (v/v) glycerol; stabilisers.

(5) Sterile paraffin oil.

(6) Agarose gel: 2% agarose gel in TBE x 1 (Sea Kem Agarose), section 7.

(7) Loading Buffer contained: Bromophenol blue, 0.05 %; sucrose, 50 %; EDTA, 0.1 M.

(8) ART^R tips: Aerosol resistant tips (Biotek).

11.2 Method

Gloves were worn throughout this procedure. Dilutions of the stock solutions were made on the day that they were used and kept on ice. Aerosol resistant tips were used at all times. The following reaction mixture was prepared: Taq Polymerase buffer X 10 ,2 µl; dNTPs , 4µl; Primer Y1, 1 µl; Primer Y2, 1 µl; Taq Polymerase, 0.4 µl; Milli-Q water, 11.6 µl. The DNA template was diluted 1:10 in sterile water and 2 µl was added to 18 µl of the reaction mixture in a sterile 1.5 ml Eppendorf tube. A negative control (sterile Milli-Q water in place of the DNA template) was also prepared. Sterile paraffin oil 20 µl was added, the tube centrifuged briefly and loaded in the thermal cycler (Techne type PHC-3) running the following programme:

Stage 1: 95°C, 3:00 minutes (template denaturation); 65°C, 30 seconds (primer annealing); 72°C, 30 seconds (extension of the annealed primers); number of cycles, 1:

Stage 2: 95°C, 45 seconds; 65°C, 30 seconds; 72°C, 30 seconds; cycles 35:

Stage 3: 95°C, 45 seconds; 65°C, 45 seconds; 72°C, 3:00 minutes; cycles 1.

PCR product (2 μ l) mixed with loading buffer (5 μ l) was examined by electrophoresis in a 2% agarose gel (1 x TBE buffer) as outlined in section 7. This was to check that a fragment of the correct size had been amplified in all tubes except the negative control. The PCR product was purified by the method described in section 12.

12. PURIFICATION OF DNA FRAGMENTS

The DNA fragment amplified by PCR was purified using Promega Magic PCR PrepsTM. The materials listed are supplied in the kit.

12.1 Materials

(1) Direct purification buffer contained (mM): KCl, 50; Tris-HCl (pH 8.8), 10; MgCl₂, 1.5; and Triton X-100 0.1%.

(2) PCR preps resin. The resin included in the Promega Magic PCR Preps was incubated at 37°C before use to dissolve the crystals.

(3) Magic PCR Preps mini-column.

(4) Column wash solution: 80% isopropanol

(5) Syringe

12.2 Method

Direct purification buffer (0.1 ml) was mixed in an Eppendorf tube with 15 - 20 μ l of PCR product (section 11). 1 ml of Magic PCR Preps resin was added and vortexed 3 times over a 1 minute period. This mix was then loaded into a syringe barrel and gently forced through the mini-column. The mini-column was washed with 2 ml of Magic PCR Preps column wash solution, then transferred to an Eppendorf tube and centrifuged for 20 seconds at 14000 x g

to dry the resin. The column was dried at room temperature for 10 minutes, and then removed to a new tube. TE buffer (section 5) (30 μ l) was applied to the column, left for one minute, then centrifuged for 20 seconds to elute the DNA. The purified DNA was stored in the Eppendorf at 4°C or -20°C.

13. PREPARATION OF A *NOD*A PROBE.

Base primers (24) were selected on the basis that they would hybridise efficiently to the sequence of interest with negligible hybridisation to other sequences present in the sample. The primers for the amplification of *nodA* sequence was selected using Primer Designer (Version 1.01; 1990; Scientific and Education Software) on the published sequences of Rossen *et al.*, (1984) and synthesised at Separation Science Unit - Molecular Genetics Unit, Massey University, Palmerston North.

<i>NodA</i> left	5'	TCA TAG TTC CGA CCC GTT TCG TTC	3'
<i>NodA</i> right	5'	ATG TCT TCT GAA GTG CGA TGG AAA	3'

A 590 bp sequence was amplified by the polymerase chain reaction (section 11, substituting Y1 and Y2 primers for the *nodA* primers) using total DNA extracted from strain ICMP2163::Tn5 as the template. The amplified product was checked for correct size (section 7), purified (section 11), and labelled with (α -P³²)dCTP by the Megaprime DNA labelling system (section 14).

14. MEGAPRIME DNA LABELLING SYSTEM

14.1 Materials

(1) Megaprime DNA labelling kit (Amersham). The kit contained: Primer solution: random primers in an aqueous solution; reaction buffer - dATP, dGTP and dTTP in a concentrated buffer; enzyme solution - (1 unit per μl DNA polymerase), 1 'Klenow' fragment.

(2) (α -P³²) dCTP (Amersham). Deoxycytidine 5' (α -P³²) triphosphate. 3000 Ci/mmol in 10 mM tricine.

(3) Column buffer contained: 1.0 M Tris - HCl (pH 8.0), 1 ml; 0.2 M EDTA, 50 μl ; β - mercaptoethanol, 30 μl ; in Milli-Q water, 100 ml.

(4) Sephadex G - 50 column: glass wool was packed in the bottom of a 1 ml syringe barrel. Sephadex G - 50 slurry (1 g Sephadex in 20 ml column buffer equilibrate overnight) was added, centrifuged at 1768xG for 5 min (Heraeus Megafuge 1.0, Sepatech) and the liquid discarded. This was repeated until the syringe barrel contained 0.8 - 0.9 ml of Sephadex G - 50 resin.

(5) Polyethyleneimine cellulose ion exchange resin (PEI) paper

(6) Scintillation vials

(7) Scintillation counter

(8) EDTA. 0.2M

(9) Hydrochloric acid (HCl): 2M

(10) All protective devices deemed necessary (by the university) for safe use of radioactive materials; perspex shields, special discard bins, the wearing of two pairs of gloves at a time.

14.2 Method

Template DNA (10 μ l of 25 - 50 ng), 5 μ l of primer solution and 20 μ l of sterile Milli-Q water was added to an Eppendorf tube. The tube was boiled for 2 minutes, immediately put on ice to cool rapidly, and briefly centrifuged at 15,600 x g. Megaprime reaction buffer (10 μ l), 3 μ l of (α -P³²)dCTP and 2 μ l of enzyme solution were added to the tube and incubated at 37°C for 30 minutes. The reaction was stopped by adding 5 μ l of 0.2 M EDTA. Incorporation of (α -P³²) dCTP was checked with Polyethyleneimine cellulose ion exchange resin (PEI) paper. Reaction mix (1 μ l) was spotted 1cm from the base of the paper, the PEI paper was placed upright in a beaker containing a 0.5 cm of 2 M HCl and left for 5 - 10 min to allow the HCl to reach the top of the paper by capillary action. The paper was cut in half, and the radioactive emission of each portion was measured by using a scintillation counter (Beckman LS7000). The lower portion of the PEI strip had the incorporated label, the upper portion had the unincorporated label. The two readings were compared both to give an indication of the percent incorporation.

If more than 50% of the label was incorporated into the DNA, the probe was used directly for hybridisation. If the incorporation was low, the reaction mixture was loaded onto a Sephadex G-50 column equilibrated with column buffer. The column was centrifuged at 1768 x g for 5 min (Heraeus Megafuge 1.0, Sepatech) and the labelled probe collected and stored in a 1.5 ml Eppendorf tube at -20°C. DNA probes were usually labelled to a specific activity of 1 - 5 X10⁷ cpm per μ g of DNA.

15. DNA HYBRIDISATION (Sambrook et al., 1989).

15.1 Materials

(1) Hybridisation buffer contained (ml/500ml): 1M HEPES buffer (pH 7), 25; 20 x SSC (section 9), 75; Herringsperm DNA (purified by phenol/chloroform extraction), 3 mg/ml, 3 ml; 20% SDS, 2.5; and 1 g of the following: Ficoll 70 000 MW; Bovine Serum Albumin (BSA); Polyvinylpyrrolidone (PVP) 10; in Milli-Q water, 500 ml. The buffer was stored at 4°C, then warmed to 37°C before use.

(2) Hybridisation tubes

(3) Plastic box : large enough to fit the membrane.

15.2 Method

The nylon membrane from the Southern blot procedure (section 9) was placed in a clean hybridisation tube with 30 ml of hybridisation buffer and pre-hybridised at 65°C for 2 hours in a hybridisation oven. Most of the buffer was decanted and the boiled probe (10^7 cpm per μg of DNA) was added. The probe was allowed to hybridise to the membrane at 65°C overnight (18 hours). The following day the membrane was placed in a plastic box and washed twice in 2 x SSC for 15 minutes at room temperature then washed in 0.1 x SSC at 50°C (using a shaking water bath). The membrane was dried on a sheet of Whatman 3MM, covered with Gladwrap and exposed to X - ray film (Fuji medical X - ray film), in the presence Cronex (DuPont) intensifying screens, at -70°C for 1 - 5 days. The film was developed using a Kodak X - Omat automatic processor.

16. 16S rDNA SEQUENCE DETERMINATION.

Direct sequencing of 16S rRNA gene fragments was performed with a Promega *FmoI*TM DNA Sequencing kit using the chain termination method (Sanger *et al.*, 1977). Gloves were worn throughout the procedure and the usual safety precautions were followed for handling radioactive isotopes and acrylamide.

16.1 Preparation of Acrylamide Gels for Sequencing.

16.1.1 Materials

(1) Tris borate buffer (TBB) x 10 contained: Boric acid, 55 g; EDTA, 19 g; Trizma base (Sigma), 324 g; Milli-Q water, 2 litres. The pH was adjusted to 8.9 and the buffer stored at 4°C.

(2) Urea solution contained: Urea, 288 g; Milli-Q water, to 450 ml.

(3) 40 % acrylamide contained: Acrylamide, 38 g; bis-acrylamide, 2 g in 100 ml Milli-Q water.

(4) Acrylamide mix contained (ml): Urea solution, 450; acrylamide (40%), 90; TBB (x 10), 60.

(5) TEMED: (N,N,N',N' - Tetramethylethylenediamine).

(6) Ammonium persulphate (AMPS) (10%)

(7) Mixed bed resin. (AG 501-X8), 20 -5 mesh.

(8) Glass plates dimensions: large, 31 cm x 38.5 cm; small, 31 cm x 36.5 cm

(9) 0.4 mm thick vinyl spacer.

(10) Vinyl sharktooth combs

(11) Silicone. 2% Dimethyldichlorosilane in carbon tetrachloride.

(12) Sleek tape. (Smith and Nephew)

16.1.2 Method

Acrylamide mix was prepared by adding urea solution to 40% acrylamide and stirring for one hour with a tablespoon of mixed bed resin to de-ionise the mixture. The solution was then filtered through a No. 1 scintillation filter, then the TBB was added. Acrylamide mix (80 ml), TEMED (50 μ l), and AMPS (500 μ l) was swirled together in a clean 250 ml Duran (Schott) bottle and used immediately. The small and large glass plates were cleaned with acetone. Two films of silicone were applied to the small plate which was then placed on the spacers (0.4 mm thick) running down the length of the large plate (the siliconised edge inner most). The edges were sealed with tape and secured with bulldog clips. The plates were tilted and the acrylamide mix was loaded from one side into the gap between the plates and filled to the top of the small glass plate, allowing a small overhang. The base of the gel comb was inserted 1 cm into the gel with the points of the teeth uppermost. After one hour the area around the comb was wrapped in Gladwrap to prevent the gel drying out, and the gel was used within 18 hours.

16.2 **Cycle Sequencing**

16.2.1 Materials

(1) Polymerase buffer: 5 X, contained : Tris-HCl (pH 9.0), 250 mM; MgCl₂, 10 mM.

(2) [α - S³⁵]dATP (> 1,000 Ci/mmol, 10 μ Ci/ μ l).

(3) Taq polmerase: (sequencing grade) Isolated from *Thermus aquaticus* YT1. The storage buffer contained Tris-HCl (pH 8), 20 mM; EDTA, 0.1 mM; dithiothreitol, 1 mM; 50 % (v/v) glycerol; stabilisers.

(4) Deoxy/dideoxy bases. d/ddA, d/ddG, d/ddA, d/ddT.

(5) Stop buffer. contained: NaOH, 10 mM; formamide, 95%; bromophenol blue, 0.05%; xylene cyanide, 0.05%.

(6) Paraffin oil.

16.2.2 Method

Both strands of the 16S rDNA gene fragment (section 11) were amplified using Y1 and Y2. For each primer, this reaction mixture was set up and kept on ice: primer (Y1 or Y2, section 11), 1 µl; polymerase buffer, 5 µl; sterile Milli-Q water, 8.5 µl; undiluted DNA template (purified PCR product), 1 µl; S³⁵, 5 µl; Taq polymerase, 1 µl. NOTE: tips used for the S³⁵ were discarded separately and gloves worn at all times. Aerosol resistant tips were used at all times.

The reaction mixture was mixed and pulsed in a microcentrifuge, then 4 µl dispensed into each of 4 Eppendorf tubes containing 2µl of one of the deoxy/dideoxy bases (d/ddA, d/ddG, d/ddA, d/ddT), remixed, and PCR grade paraffin oil (10µl) added before being finally pulsed in a microcentrifuge. The 4 tubes containing the reaction mixture and the deoxy/dideoxy bases were loaded on to the thermal cycler (Techne, type PHC-3) when it was between 80°C and 90°C and the following programmes were run:

Stage 1: 93°C, 2:30 minutes; 53°C, 30 seconds; 70°C, 30 seconds, cycles 1.

