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DNA HOMOLOGY WITHIN THE *RHIZOBIACEAE*

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

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1984

ABSTRACT

The relationship of rhizobia that nodulate *Galega officinalis* to the known species of *Rhizobium* and *Bradyrhizobium* was investigated. Similarly, the recently discovered fast-growing soybean nodulating group of rhizobia was studied. Both groups were investigated using DNA:DNA hybridization as well as nodulation on legumes and phage-typing.

The *Galega* nodulating rhizobia were found to form a distinct DNA homology group. The mean relative homology of 11 strains of *Galega* nodulating rhizobia with the reference strains gal 1 and gal NW 3, which effectively nodulate *Galega officinalis*, was significantly higher than the mean relative homology of other groups of rhizobia.

The *Galega* rhizobia only nodulated *Galega officinalis* and formed a distinct phage-typing group in agreement with the DNA homology results. These rhizobia therefore appear to form a unique taxonomic group within the genus *Rhizobium*.

The fast-growing soybean nodulating rhizobia formed a distinct DNA homology group with at least two subgroups. The mean relative homology of 11 of these strains with the reference strains USDA 208 and USDA 191 which nodulate *Glycine max*, was significantly higher than the mean relative homology of other groups of rhizobia. Low DNA homologies were found between the fast-growing soybean strains and *Bradyrhizobium japonicum* ATCC 10324.

The fast-growing soybean nodulating rhizobia nodulated *Glycine max* and formed ineffective nodules on *Lotus pedunculatus*. None of these strains were lysed by the bacteriophages used in the study, but as yet, no bacteriophage specific for this group of rhizobia has been isolated. The fast-growing soybean nodulating rhizobia were concluded to be taxonomically distinct from other species of *Rhizobium* .

The thermal stability of reassociated DNA duplexes was examined for both the *Galega* and fast-growing soybean rhizobia and further indicated the uniqueness of both groups.

The use of colony hybridization as a means of identifying different strains of *Rhizobium* was investigated and was found to be useful in distinguishing between genetically distinct rhizobia and to identify rhizobia within root nodules.

ACKNOWLEDGEMENTS

I wish to thank the Department of Microbiology and Genetics, Massey University, for the facilities and the opportunity for this research project.

In particular I would like to thank my supervisor, Dr B.D.W. Jarvis for his constant interest and encouragement.

I would also like to thank:

Dr A.W. Jarvis (D.R.I.) for the demonstration of the colony hybridization methods and for the use of their facilities and the gift of *Streptococcus lactis* H1 DNA.

Miss C.P. Liddane (D.S.I.R.) for the use of the glasshouse facilities for plant nodulation testing.

Dr H.H. Keyser (U.S. Department of Agriculture) for the gift of eleven strains of fast-growing soybean nodulating rhizobia.

Dr J.J. Patel (D.S.I.R.) for the gift of bacteriophages ϕD2, ϕ2037/1 and ϕgal 1/R.

Mrs V. Fieldsend for the typing.

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INTRODUCTION

1. Significance of the genus *Rhizobium*

Bacteria belonging to the genus *Rhizobium* form nodules on the roots of legumes (Jordan and Allen 1974). Members of this genus are gram negative aerobic non-sporing rods. They occur free-living in the soil or in the rhizosphere of plants.

Rhizobia in the nodules fix atmospheric nitrogen, converting it into a form available to the plant and thus are of great importance agronomically. Biological nitrogen fixation is receiving increasing attention, since the production of nitrogenous fertiliser by chemical means is energy demanding. Of all the nitrogen-fixing bacteria, the genus *Rhizobium* is the most important. This genus is estimated to carry out 50-70% of world biological nitrogen fixation (Quispel 1974). Many aspects of agriculture, especially pastoral farming, are dependent on this process for the maintenance of productivity. MacKinnon *et al* (1975) estimated that pastures in New Zealand used 800,000 tonnes of fixed nitrogen per annum and that 97% of this is biologically fixed by rhizobia in association with leguminous plants.

