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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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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 *nod*A 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 *nod*A 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 postions 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 x 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->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 *nod*AB 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^{o}C and 49 - 94% and 1.3 - 7.0^{o}C respectively. $\Delta T_{m(e)}$ is a statistic which expresses the base sequence homology in the fraction of DNA which hybridises. Each 1^{o}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).

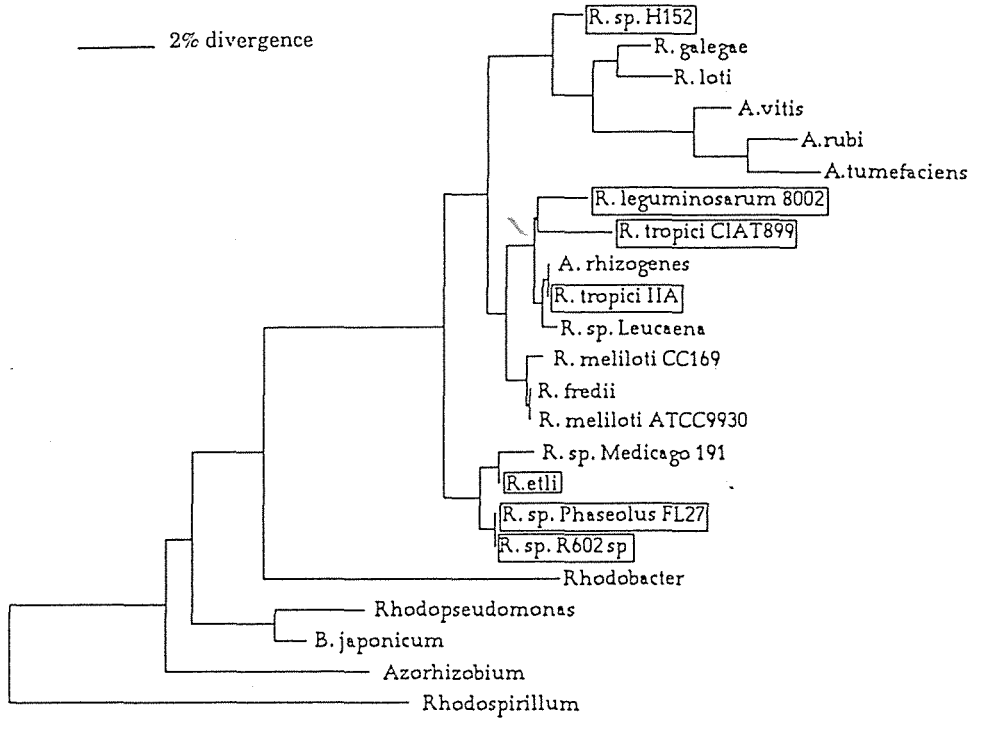
Jarvis et al. (1989) suggested that *Rhizobium* classification should be defined in terms of DNA-DNA or rRNA-DNA homology to accepted reference bacteria. In addition, it may be useful to use the 16S ribosomal DNA sequence to determine what is a 'true' rhizobia. PCR-RFLP analysis has been described as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


2% divergence
R. sp. H152
R. galegae
R. loti
A.vitis
A.rubi
A.tumefaciens
R. leguminosarum 8002
R. tropici CIAT899
A. rhizogenes
R. tropici IIA
R. sp. Leucaena
R. meliloti CC169
R. fredii
R. meliloti ATCC9930
R. sp. Medicago 191
R.etli
R. sp. Phaseolus FL27
R. sp. R602 sp
Rhodobacter
Rhodopseudomonas
B. japonicum
Azorhizobium
Rhodospirillum

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 plamid (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