Stage 2: 93°C, 30 seconds; 53°C, 30 seconds; 70°C, 30 seconds; cycles 30.

The tubes were stored at -20°C as soon as the programme was finished or else 4 µl of stop buffer was added.

16.3 Separation of Cycle Sequencing Products.

16.3.1 Materials

- (1) Tris borate buffer (TBB x 1). section 16.1.1
- (2) Stop reaction. section 16.2.1
- (3) Polymerised acrylamide gel. section 16.1.1
- (4) Acrylamide gel electrophoresis chamber. Model SA or S2, BRL
- (5) Power pack. ECPS 3000/150, Pharmacia
- (6) Fixative. contained (%): methanol, 10; acetic acid, 10.
- (7) Gel drier.
- (8) X-ray film. Kodak.
- (9) Sterile loading tips

16.3.2 Method

The cycle sequencing products were placed in a heating block at 75°C until the gel was ready to be loaded. The Gladwrap and the bulldog clips were removed from the gel. Excess gel from around the comb area was washed away with water and the comb removed. The sealing tape at the bottom of the gel was cut and the gel was placed in the electrophoresis chamber with the large plate facing outermost. The drains were closed and the gel clamped tightly in place. The buffer (TBB x 1) was added to the top and bottom buffer chambers. The comb was placed so that the points of the teeth just rested on the edge of the gel to create wells for the samples. Stop reaction (4 µl) was loaded into a well and allowed to run for a few centimetres to check for leaks. Bubbles and excess urea were expelled from the wells area using a Pasteur pipette. An aliquot (4µl) of sequencing products from each tube was loaded into a well on one side of the gel (long run) through the buffer. The remainder

of the PCR sample was stored at -20°C for later use. The power was then switched on and the gel run at 65 W until the blue dye band reached the bottom of the gel. Then power to the apparatus was turned off, and the remainder of the thawed samples were loaded in the same manner as before. Once this side had run to the bottom of the gel, the power was turned off and the buffer was drained away. The spacers, comb and the small plate were carefully removed. The large plate with the gel sitting on it was placed in fixative for 30 minutes, then carefully placed on a bench. Whatman paper (3MM) and then blotting paper was placed on top of the gel, after which the large plate was carefully turned over. The large plate was raised from one end allowing the gel stuck to the Whatman paper to be peeled off and dried at 80°C for 2 hours under vacuum on a slab drier (model 483) or a gel drier (model 583, Bio-Rad).

The dried gel adhered to the Whatman paper and was loaded into a cassette with X-ray film in direct contact with the gel surface. The film was exposed at room temperature for about 24 hours and developed in a Kodak (XY2) processor.

16.4 16S rDNA Sequence from the Autoradiograph.

The developed autoradiograph was placed on a light box (CustomLight, Christchurch) and the 16S rDNA sequence was read manually. The sequence obtained from the Y1 strand was checked against the complementary Y2 strand. Sequences were double checked against the original autoradiograph.

17 SEQUENCE ANALYSIS BY COMPUTER SOFTWARE.

17.1 Identification of Bacterial Strains.

The 16S rDNA sequence was entered in an IBM computer as a text file. A BLAST search (Altschul *et al.*, 1990) was conducted by sending an E-mail request to the following address at the National Center for Biotechnology Information (National Centre for Medicine, NIH, Bethesda, USA):

blast@ncbi.nlm.nih.gov.

The 16S rDNA sequences were compared to sequences lodged in the GenBank/EMBL data library.

17.2 Phylogenetic Analysis.

The following GenBank sequences were used: *Rhizobium leguminosarum* bv *trifolii* ATCC14480, X67227; *Rhizobium meliloti* NZP4017, M55495; *Rhizobium loti* NZP2213, X67229; *Rhizobium tropici* CFN299, X67233; *Rhizobium etli* Or191, M55236; *Bradyrhizobium japonicum* USDA31, M55487; *Azorhizobium caulinodans*, M55491; *Agrobacterium tumefaciens* DMS30105, M11223; *Rhodospirillum rubrum*, M34127; *Rhodospirillum rubrum*, M34129; *Sphingobacterium thalophilum*, M58779; *Sphingobacterium mizutae*, M58796.

The *Caulobacter crescentus* ATCC15252 sequence was provided by Christine Fenton.

The 260 bp 16S rDNA nucleotide sequences were aligned by using the PILEUP programme of the Genetics Computer Group Sequence Analysis Package (Devereux *et al.* 1984), version 7, on a VAX computer. The DNADIST programme was used to determine the Jukes-Cantor distances of the aligned

sequences (Jukes and Cantor, 1969). The NEIGHBOR programme was used to construct a phylogenetic tree based on the Neighbor-Joining method of Saito and Nei (1987). J. Felsenstein's (1982) PHYLIP 3.5c SEQBOOT programme provided 100 data sets for bootstrap analysis.

18. NUCLEOTIDE SEQUENCE ACCESSION NUMBER.

The *Sphingobacterium multivorum* sequence was deposited in the GenBank Data Library under accession no.U01879.

RESULTS

1. CONJUGATION IN SOIL.

Conjugation between an effective strain of *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5 and the non-nodulating soil bacterium NR40 (*Rhizobium loti* on the basis of its fatty acid profile, Materials and Methods section 4) was tested in sterile soil microcosms at 50% water holding capacity. Ramiha hill soil and Ashurst silt loam soil, taken from ryegrass/white clover pastures, were found to have a pH of 5.0 (Materials and Methods section 2.2). Calcium hydroxide was used to amend the pH from a minimum of 5.0 to a maximum of 8.0.

1.1 Factors Affecting pSym Transfer.

1.1.1 pH.

In both Ramiha hill soil and Ashurst silt loam soil, the *Rhizobium* strains introduced to soil at pH 5.0 became non-viable within 7 days (Figure 3 and Figure 4) but remained viable for a maximum of 21 days in soil at pH 5.5. No transconjugant NR40 were recovered. In soil at pH 6.0 or higher cell numbers increased by approximately 100-fold within 7 days and remained stable until the end of the 21 day trial. Typically, the concentration of transconjugant NR40 increased five fold in eighteen days, from 2 ± 1 CFU/g of soil to 10 ± 3 CFU/g of soil. It is concluded that the viability of *Rhizobium* strains ICMP2163::Tn5 and NR40 was significantly affected by soil at pH 5.5 or less, within 21 days, when compared to the other treatments (Student's-t test, $P < 0.001$; Materials and Methods, section 2).

1.1.2 Presence of plants.

The addition of sterile white clover plants to the microcosms containing Ramiha hill soil significantly increased the frequency of NR40 transconjugants formed (Figure 5a) when compared to the frequency of transconjugants formed in soil alone (Student's-t test, $P < 0.001$). The same effect was observed if ryegrass was substituted for the white clover plants (Figure 5b). Typically, the concentration of transconjugant NR40 increased ten fold in eighteen days, from 4 ± 2 CFU/g of soil to 44 ± 5 CFU/g of soil. In microcosms containing Ashurst silt loam soil the same effect of the presence of plants was observed (Figure 6). It is concluded that the presence of ryegrass or clover seedlings significantly increases the frequency of transconjugants formed in both soil types used.

1.1.3 Soil type.

The characteristics of the two soil types used are outlined in Table 2. Despite differences in structure and chemical composition, the results obtained for each treatment with the two soils were almost identical (Figure 3 - Figure 6). Student's-t test analysis confirmed that there was no significant difference when comparing the effect of using Ashurst silt loam soil instead of Ramiha hill soil in the experiments described above.

TABLE 2. Soils used in this study^a

Name	Type	Composition	Comment
Ramiha hill soil	Hill country soil of the Tararua Ranges	Yellow-brown earth (fulvic soil) from slope deposits.	Strongly leached by rainfall (1270 - 1780 mm/year). Well drained. Altitude, 300-600 metres.
Ashurst silt loam soil	Flat terrace land in Palmerston North	Yellow-brown shallow and stoney soil (co-pallic fulvic soil) from thin deposits of colluvium & alluvium.	Weakly leached. Rainfall, 890-1140 mm/year. Well drained. Altitude, 30-90 metres.

^a Taken from Cowie (1972).

Figure 3. THE EFFECT OF pH ON THE VIABILITY OF RHIZOBIUM STRAINS IN RAMIHA HILL SOIL.

Bars indicate 95% confidence limits.

A) *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5.

B) *Rhizobium loti* soil strain NR40.

Figure 3a

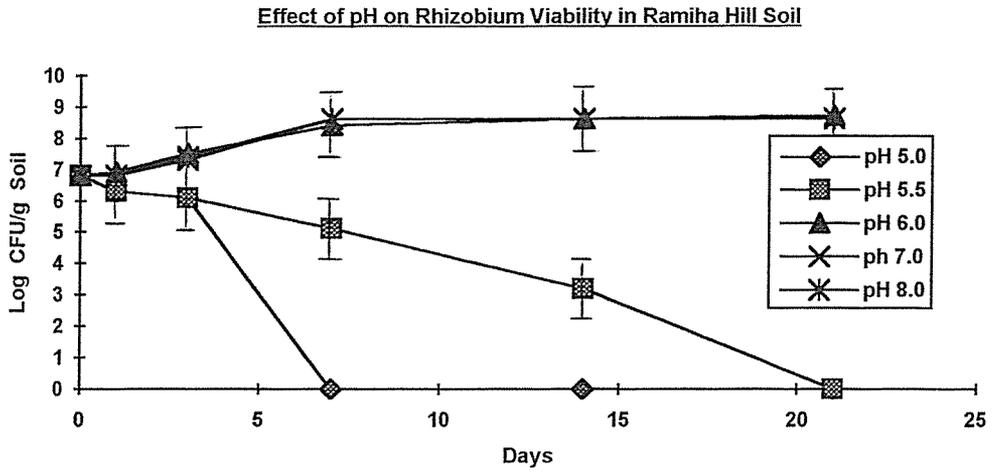


Figure 3b

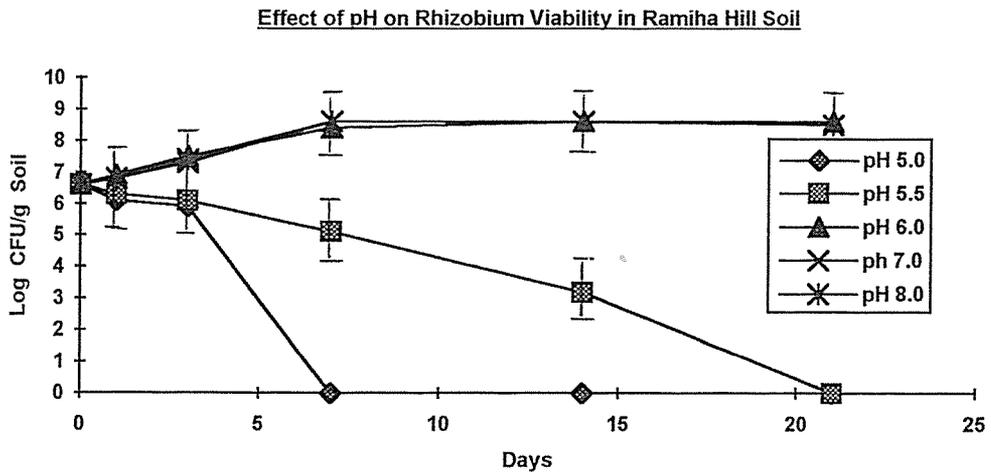


Figure 4. THE EFFECT OF pH ON THE VIABILITY OF RHIZOBIUM STRAINS IN ASHURST SILT LOAM SOIL.

Bars indicate 95% confidence limits.

A) *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5.

B) *Rhizobium loti* soil strain NR40.