2. Classification of *Rhizobium* by cross-inoculation groups

Species designation within the genus *Rhizobium* as presented in the eighth edition of Bergey's Manual of Determinative Bacteriology (Jordan and Allen 1974) is based on the host specificity of the bacteria. The plant hosts for a given species comprise a cross-inoculation group. A cross-inoculation group is defined as a group of plants within which the root nodule organisms are mutually interchangeable (Fred *et al* 1932). The Bergey classification recognises

six such cross-inoculation groups and hence six species of *Rhizobium* are described (Table I).

This classification has long been considered unsatisfactory for a number of reasons. Many legumes are not included in the six cross-inoculation groups which define the recognized species (Dixon 1969). The cross-inoculation groups are not mutually exclusive and strains of *Rhizobium* are found which can nodulate legumes from more than one cross-inoculation group (Wilson 1944, Graham 1964). The slow-growing group of rhizobia, known as the cowpea rhizobia, can not be classified by this method due to their ability to nodulate a wide range of legume hosts (Allen & Allen 1940, Norris 1956). Rhizobia can lose the ability to effectively nodulate specific legumes (Labandera 1975) and then become unclassifiable using cross-inoculation groups. The ability to nodulate specific legumes can be transferred between different species of *Rhizobium*(Higashi 1967, Johnston *et al* 1978, Brewin *et al* 1980).

Plasmid control of symbiotic characteristics has been suggested (Higasti (1967), Dunican & Cannon (1971), Nuti *et al* (1977). Host specificity genes in certain strains of *R. leguminosarum* (Johnston *et al* 1978, Brewin *et al* 1980, Buchanan-Wollaston *et al* 1980) and *R. trifolii* (Scott & Ronson 1982) and *R. phaseoli* (Beynon *et al* 1980) have been shown to be borne on specific plasmids. Plasmid control of nodulation on legumes has important consequences for the classification of rhizobia by cross-inoculation groups. Both the possibility of the loss of plasmids, on the transfer of plasmids between different strains of rhizobia, would make this method of classification untenable.

TABLE I: CLASSIFICATION OF RHIZOBIUM BY CROSS-INOCULATION GROUPS

Rhizobium leguminosarum	nodulates	*Lathyrus* (sweet peas) *Pisum* (garden peas) *Vicia* (vetches) *Lens* (lentils)
Rhizobium trifolii	nodulates	*Trifolium* (clovers)
Rhizobium phaseoli	nodulates	*Phaseolus* spp. (beans)
Rhizobium meliloti	nodulates	*Melilotus* (sweet clover) *Medicago* (lucerne) *Trigonella* (leguminous herbs)
Rhizobium lupini	nodulates	*Lupinus* (lupines) *Ornithopus* (birdsfoot)
Rhizobium japonicum	nodulates	*Glycine* spp. (soybeans)

3. Alternative approaches to the classification of the genus *Rhizobium*

Norris (1965 examined 717 strains of rhizobia isolated from 278 species of legumes and concluded that they could be divided into two groups: those that grew fast and produced acid on laboratory media and those that grew slowly and produced a neutral or alkaline reaction. Martinez-De Drets *et al* (1972, 1974) distinguished the fast and slow-growing rhizobia on the basis of enzymatic differences. Fast-growing strains were found to contain an invertase able to metabolise sucrose, whereas both this enzyme and sucrose phosphorylase were absent from slow-growing strains. Only strains of the fast-growing group of *Rhizobium* were found to contain a nicotinamide adenine dinucleotide phosphate (NADP)-6-phosphogluconate dehydrogenase.

Numerical analysis was applied to the root nodule bacteria by Graham (1964). He concluded that *R. trifolli*, *R. leguminusarum* and *R. phaseoli* should be consolidated into a single species, *R. leguminosarum* . *R. meliloti* however, should be maintained as a single species. He proposed that *Agrobacterium radiobacter* and *A. tumifaciens* be united and included as *R. radiobacter* within the genus *Rhizobium*. Slow-growing strains of root nodule bacteria would be classified within the genus *Phytomyxs*.

t'Mannetje (1967) reanalysed Graham's data using different sorting techniques. He concluded that *Agrobacterium radiobacter* and *A. tumefaciens* be combined but not included in the genus *Rhizobium*. Slow-growing root nodule bacteria should be retained as a single species *R. japonicum* within the genus *Rhizobium*.