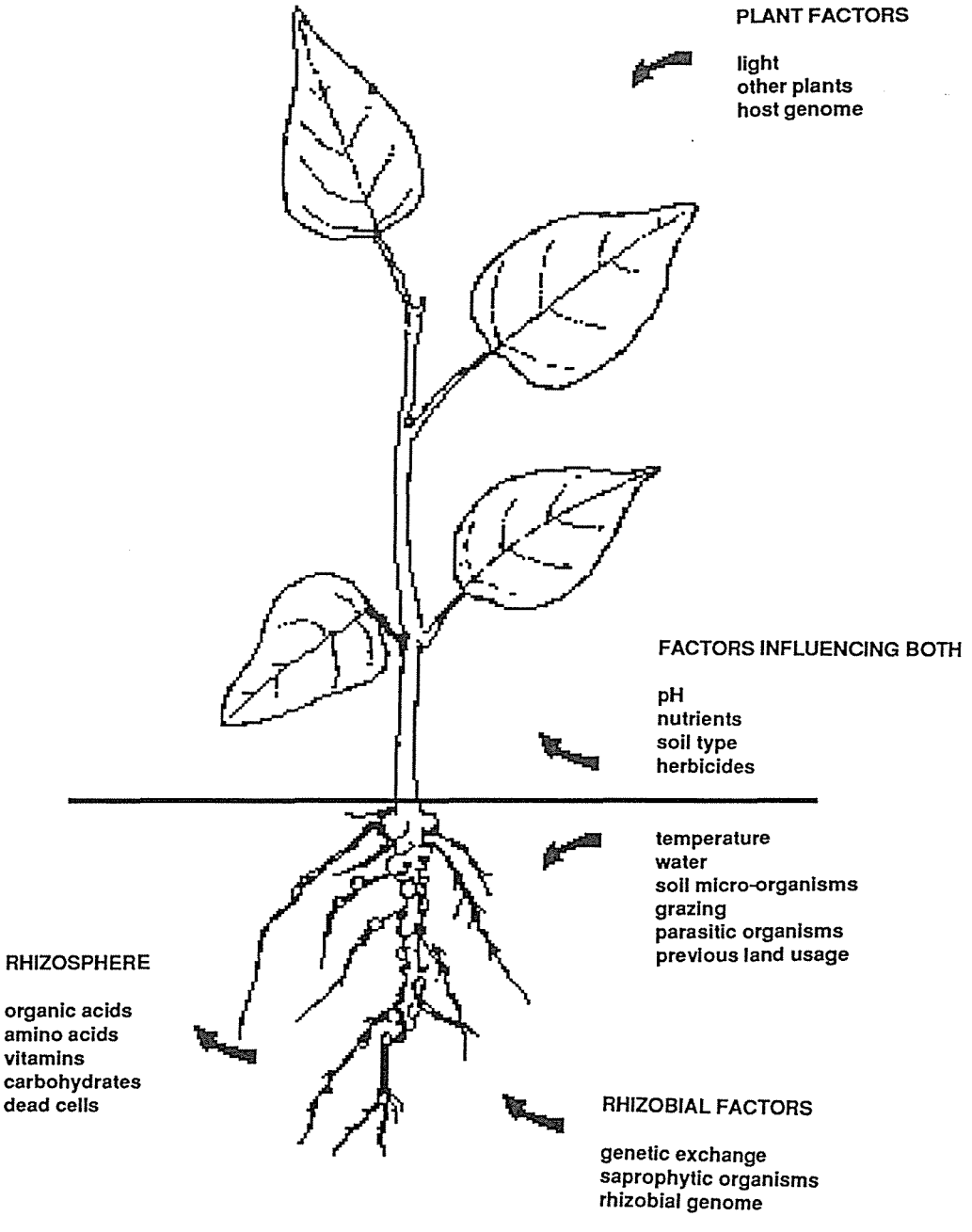
inoculant strain may need to be supplied at 1000X the level of the indigenous *Rhizobium* population in order to form 50% of the nodules. For the inoculation industry this may yield unexpected benefits if it were possible to isolate indigenous soil bacteria able to nodulate and fix nitrogen better than 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 phosporous 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, epiphtyic 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).


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

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*.