Figure 4a

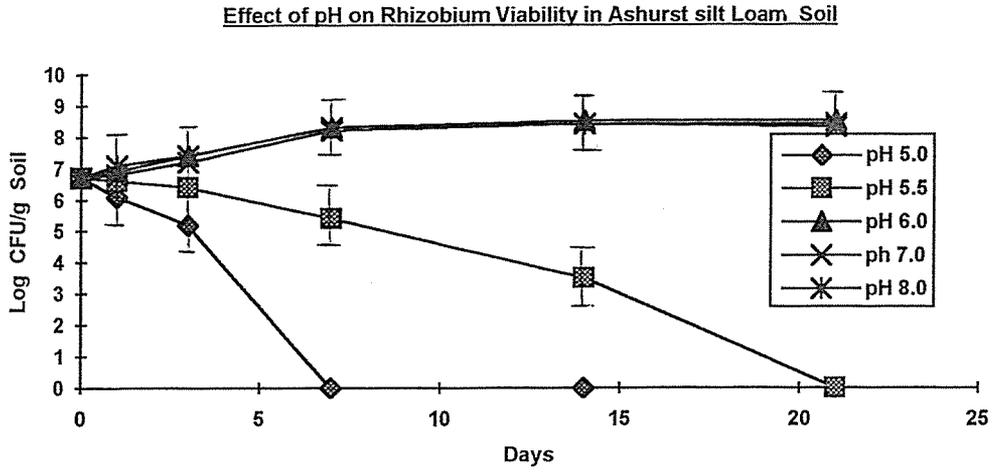


Figure 4b

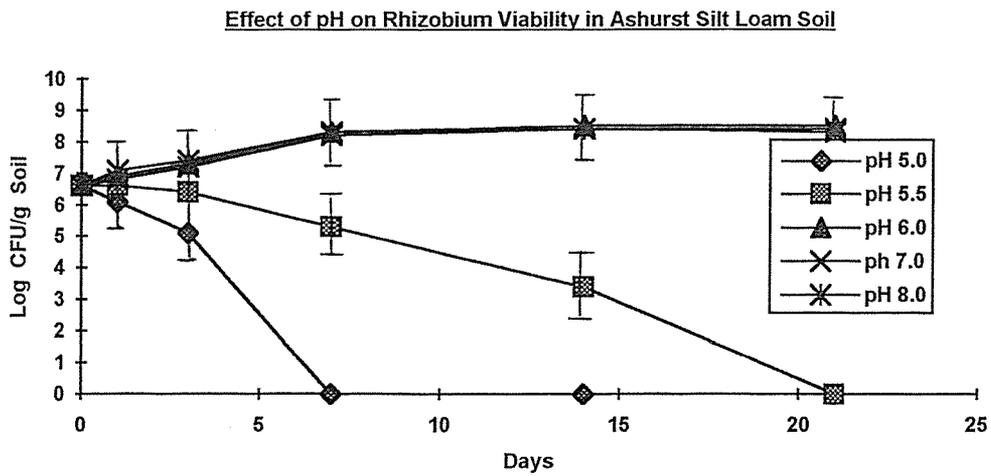


Figure 5. THE EFFECT OF THE PRESENCE OF PLANTS ON
THE TRANSFER OF THE SYMBIOTIC PLASMID FROM
RHIZOBIUM LEGUMINOSARUM BV TRIFOLII STRAIN
ICMP2163::Tn5 TO RHIZOBIUM LOTI SOIL ISOLATE
NR40 IN RAMIHA HILL SOIL.

Bars indicate 95% confidence limits.

A) White clover seedlings

B) Ryegrass seedlings

Figure 5b

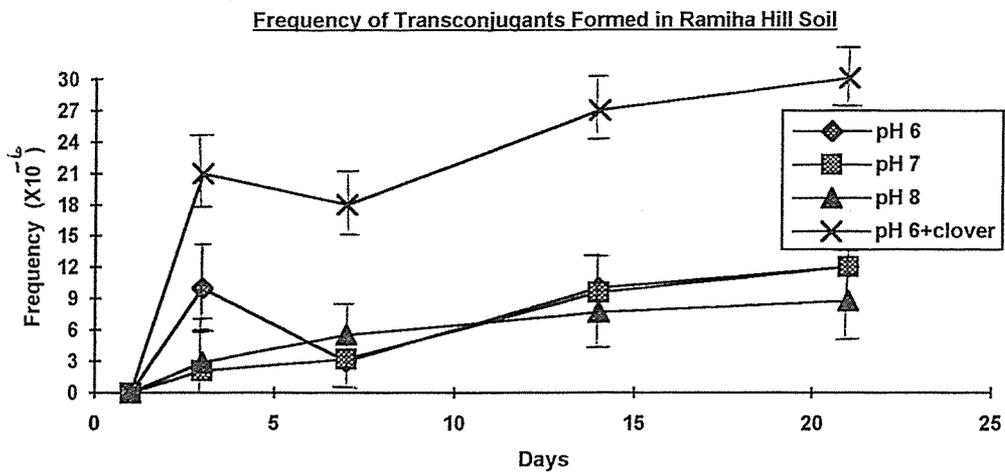


Figure 5a

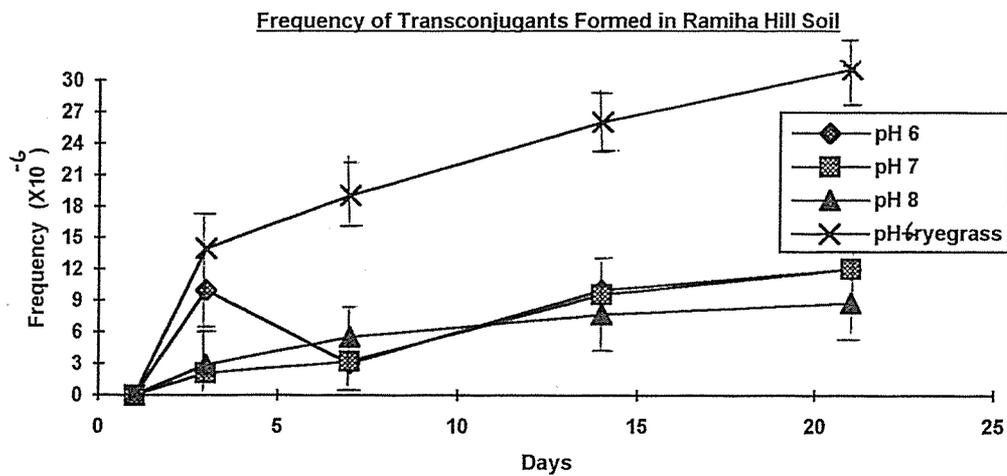


Figure 6. THE EFFECT OF THE PRESENCE OF PLANTS ON
THE TRANSFER OF THE SYMBIOTIC PLASMID FROM
RHIZOBIUM LEGUMINOSARUM BV TRIFOLII STRAIN
ICMP2163::Tn5 TO *RHIZOBIUM LOTI* SOIL ISOLATE
NR40 IN ASHURST SILT LOAM SOIL.

Bars indicate 95% confidence limits.

A) White clover seedlings

B) Ryegrass seedlings

Figure 6b

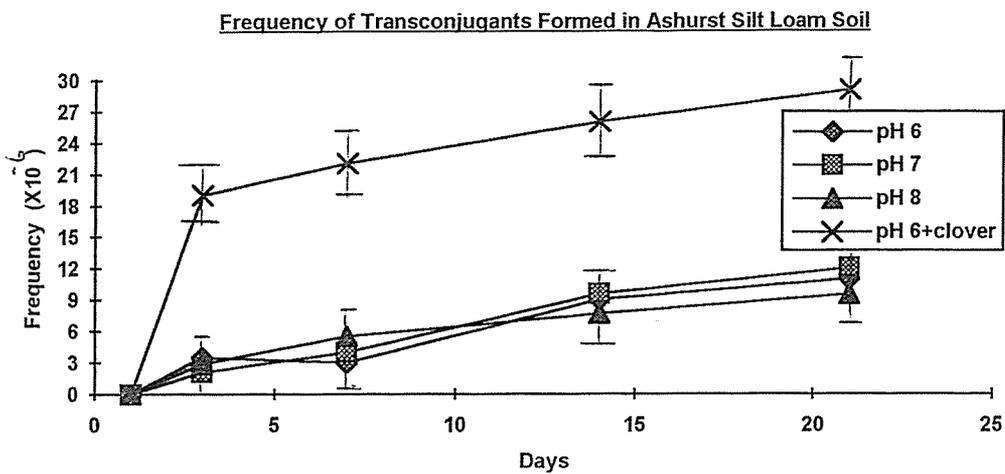
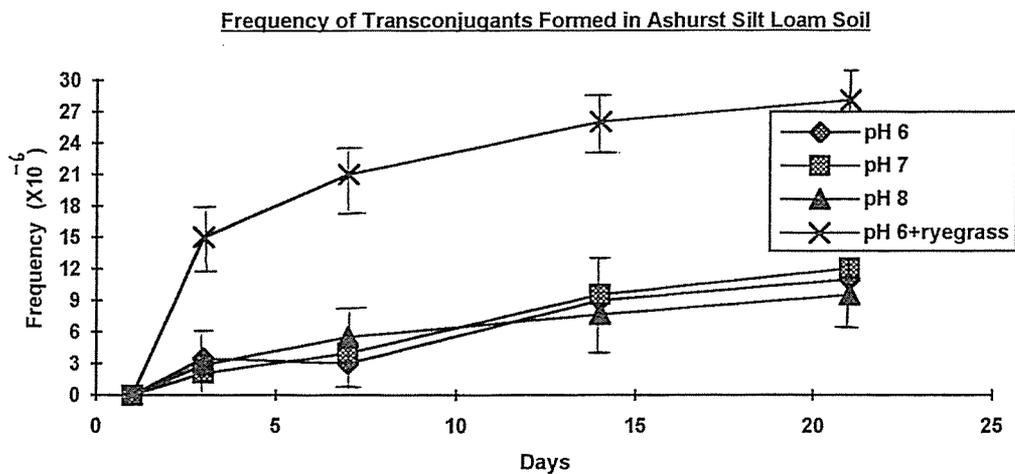


Figure 6a



1.2 Confirmation of pSym Transfer to NR40.

1.2.1 Restriction Endonuclease Digests

DNA obtained from bacteria arising on TY.Rif.Neo plates was used to verify that the bacteria isolated from the soil microcosms were similar to NR40 and not ICMP2163::Tn5. Figure 7a shows the restriction fragment patterns obtained from genomic DNA of the Sym plasmid donor (ICMP2163::Tn5), the recipient (NR40), and five putative transconjugant strains (ICMP2163::Tn5 X NR40) selected at random. Comparison of the series of restriction fragment bands that occur from the 23.1 kb to 9.4 kb, and from 4.4 kb to 2.3 kb, indicate that the transconjugants (MFNR series) are similar to the recipient strain NR40 and not the donor strain ICMP2163::Tn5.

1.2.2 nodA Probe

Southern blots were made of gels used for restriction fragment analysis and probed with a 590 bp fragment of *nodA* DNA. Figure 7b shows that the *nodA* probe hybridised to a fragment of between 2 kb and 564 bp from the Sym plasmid donor strain ICMP2163::Tn5, and not to the recipient strain NR40. The probe also confirmed that the putative NR40 transconjugants (MFNR series) isolated from soil microcosms contained *nod* genes.

1.2.3 Plant Nodulation By Transconjugant Bacteria

NR40 transconjugants (MFNR series) were used to inoculate white clover seedlings in nitrogen-deficient media to confirm that they could incite nodule formation (Figure 8). Plants nodulated by the transconjugant typically carried 10 ± 3 small white nodules and the plants were 4-7 cm in length, the same height as the negative control plants. Plants nodulated by *Rhizobium*

leguminosarum biovar *trifolii* strain ICMP2163::Tn5 typically carried 3-5 healthy pink large nodules and were 9-12 cm in length. It is concluded that NR40 transconjugants (MFNR series) were able to incite nodule formation but in all cases the nodules appeared ineffective at promoting plant growth.

Figure 7. HYBRIDISATION OF A *NODA* PROBE TO TOTAL GENOMIC DNA FROM TRANSCONJUGANT NR40.

(A) Genomic DNA from *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5 (1), *Rhizobium loti* soil isolate NR40 (2) and transconjugant (ICMP2163::Tn5 X NR40) strains (3-7) digested by *Eco*RI and separated by electrophoresis. The left hand lane contains Lamda *Hind*III cut DNA from 564 bp to 23,130 bp.

(B) Autoradiograph from a Southern blot of genomic DNA digested with *Eco*RI probed with a 590 bp fragment of *nodA* DNA.

Lane 1, Lamda *Hind*III cut DNA ladder from 564 bp to 23,130 bp.;

Lane 2, *Rhizobium leguminosarum* biovar trifolii strain
ICMP2163::Tn5;

Lane 3, *Rhizobium loti* soil isolate NR40;

Lane 4-8, transconjugant (ICMP2163::Tn5 X NR40) MFNR strains.

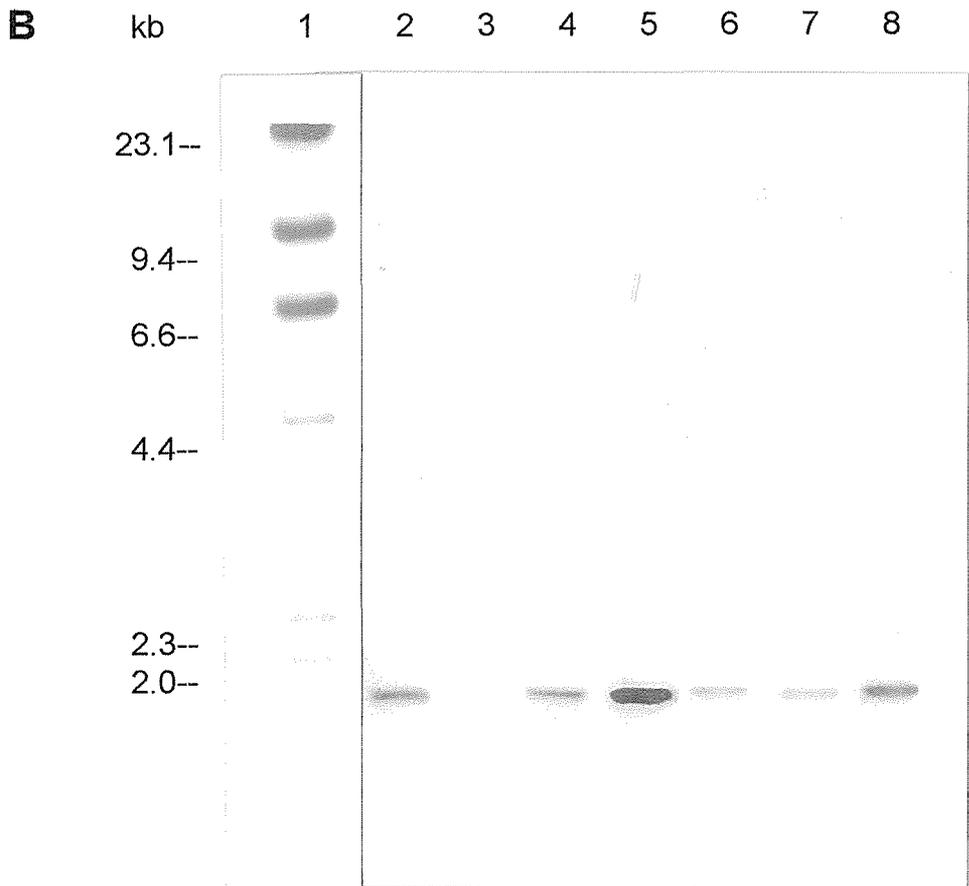
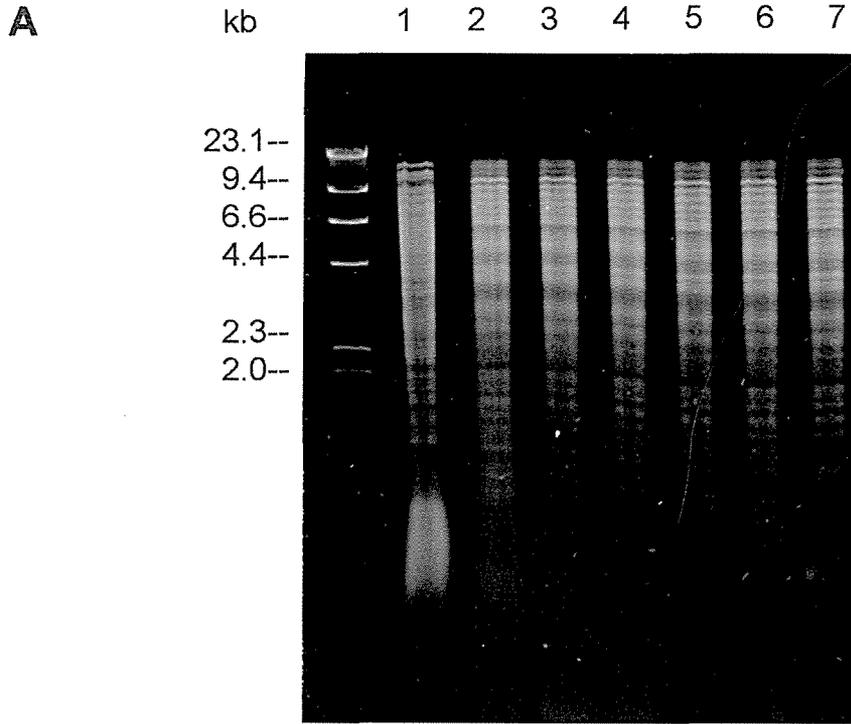


Figure 8. PLANT NODULATION BY TRANSCONJUGANT NR40

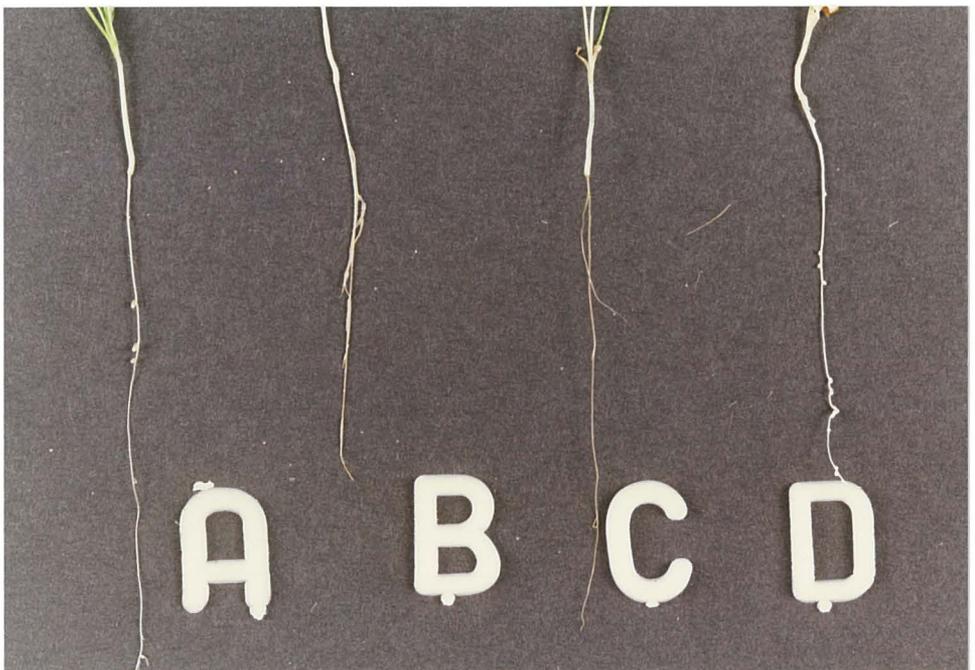
Nodule formation on white clover (*Trifolium repens*)
seedlings thirty days after inoculation.

(A) and (B): Plants inoculated with (left to right): *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5; sterile water; *Rhizobium loti* soil isolate NR40; transconjugant (ICMP2163::Tn5 X NR40) MFNR10

A



B



2. EXPRESSION OF SYMBIOTIC GENES IN SOIL BACTERIA

In the first series of experiments, conjugation was tested between *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5 and *Sphingobacterium multivorum* strain NZRM1228.

In the second series of experiments, conjugation was tested between *Escherichia coli* strain PN200 and *Caulobacter crescentus* strain MCDF23.

2.1 **Transfer of pSym From *Rhizobium leguminosarum* biovar trifolii to *Sphingobacterium multivorum*.**

Conjugation between an effective strain of *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5 and *Sphingobacterium multivorum* strain NZRM1228 was carried out on artificial media using the membrane filter technique.

2.1.1 Plant nodulation by transconjugant *S. multivorum*.

It was not possible to use antibiotic markers to select for transconjugants on selective media as strain NZRM1228 was spontaneously resistant to a number of antibiotics (Table 1), including neomycin normally used to select for the presence of Tn5. Consequently the mix of putative transconjugants and residual recipients were isolated on LB Rif.Neo.Str media to select against the *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5 donor. Growth from the LB Rif.Neo.Str plates was used to inoculate clover seedlings growing on nitrogen deficient media and after 8 weeks 2 out of 20 plants formed nodules (Table 3). The small proportion of nodulated plants may have been due to the low number of transconjugants in the inoculum compared with the number of residual recipients. After isolating and colony purifying the nodule

occupants (MF100 series) another round of plant tests was performed with MF100 and 10 out of 10 plants formed nodules after 2 weeks (Figure 9b). In all cases the nodules formed appeared ineffective at promoting plant growth (Figure 9a and Table 3). The symbiotic plasmid was lost from nodule isolates after 2-3 rounds of single colony purification, perhaps due to the inability to use antibiotic resistance markers to select for retention of pSym::Tn5.

TABLE 3. Evaluation of nodule formation on white clover seedlings

Inoculum	Plants nodulated	Nodules per plant		First appearance of nodules (weeks)
		Mean	range	
1st test				
ICMP2163::Tn5	10/10 ^a	4	3-7	2
NZRM1228 cross ^b	2/20 ^c	2	1-3	8
2nd test				
ICMP2163::Tn5	10/10 ^a	4	3-8	2
MF100	10/10 ^c	10	6-18	2

^a fully effective nitrogen fixation.

^b mix of presumptive transconjugants and residual recipients.

^c ineffective nitrogen fixation.

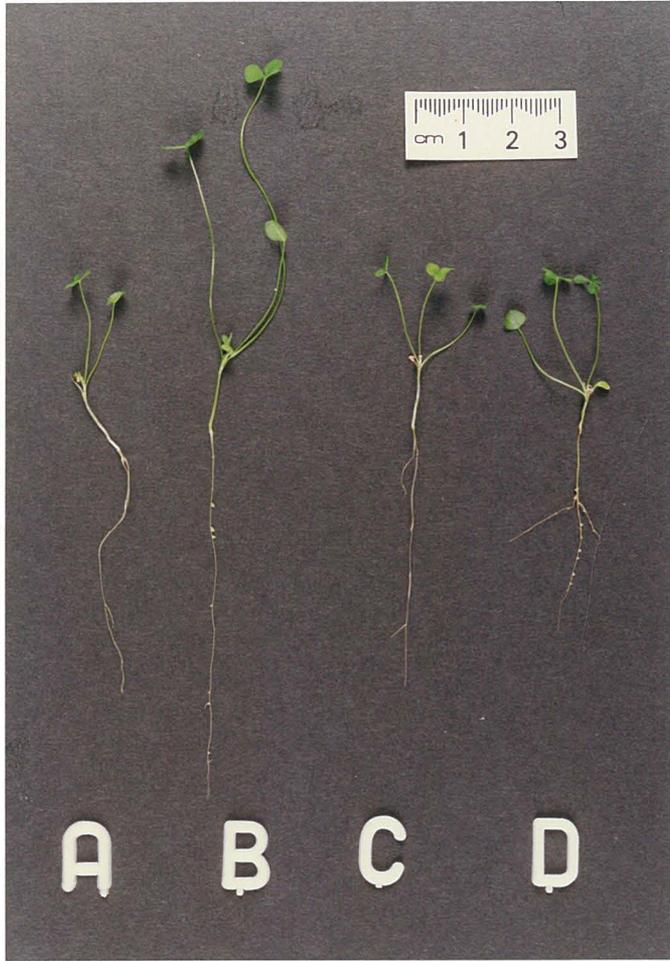
Figure 9. PLANT NODULATION BY TRANSCONJUGANT
SPHINGOBACTERIUM MULTIVORUM MF100.

Nodule formation on white clover (*Trifolium repens*)
seedlings thirty days after inoculation.

(A) Plants inoculated with (left to right): sterile water; *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5 (5 nodules); *Sphingobacterium multivorum* strain NZRM1228 (no nodules); transconjugant MF100 obtained by crossing ICMP2163::Tn5 with NZRM1228 (18 nodules). A nodule from this plant was sectioned for Figure 9.

(B) Seedling roots after inoculation with (left to right): *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5; transconjugant MF100.

A



B

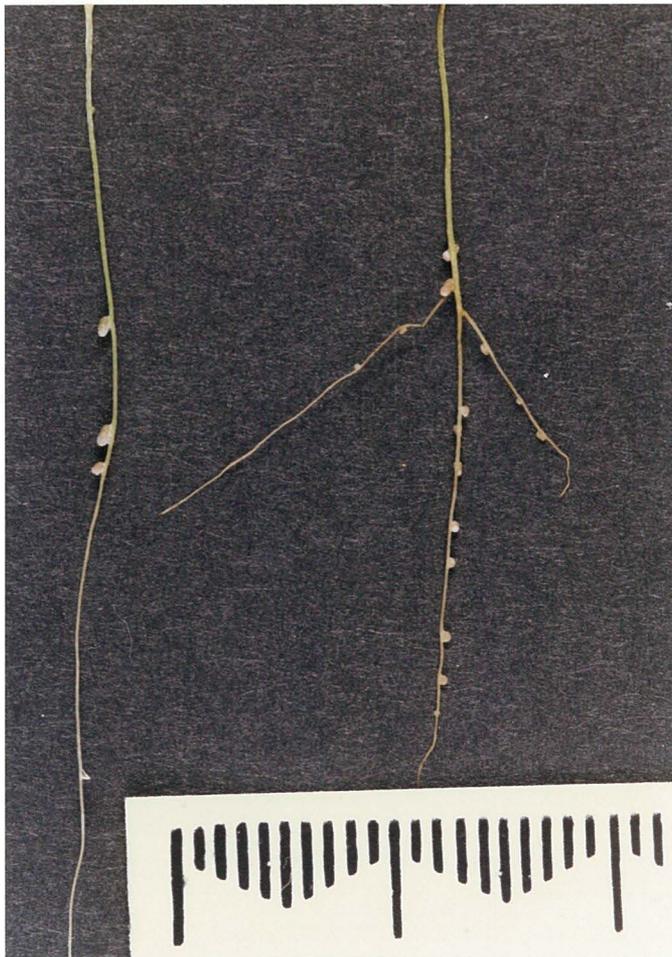


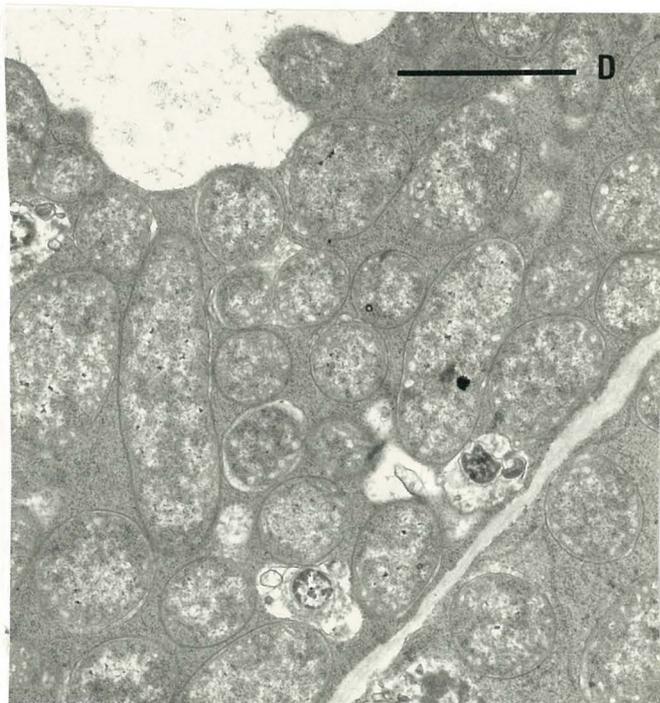
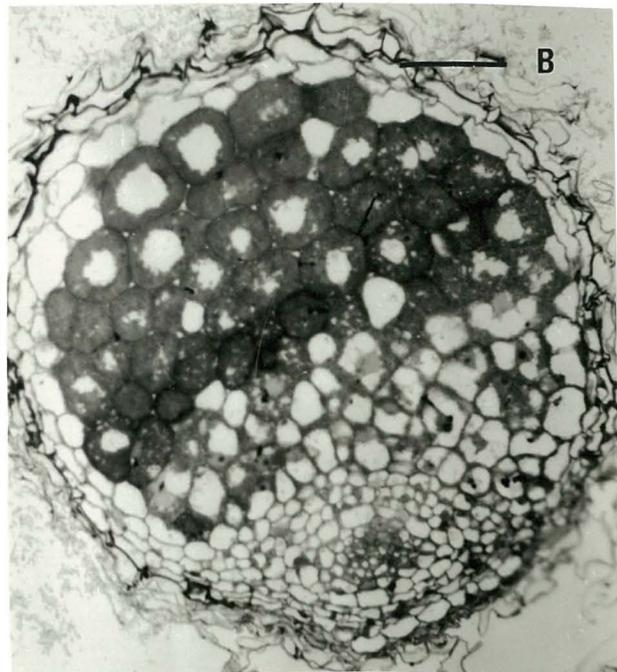
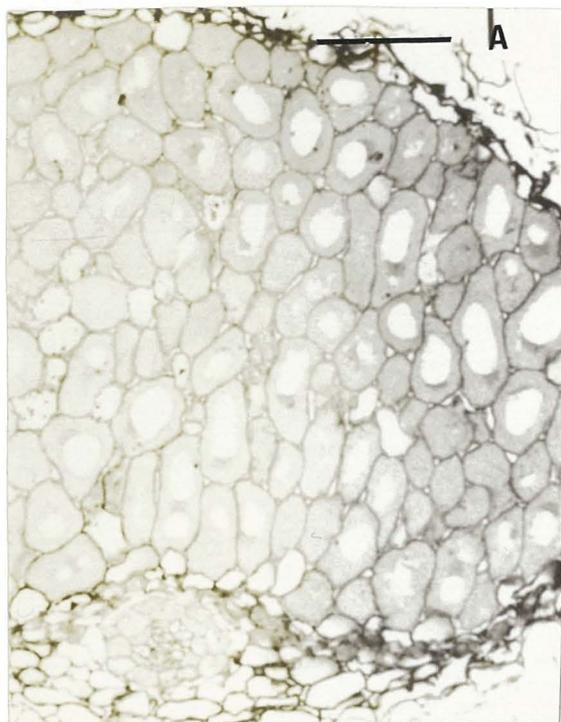
Figure 10. SECTIONS FROM WHITE CLOVER (*TRIFOLIUM REPENS*)
NODULES INFECTED BY *RHIZOBIUM* STRAIN ICMP2163::TN5
AND *SPHINGOBACTERIUM* TRANSCONJUGANT MF100
(ICMP2163::TN5 X NZRM1228) EXAMINED BY LIGHT AND
ELECTRON MICROSCOPY.

(A) Light micrograph section of a nodule formed by *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5. It has a symbiotic zone in which cells are filled with bacteroids. Bar = 100 μm .

(B) Light micrograph section of a nodule formed by *Sphingobacterium multivorum* transconjugant strain MF100. It has a symbiotic zone in which cells are filled with bacteroids. Bar = 100 μm .

(C) Electron micrograph of a plant cell infected by *Rhizobium* strain ICMP2163::Tn5 (X11,500). Bar = 1 μm .

(D) Electron micrograph of a plant cell infected by *Sphingobacterium multivorum* transconjugant strain MF100 (X11,500). Bar = 1 μm .



2.1.2 Preliminary Identification of Nodule Isolates.

A subculture of strain MF200, processed as an unknown isolate, was identified as *Sphingobacterium spiritivorum* on the basis of its fatty acid profile and showed no relationship to 120 *Rhizobium* strains in the database representative of *R. fredii*, *R. galegae*, *R. leguminosarum*, *R. loti*, *R. meliloti* and *R. tropici*.

2.1.3 Microscopic Examination of Root Nodules.

Nodules formed by putative *S. multivorum* transconjugants were examined by light and electron microscopy. Under the light microscope strain MF200 appeared to be able to invade cortical cells as efficiently as *Rhizobium* strain ICMP2163::Tn5 (Figure 10a, 10b). Examination under the electron microscope revealed that cells invaded by strain MF200 were more densely packed by bacteroids and had a more electron dense cytoplasm than cells invaded by *Rhizobium* strain ICMP2163::Tn5 (Figure 10c, 10d).

2.1.4 Examination of *S. multivorum* Transconjugants.

The bacteria occupying the nodules of plants inoculated with strain MF100 were isolated and single colony purified (MF200 series). Restriction fragment patterns of total DNA digested with *EcoRI* (Figure 11a) indicated that the nodule isolates were similar to *Sphingobacterium multivorum* and not *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5. Southern blots were probed with a 590 bp fragment of *nodA*. Figure 10b shows that the *nodA* probe hybridised to a fragment of between 2 kb and 564 bp from the Sym plasmid donor strain ICMP2163::Tn5, and not to the recipient strain NZRM1228. The probe also confirmed that the nodule isolate MF200 contained *nod* genes (Figure 11b).

2.1.5 Sequence Data.

The Polymerase Chain Reaction was used to amplify a 16S rRNA gene fragment from total DNA from *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5, *Sphingobacterium multivorum* strain NZRM1228 and the nodule isolate MF200. Figure 12a shows that two PCR products were formed for strain NZRM1228 and the nodule isolate strain MF200 under the conditions used. Increasing the annealing temperature did not result in only one product being formed. The presence of two PCR products did not appear to cause problems when sequencing the fragments (Figure 12b).

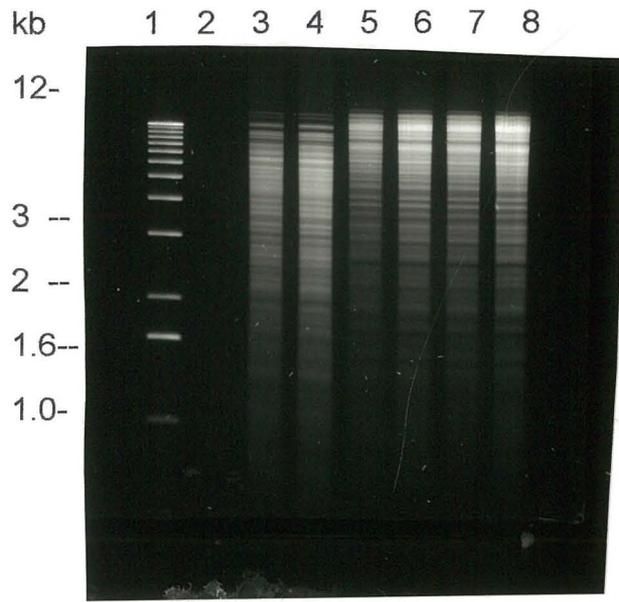
The nodule isolate strain MF200 was identical in sequence to that of *S. multivorum* NZRM1228 (Figure 13). *Sphingobacterium thalpophilum* shows the most homology to the *S. multivorum* sequence. For reference organisms outside this genus there are 2 main regions where sequence gaps occur when aligned with the *S. multivorum* sequence. Figure 14 shows schematic 2D models of part of the 16S rRNA molecule for *E.coli*, *Rhizobium leguminosarum* bv trifolii, and *Sphingobacterium multivorum*. The areas labelled 1 and 3 correspond to the 2 main gaps in the aligned sequences as mentioned above. There are also a number of regions where base mismatches occur reflecting the different lines of descent for these organisms. The phylogenetic tree obtained from the Jukes-Cantor distances of the aligned sequences shows that *Sphingobacterium multivorum* is distantly related to *Rhizobium* and the other reference strains used (Figure 20).

Figure 11. HYBRIDISATION OF A *NODA* PROBE TO TOTAL GENOMIC DNA FROM TRANSCONJUGANT *SPHINGOBACTERIUM MULTIVORUM*

(A) Genomic DNA from *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2163::Tn5, *Sphingobacterium multivorum* strain NZRM1228 and transconjugant MF200 (ICMP2163::Tn5 X NZRM1228) digested by *Eco*RI and separated by electrophoresis. Lane 1, BRL 1kb DNA ladder from 1018 bp to 11,198 bp; Lanes 3 & 4, ICMP2163::Tn5; Lanes 5 & 6, NZRM1228; Lanes 7 & 8, MF200.

(B) Autoradiograph from a Southern blot of genomic DNA digested with *Eco*RI probed with a 590 bp fragment of *nodA* DNA. Lane 1, Lamda *Hind*III cut DNA ladder from 564 bp to 23,130 bp.; Lane 2, *Rhizobium leguminosarum* biovar *trifolii* strain ICMP2163::Tn5; Lane 3, *Sphingobacterium multivorum* NZRM1228; Lane 4, transconjugant MF200. The Lamda ladder shows up more strongly due to the the higher concentration of DNA for hybridisation.

A



B

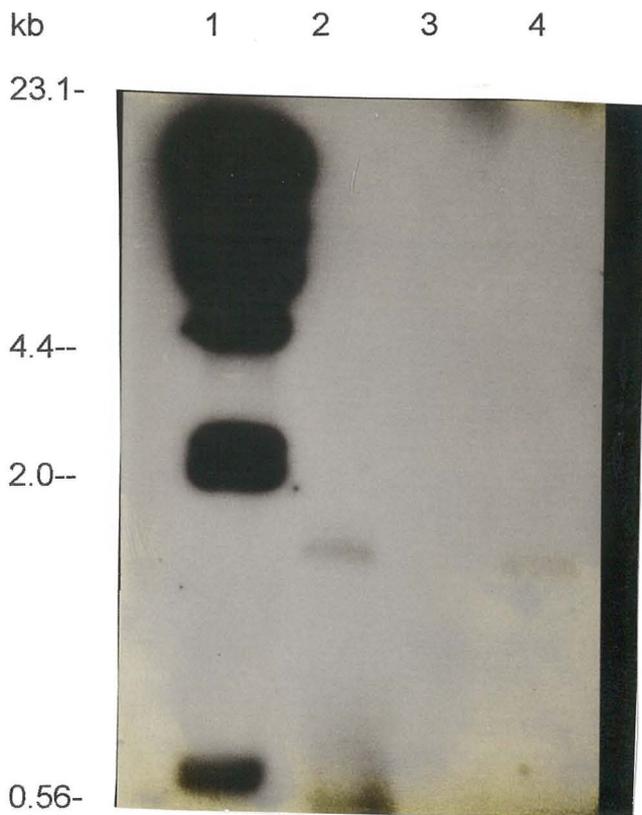
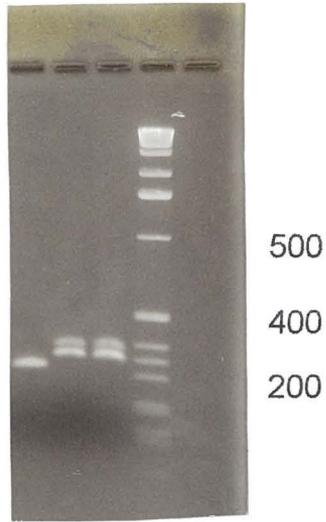


Figure 12. SEQUENCING OF 16S rDNA GENE FRAGMENTS FROM
SPHINGOBACTERIUM MULTIVORUM STRAIN NZRM1228
AND THE NODULE ISOLATE MF200.

(A) 2% agarose gel showing the PCR products amplified from total genomic DNA . Lane 1, *Rhizobium leguminosarum* biovar trifolii strain ICMP2163::Tn5; lane 2, *Sphingobacterium multivorum* strain NZRM1228; lane 3, nodule isolate MF200; lane 4, BRL 100 bp DNA ladder from 100 bp to 1000 bp; lane 5, negative control.

(B) Part of an acrylamide sequencing gel of an amplified 16S rDNA fragment. Lanes 1-4, sequence obtained by using the Y1 primer (from left to right, A G C T); Lanes 5-8, sequence obtained by using the Y2 primer (from left to right, A G C T).

A 1 2 3 4 5 bp



B

1 2 3 4 5 6 7 8



Figure 13. ALIGNED SEQUENCES OF PART OF THE 16S RNA GENE OF SIX BACTERIAL STRAINS.

Dots indicate that all the sequences are identical to the consensus (shown at top); blanks indicate alignment gaps. The sequence shown corresponds to positions 50 to 337 in the *E. coli* 16S rRNA sequence (Young *et al.*, 1991). Genus abbreviations used: S., *Sphingobacterium*; R., *Rhizobium*; B., *Bradyrhizobium*; C., *Caulobacter*.

1

50

100

<i>S. multivorum</i>	AATACATGCA	AGTCGGACGG	GATCCATCGG	AAGCTTGCTC	GAGATGGTGA	GAGTGGCGCA	CGGGTGC GTA	ACGCGTGAGC	AACCTACCTC	TATCAGGGGG
MF200
<i>S. thalpopophilum</i>	T.....A	TCA.....G.....	C.....
<i>S. mizutae</i>	T.....A	CCN.....G..NA
<i>R. leguminosarum</i>	..C.....	CCCGCAAG.G	..C...AG.A...G.	..T.....CT	.GA.TAC..A
<i>B. japonicum</i>	..C.....GG..AT	AGC.ATA..T	C..C...AG.A...G.	..G.....T	.TGGTTC..A
<i>C. crescentus</i>	..C.....A.GGAT	CCTTC..GAT	T.....G.A...	..A...G.	..G.G..CT	.TGGTTC..A

101

150

200

<i>S. multivorum</i>	ATAGCCTCTC	GAAAGAGAGA	TTAACACCGC	ATAACATCAA	CAGTTCGCAT	GTTTCGGTTGA	TTAAATATTT	ATAGGATAGA	GATGGGCTCG	CGTGACATTA
MF200
<i>S. thalpopophilum</i>A.T	ATTCCG....	CGGGATA.T.G..
<i>S. mizutae</i>CGGATCCG..G...CTGC	TTTCCG....	CGGGA.G NG	.C.....CTG
<i>R. leguminosarum</i>	..A.GCAGGCTTGTG	C...T...T	G..C.TTCG.G	AG...G....	..C..TC.AGA..C..	...TGG....
<i>B. japonicum</i>	.C.A.TGAGGCTTCAG	C...T...GA..CC.TTAC.G	GG...G....	..C.CCG.A.	...C...C..	...CTG....
<i>C. crescentus</i>	.C.A.TCAGGCTTGAG	C...T...G	G..CC.TTCG.G	GG...G....	..C.CCATTG	..GC...C..	...CTG....

201

250

291

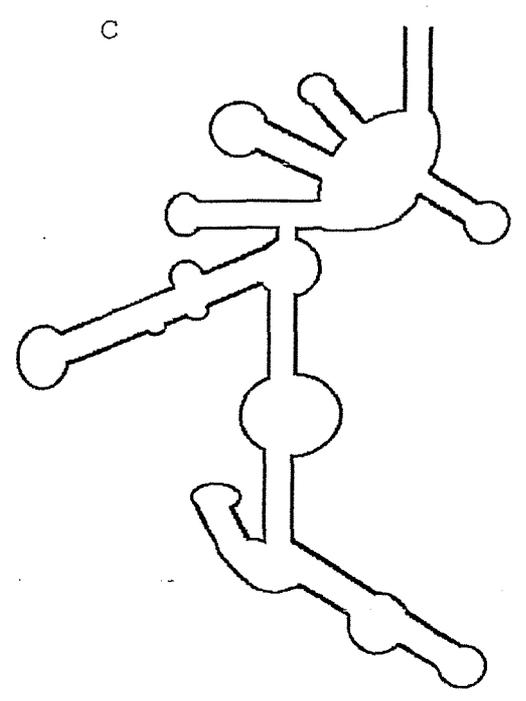
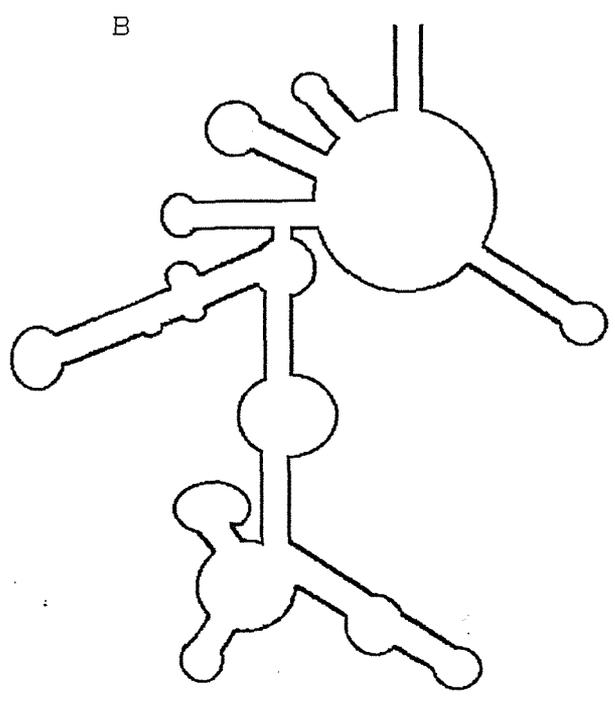
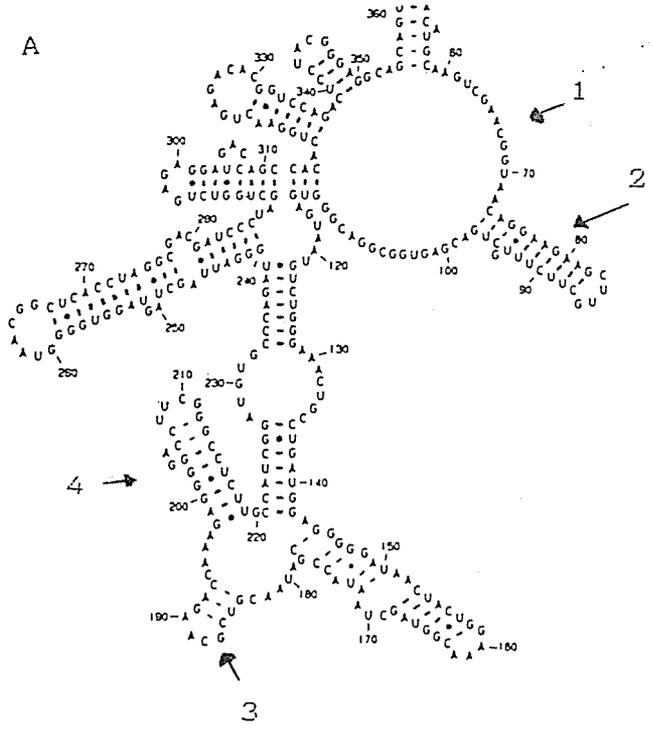
<i>S. multivorum</i>	GCTAGTTGGT	AGGGTAACGG	CCTACCAAGG	CGACGATGTC	TAGGGGCTCT	GAGAGGAGAA	TCCCCACAC	TGGTACTGAG	ACACGGACCA	G
MF200
<i>S. thalpopophilum</i>	R.....	..Y..N...NNN...
<i>S. mizutae</i>	...N.....	G.....	..C..N...NN.....
<i>R. leguminosarum</i>	G.....A..CCA	...CT.G...TG.	..AG.....T	..G.....C...A
<i>B. japonicum</i>	GA.....T..	..TC.....CAG	...CT.G...TG.	..AG.....T	..G.....C...A
<i>C. crescentus</i>	GA.....A..	..TC.....CAG	...CT.G...TG.	..AG.....T	..G.....C...A

Figure 14. SECONDARY STRUCTURE MODELS OF PART OF THE EUBACTERIAL 16S rRNA MOLECULE.

(A) *Escherichia coli*. Secondary structure model from position 53 to 358 (taken from Stackebrandt and Goodfellow, 1991). Numbered arrows indicate areas of greatest divergence compared to the other species shown.

(B) *Sphingobacterium multivorum*. Schematic 2D representation derived from the sequence in figure 13.

(C) *Rhizobium leguminosarum* bv trifolii ATCC14480. Schematic 2D representation derived from published sequence data.



2.2 Transfer of the Co-integrate Plasmid pPN1 to *Caulobacter*.

Conjugation between *E. coli* strain PN200 carrying the co-integrate plasmid pPN1 and *Caulobacter* strain MCDF23 was carried out on artificial media using the membrane filter technique. Ronson and Scott (1982) obtained the 770 Mda pPN1 by co-integrating the Sym plasmid from *Rhizobium leguminosarum* bv trifolii strain NZP514 (pRtr514) with the broad-host-range plasmid R68.45. The plasmid confers neomycin resistance to its host.

2.2.1 Maintenance of pPN1 by Transconjugant *Caulobacter*.

TY.Neo.Rif media was used to select against the Rif^S Neo^R *E.coli* PN200 donor strain and the Rif^R Neo^S *Caulobacter* recipient. Only transconjugant *Caulobacter* carrying pPN1 were able to grow. The plasmid was transferred at frequencies of 10⁻⁵ to 10⁻⁴. After 6 rounds of single colony purification, *Caulobacter* strain MCDF100 appeared able to stably maintain the plasmid, as confirmed by Eckhardt gel analysis.

2.2.2 Eckhardt Gel Analysis of *Caulobacter* Transconjugant MCDF100

To confirm that the co-integrate plasmid pPN1 had been transferred to the *Caulobacter* strain MCDF23, plasmids present in the transconjugant were analysed by the Eckhardt method. Figure 15 shows *E.coli* strain PN200 carrying pPN1 (lane 1). *Caulobacter* strain MCDF23 does not carry mega plasmids and is the negative control (lane 3). Lane 2 confirms that *Caulobacter* transconjugant strain MCDF100 carried the pPN1 plasmid from *E.coli* PN200.

2.2.3 Plant Nodulation by *Caulobacter* Transconjugant MCDF100

Colonies arising on TY.Rif.Neo plates were used to inoculate clover seedlings growing on nitrogen deficient media (Throntons Agar). After 8 weeks 3 plants out of 10 had a tumour-like growth (1 mm in length) on the roots (Figure 16). Examination of the growth showed zones of degeneration (Figure 17). Attempts to isolate the growth occupants from plants inoculated with *Caulobacter* strain MCDF100 were unsuccessful.

2.2.4 Microscopic Examinations Root Growths

The growths formed by transconjugant *Caulobacter* strain MCDF100 were examined by electron microscopy. The structure did not appear to be invaded by bacteria; however, a few bacteria were found within the intercellular spaces of the outermost cells of the structure. The plant cells in this region had degenerated (Figure 18 and Figure 19). Plazinski and Rolfe (1985) reported similar results with *Pseudomonas* strain PAO5.

Figure 15. ECKHARDT GEL ANALYSIS OF TRANSCONJUGANT
CAULOBACTER STRAIN MCDF100.

Lane 1, *E.coli* strain PN200 carrying pPN1;

Lane 2, *Caulobacter* transconjugant strain MCDF100;

Lane 3, *Caulobacter* recipient strain MCDF23.

1 2 3

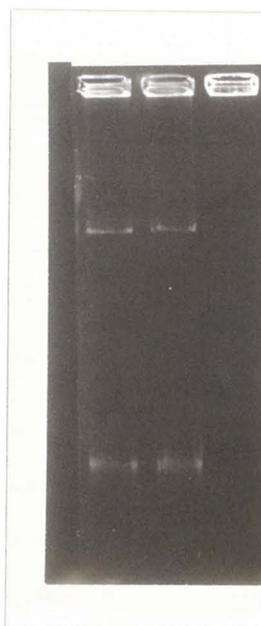


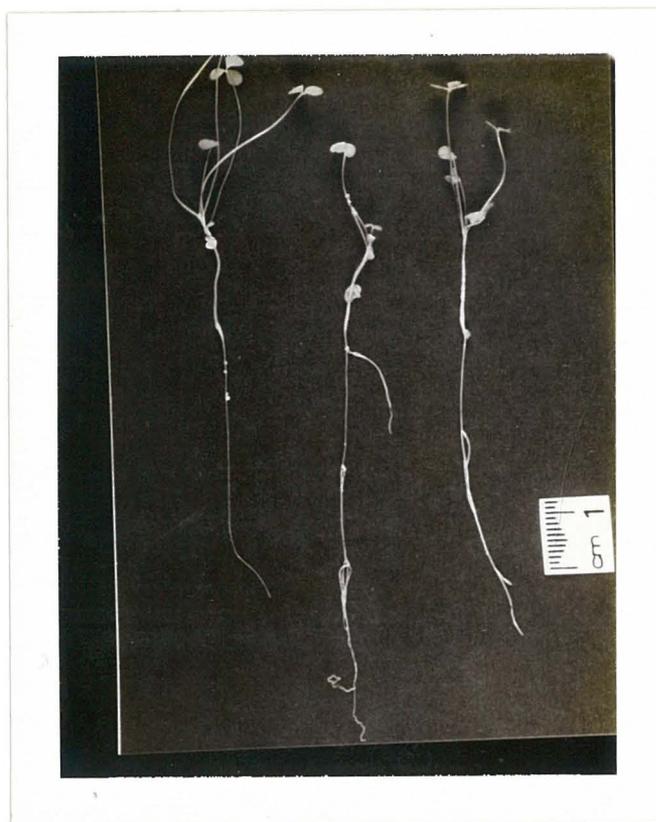
Figure 16. PLANT NODULATION BY TRANSCONJUGANT
CAULOBACTER STRAIN MCDF100

Nodule formation on white clover (*Trifolium repens*)
seedlings thirty days after inoculation.

(A) Plants inoculated with (left to right): *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5; *Caulobacter* transconjugant strain MCDF100 (*E.coli* X MCDF23), *E.coli* strain PN200.

(B) Plants inoculated with (left to right): *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5; *Caulobacter* transconjugant strain MCDF100 (*E.coli* X MCDF23).

A



B

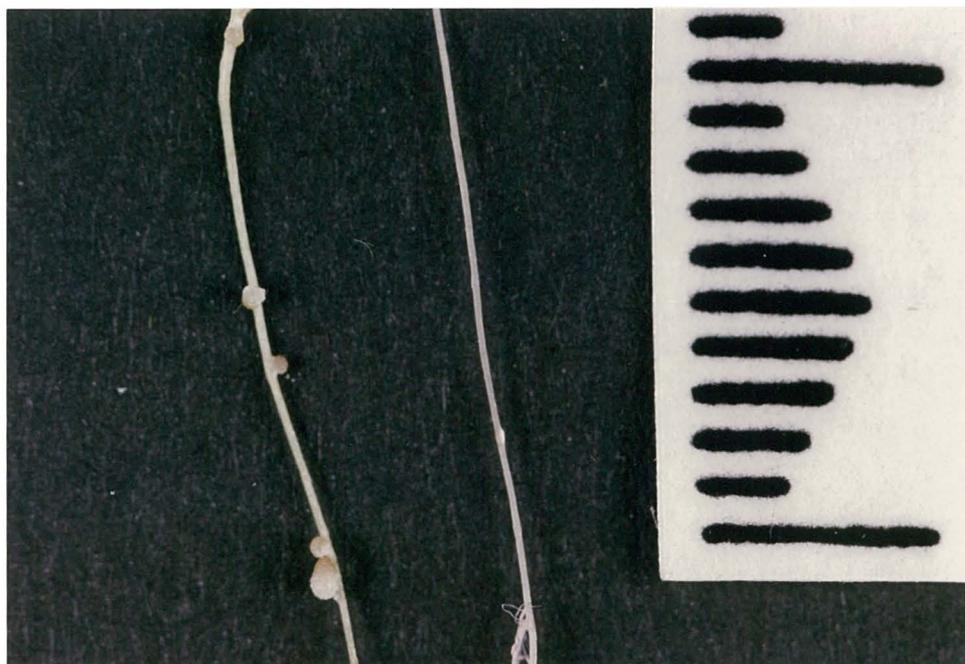


Figure 17. ZONES OF DEGENERATION ON WHITE CLOVER ROOTS
INOCULATED WITH TRANSCONJUGANT CAULOBACTER
STRAIN MCDF100

White clover (*Trifolium repens*) seedlings thirty days after inoculation.

Bar=1 mm

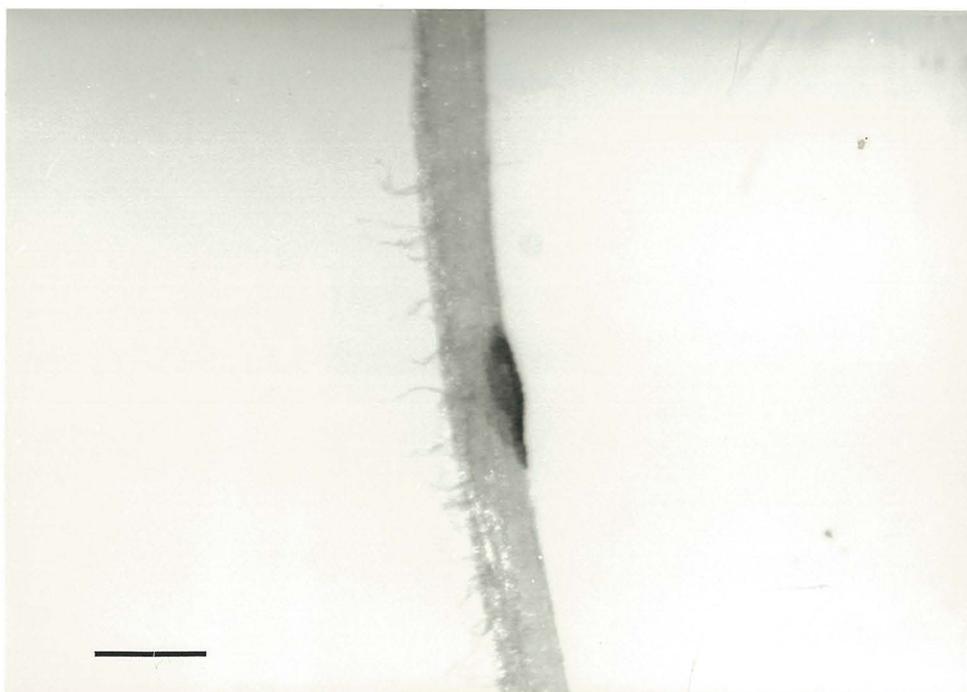
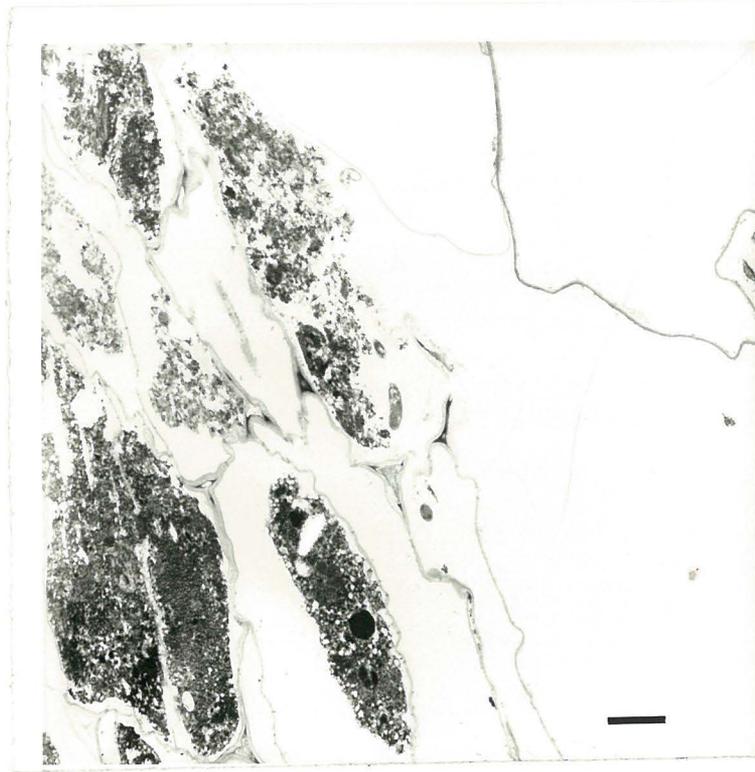


Figure 18. SECTIONS FROM WHITE CLOVER (*TRIFOLIUM REPENS*)
NODULE-LIKE STRUCTURES FORMED BY
TRANSCONJUGANT *CAULOBACTER* STRAIN MCDF100

(A) Electron microscope section of a nodule like growth formed by transconjugant *Caulobacter* strain MCDF100 (X 4000). Bar = 1 μ m.

(B) Electron microscope section of a nodule like growth formed by transconjugant *Caulobacter* strain MCDF100 (X 16100). Bar = 1 μ m.

A



B

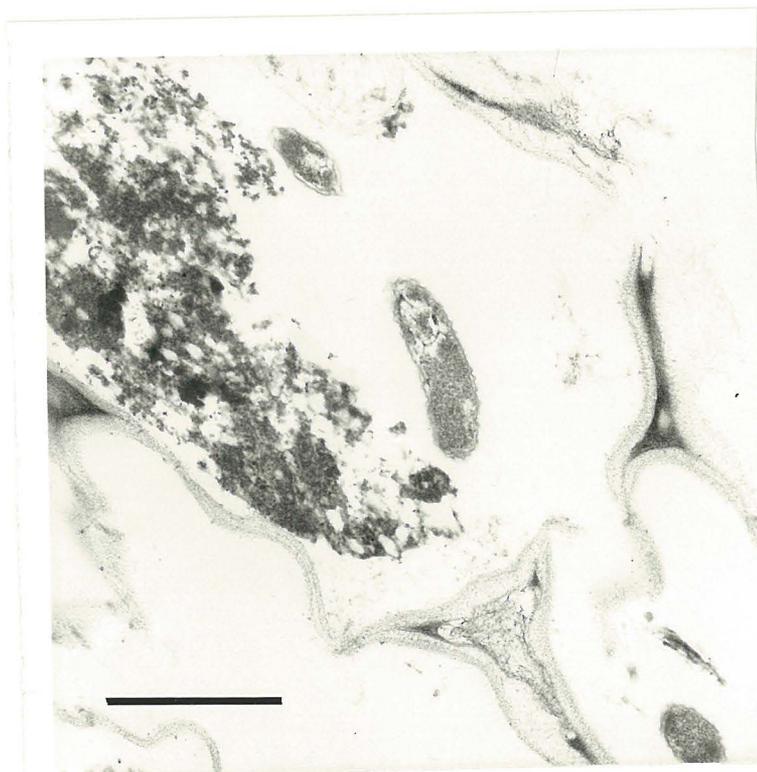
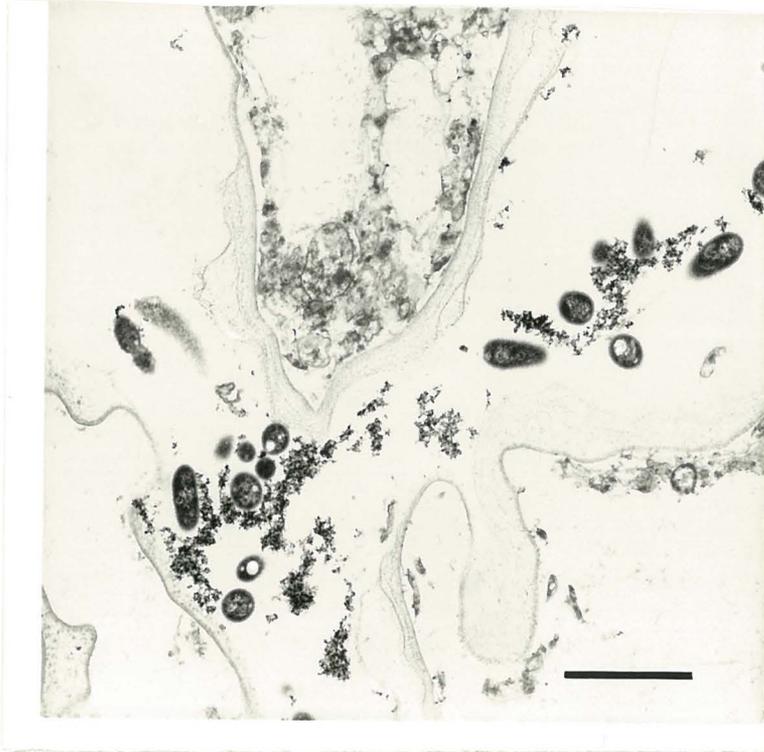


Figure 19. SECTIONS FROM WHITE CLOVER (*TRIFOLIUM REPENS*)
NODULE-LIKE STRUCTURES FORMED BY
TRANSCONJUGANT *CAULOBACTER* STRAIN MCDF100

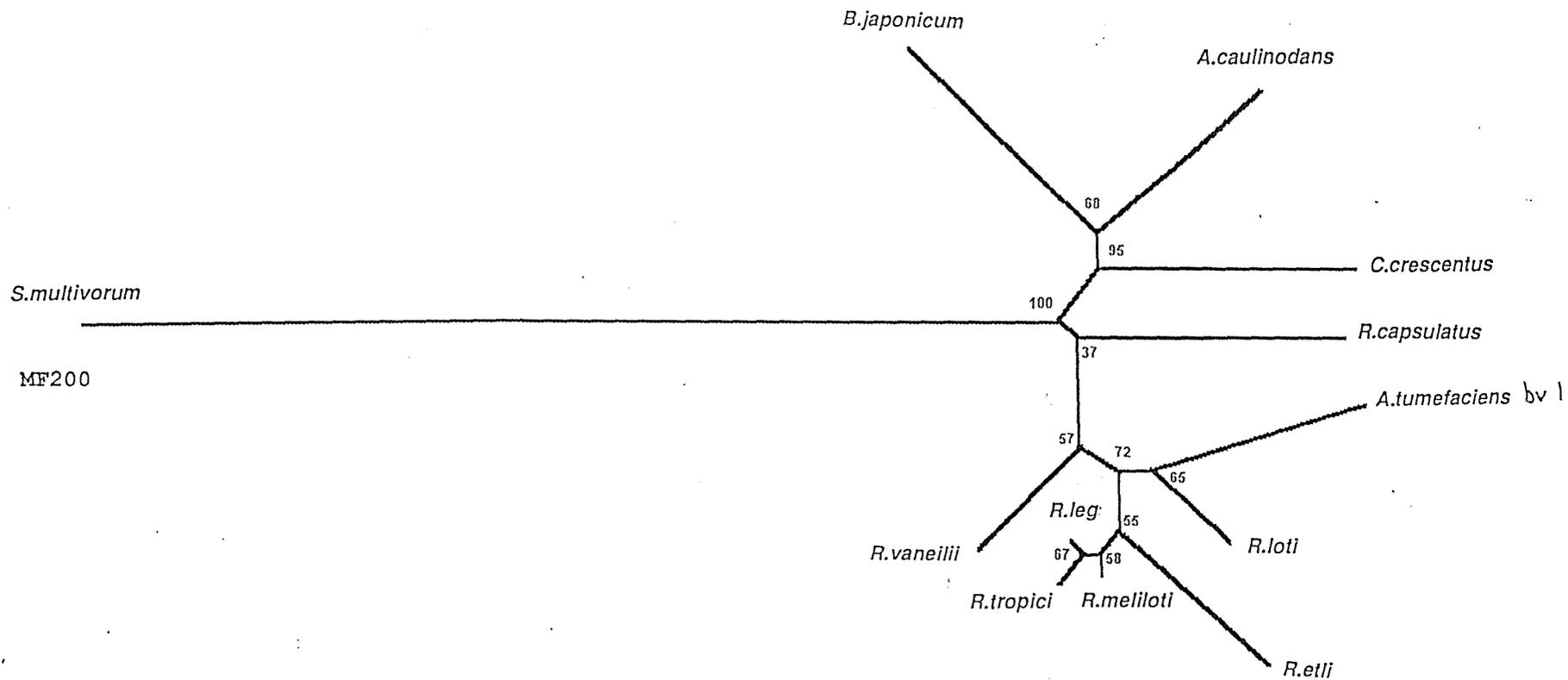
Electron microscope section of a nodule like growth formed by
transconjugant *Caulobacter* strain MCDF100 (X 9275). Bar = 1 μm .



3. PHYLOGENETIC ANALYSIS OF BACTERIA THAT CAN EXPRESS SYMBIOTIC PLASMID GENES

A phylogenetic tree was constructed from the Jukes-Cantor distances of the aligned sequences of bacteria used in this study and suitable reference strains (Figure 20). *Rhizobium loti* clusters with the other species of *Rhizobium* but on a different branch. *Caulobacter*, a member of the budding and prosthecate group of organisms, is distantly related to *Rhizobium leguminosarum* biovar trifolii, and *Sphingobacterium* is the most distantly related. It is concluded that the ability to carry or express symbiotic genes is not dependant on having a phylogenetic relationship with *Rhizobium*.

Figure 20. UNROOTED PHYLOGENETIC TREE CONSTRUCTED BY THE NEIGHBOR-JOINING METHOD FROM A DISTANCE MATRIX (WITH JUKES-CANTOR CORRECTIONS).



DISCUSSION

1. Conjugation in sterile soil microcosms.

1.1 Strains used.

Rhizobium leguminosarum bv trifolii strain ICMP2163 is used as a white clover inoculant strain in New Zealand. The symbiotic plasmid was labelled by insertion of the transposon Tn5 which confers neomycin resistance to its host (Rao *et al.*, 1994). The Sym plasmid from strain ICMP2163::Tn5 used in this study was shown to be fully effective for strain PN165, a pSym cured derivative of strain ICMP2163. This demonstrated that the pSym was self-transmissible and that insertion of Tn5 did not affect the expression of nodulation genes. Our laboratory had been investigating the transfer of pSym from New Zealand inoculant strains to native non-nodulating soil isolates. The soil isolate NR40 was identified by its fatty acid profile, a method shown to be reliable (Jarvis and Tighe, 1994), as *Rhizobium loti*. Growth requirements, cell morphology and colony morphology of NR40 on TY agar are consistent with those of *R. loti*, however a detailed study comparable to Segovia *et al.*, (1991) would confirm this identification. The observation that transconjugant NR40 forms ineffective nodules on white clover seedlings would not be surprising for *R. loti* transconjugants.

1.2 Factors affecting soil rhizobia.

A number of factors affect the survival of micro-organisms in soil. Investigators tend to concentrate on factors that lend themselves to study in the laboratory, such as temperature, pH, moisture content and organic matter content (reviewed by Dowling and Broughton, 1986). *Rhizobium* strains vary

in their acid tolerance. In soil, pH not only directly affects the growth of micro-organisms, but also affects the solubility of many cations which may indirectly alter growth patterns. A strain of *Rhizobium fredii* did not survive in soil below pH 5.25 (Richaume, 1989). Dughri and Bottomley (1984) were able to alter the outcome of competition between indigenous rhizobia in the soil by changing the acidity.

Rhizobium leguminosarum bv trifolii strain ICMP2163::Tn5 was able to transfer its Tn5-marked symbiotic plasmid to the pSym deficient *Rhizobium* soil isolate NR40, in sterile soil at a pH greater than 5.5. NR40 was not able to incite root nodule formation but transconjugant bacteria were able to form ineffective nodules on white clover seedlings. The parent strains survived for a maximum of 21 days in soil at pH 5.5 or less. It is concluded that the inoculant strain ICMP2163 would not be suitable for use in acidic soils.

There was a significant increase in the frequency of plasmid transfer in the presence of ryegrass or white clover plants; from 1×10^{-6} to 3×10^{-6} . This may be due to stimulating factors associated with the rhizosphere. Bacteria associated with the rhizosphere will have access to attachment sites, nutrients and minerals at high concentrations and as a consequence, will be metabolically more active than their free-living counterparts. Overall, there will be much greater opportunity for genetic exchange to occur. In sterile Ramiha hill soil at 50% water holding capacity (pH 6.0) with clover seedlings present, the number of transconjugants present per gram of soil increased 10-fold over an 18 day period; from 4 CFU/g to 44 CFU/g. The significance of these results are two-fold. Firstly, Theis *et al.*, (1919a,1991b) have shown that as few as 50 indigenous rhizobia per gram of soil eliminated the inoculum

response to 10^6 to 10^7 rhizobia per seed. The inoculant strain ICMP2163 has the potential to transfer its pSym to indigenous soil bacteria at a high enough frequency to eliminate future inoculum responses. Secondly, in order to get meaningful results, laboratory simulations should be as close to conditions in natural environments as possible. These experiments involved one potential recipient strain. In non-sterile soil there are a great number of species that could be involved, although there is no indication as to how many that may be.

There appeared to be no significant difference in using Ramiha hill soil or Ashurst silt loam soil in the above mentioned experiments. This implies that what happens in one soil type may well occur in others. There may be no need to tailor bacteria for specific soil types if this is true. However, the experiments carried out so far are rather simplistic, looking at a few of the variables associated with soil. Experiments with non-sterile soil, in the manner of Kinkle and Schmit (1991), would be more convincing.

1.2 Significance of plasmid transfer in soil.

Rhizobium strains lacking symbiotic plasmids in soil may act as biological sinks for the symbiotic plasmids from inoculant strains. Strains of bacteria exist that fail to satisfy Jordan's definition but are clearly rhizobia lacking the symbiotic plasmid (Scott and Ronson, 1982; Soberon-Chavez and Najera, 1988; Segovia *et al*, 1991). This could explain the temporal loss of inoculant strains in the field and the appearance of indigenous rhizobia where there was no evidence of previous *Rhizobium* populations (Roughley *et al.*, 1976).

It is now well established that self-transmissible symbiotic plasmids can be exchanged between strains of *Rhizobium* on artificial media and there is

evidence that this exchange occurs in the natural field populations. Two independent studies, one involving *Rhizobium leguminosarum* bv trifolii (Schofield *et al.*, 1987), and the other involving *Rhizobium leguminosarum* bv viceae (Young and Wexler, 1988), have reported that similar symbiotic plasmids could be found in genetically unrelated isolates. Kinkle and Schmit (1991) observed the transfer of the symbiotic plasmid pJB5JI between strains of *Rhizobium* in sterile and non-sterile soil. It is concluded that the improved use of *Rhizobium* seed inoculants will require further study of plasmid transfer mechanisms between the inoculant bacteria and the other soil bacteria.

2. Expression of Symbiotic Genes by Non-*Rhizobium* Species.

The recognition that *Rhizobium* strains lacking Sym plasmids exist in soil has meant that soil is often screened for new *Rhizobium* strains. Isolates are usually selected on the basis of having particular growth characteristics and colony morphology on solid media compared to known reference strains. It was in this manner that NR40 was isolated. It can take some time for an isolate to be completely characterised and accurately identified. One of the soil isolates used in our laboratory (NR64) was thought to be a *Sphingobacterium* (Bianchin, 1989) but was later shown to be a strain of *Rhizobium*. A strain of *Sphingobacterium* was obtained for further study. The approach used was to cross *Rhizobium leguminosarum* bv trifolii strain ICMP2163::Tn5 with a number of known non-*Rhizobium* strains. In this manner it was hoped to gain some insight as to the distance that symbiotic genes could travel.

2.1 Transfer of pSym to *Sphingobacterium multivorum*.

Sphingobacterium multivorum is an organism that can be found in soil. It is able to grow agar at 37 °C, and is sometimes isolated from clinical samples. Table 1 (Materials and Methods) outlines some of the differences from *Rhizobium*. As strain NZRM1228 was spontaneously resistant to neomycin it was not possible to use the Tn5 antibiotic resistance marker to select for transconjugants retaining pSym on artificial media. The symbiotic plasmid was lost from nodule isolates after 2 - 3 rounds of single colony purification. Luria Rif.Neo.Str agar ensured that only the *Sphingobacterium* would grow for use in plant inoculation tests. The use of white clover seedlings to select for transconjugants and the isolation of the bacteria from the root nodules was the most effective way to obtain enough bacteria for study (Table 3). This suggests that as long as the appropriate selection pressure is applied the *Sphingobacterium* transconjugants would continue to nodulate other clover seedlings. Comparison of the electron micrographs in Figure 10 indicates that the nodule occupant of the MF100 plant shown in Figure 9 was different from the *Rhizobium* donor strain. Total genomic digest profiles of the nodule isolate were the same as strain NZRM1228 and Southern blots probed with *nodA* DNA confirmed that the nodule isolate contained symbiotic genes. 16S rDNA sequence analysis identified the nodule isolate as *Sphingobacterium multivorum* recipient strain NZRM1228.

This is the first report of the spontaneous transfer of the symbiotic plasmid from an inoculant strain of *Rhizobium leguminosarum* bv *trifolii* to *Sphingobacterium multivorum*.

2.2 Expression of pSym Genes in *Caulobacter*.

E. coli strain PN200, carrying the co-integrate plasmid pPN1, and *Caulobacter* strain MCDF23 was crossed in an alternative method to test the ability to express Sym plasmid genes. Scott and Ronson (1982) obtained the 770 Mda pPN1 by co-integrating the Sym plasmid from *Rhizobium leguminosarum* bv trifolii strain NZP514 (pRtr514) with the broad-host-range plasmid R68.45. The plasmid confers neomycin resistance to its host. *Caulobacter* belongs to the budding and prosthecate group of organisms and is found in soils and waterways. A transconjugant *Caulobacter* isolate MCDF100 containing the co-integrate plasmid pPN1 (Scott and Ronson, 1982) was able to induce a tumour-like growth within 12 days of inoculation onto sterile 3 day old white clover seedlings. Attempts to isolate the nodule occupants were unsuccessful. Examination by electron microscope showed that the growth did not appear to be invaded by bacteria. A few bacteria were found within the intercellular spaces of the outermost cells of the structure and the plant cells in this region had degenerated (Figure 18 and 19). Plazinski and Rolfe (1985) reported similar results with *Pseudomonas* strain PAO5. Expression of the Sym plasmid genes carried on pPN1 may have been affected by the RP4 *tra* genes in the R68.45 section of the co-integrate plasmid (Hynes and O'Connell, 1988).

This is the first report of *Caulobacter* carrying symbiotic plasmid genes and causing tumour-like growths on white clover seedlings.

3. Consequences for Taxonomy.

The ability to nodulate leguminous plants is regarded as the characteristic function of the genus *Rhizobium* with nitrogen fixation a normal but not essential consequence of nodulation (Jordan, 1984). There are a number of *Rhizobium* species that carry the nodulation and nitrogen fixation genes on plasmids which may be transferred by conjugation. Soil bacteria other than rhizobia could be involved in the dissemination of symbiotic genes, perhaps acting as temporary hosts before passing the genes back to an appropriate *Rhizobium* strain. The existence of species of soil bacteria, outside of *Rhizobium*, capable of expressing Sym plasmid genes may have been overlooked because of the screening method or media used. Possible candidates are *Agrobacterium* (Hooykass *et al.* 1981; Kondorosi *et al.*, 1982; O'Connell *et al.*, 1987), *Enterobacter* (Dohler and Klingmuller, 1988), *Pseudomonas* and *Lignobacter* (Plazinski and Rolfe, 1985), *Caulobacter*, and *Sphingobacterium multivorum*. Sequence data for some of these other organisms was unavailable for comparison but the phylogenetic tree is shown in Figure 20. *Caulobacter* is distantly related to *Rhizobium*, and *Sphingobacterium* is the most distantly related. Schematic 2D models of the 16S RNA molecule shown in Figure 14 indicate that *Sphingobacterium* is quite unrelated to *Rhizobium*. It is concluded that the ability to express or carry symbiotic plasmid genes is not dependant on having a phylogenetic relationship to *Rhizobium*. Clearly, it is undesirable for there to be this confusion in a classification scheme. A number of authors have suggested revised descriptions of the genus *Rhizobium* and *Agrobacterium* based on 16S rDNA sequences and DNA/DNA homology studies (Willems and Collins, 1993; Sawada *et al.*, 1993; Yanagi and Yamasato, 1993).

4. Conclusion.

This study reports the isolation of a strain of *Sphingobacterium multivorum* that is able to nodulate white clover seedlings and would fit Jordan's definition of a *Rhizobium* species. It is concluded that taxonomic relationships based on characteristics carried on plasmids may not reflect real relationships amongst micro-organisms. It would be preferable to identify known *Rhizobium* species from their fatty acid profiles or specific DNA probes and define new species in terms of their 16S rRNA gene sequence and DNA-DNA relatedness with recognised reference strains.

Further work needs to be done in looking at the factors affecting pSym transfer in soil, preferably using non-sterile soil. Non-*Rhizobium* strains should continue to be tested to see if they are able to receive the Sym plasmid from New Zealand inoculant strains. This may result in new inoculant strains becoming available with unique characteristics.

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