Moffett & Colwell (1968) applied numerical analysis to the *Rhizobiaceae*. Their results agreed with those of Graham (1964). They however, included *Agrobacterium rhizogenes*

in the species *Rhizobium radiobacter* and added a fourth species *R. rubi* to the genus *Rhizobium*. Studies by White (1972) also agreed with these conclusions.

Phage-typing has been used to classify rhizobia. Staniewski (1968) divided 230 strains of *Rhizobium trifolium* , *R. leguminosarum* , *R. phaseoli* and *R. meliloti* into three phage-typing groups. Group 1 consisted of strains of *R. trifolium* , *R. leguminosarum* and *R. phaseoli* . Group 2 included some strains of *R. trifolii* and *R. leguminosarum*. Group 3 included *R. meliloti* strains. Hence *R. meliloti* formed a distinct group, distinguishable by phage-typing from the three other species which were indistinguishable by phage-typing .

Serological methods have been used in the classification of rhizobia. The technique of internal antigen analysis has been used by Vincent & Humphrey (1970) who found a closer relationship between *R. trifolii* , *R. leguminosarum* and *R. phaseoli* than between these three species and *R. meliloti* . There was an evident relationship between *R. meliloti* and the agrobacteria and a lack of relationship between these three groups and the slow-growing rhizobia.

Two-dimensional polyacrylamide gel electrophoresis has been used by Roberts *et al* (1980) to classify rhizobia. The slow-growing rhizobia were shown to be distinct from the fast-growers. Of the fast-growing rhizobia, strains of *R. leguminosarum* and *R. trifolii* formed a distinct group as did strains of *R. meliloti*.

The problem with all of the above approaches to the classification of rhizobia is they only examine a limited number of characters, which don't necessarily represent the actual amount of genetic information which is common to different strains.

Overall genetic similarity has been measured by the comparative study of rhizobial DNA. On the basis of the %GC content of DNA, De Ley & Rassel (1965) divided rhizobia into two groups. The fast-growing, peritrichously flagellated group had a low %GC content in the range 58.6 - 63.1% and was comprised of *R. leguminosarum* and *R. meliloti*. The slow-growing, subpolarly flagellated strains ranged from 62.8 to 65.5% in GC content. One species was proposed for this group, tentatively named *R. japonicum*.

Heberlein *et al* (1967) studied the relationship between *Rhizobium*, *Chromobacterium* and the genus *Agrobacterium* by the use of DNA-agar hybridization. On the basis of DNA homology, *Agrobacterium rubi*, *A. tumefaciens* and *A. radiobacter* were indistinguishable. Six other distinct genetic groups were found: *Rhizobium rhizogenes*, *R. leguminosarum*, *R. meliloti*, *R. japonicum*, *Chromobacterium* spp and *Agrobacterium pseudosugae* strain 180.

Gibbons and Gregory (1972) studied the relatedness of *Rhizobium* and *Agrobacterium* species by three methods of nucleic acid hybridization: DNA/DNA and DNA/RNA hybridization on membrane filters and by a spectrophotometric technique. They found a close relationship between *R. leguminosarum* and *R. trifolii* and in agreement with Graham (1964) concluded that these two species should be combined into a single species. However, they concluded that *R. phaseoli* should remain as a separate species. *R. lupini* and *R. japonicum* were found to be closely related which is in agreement with the conclusions of Graham (1964), Hebertein *et al* (1967) and Moffett & Colwell (1968).

Hybridization studies using the hydroxyapatite method have been carried out by Jarvis *et al* (1980) and Crow *et al* (1981). Jarvis *et al* (1980) determined the extent of DNA homology among 27 strains of *R. trifolii*, 4 strains of *R. leguminosarum* and 4 strains of *R. phaseoli*. *R. leguminosarum* and *R. trifolii* were found to be genetically

similar and it was suggested that they should be combined into a single species *R. leguminosarum* Frank with biovars designated by the species name of the legume which they effectively nodulate. They found that the average relatedness of *Rhizobium* strains from *Phaseolus vulgaris* to those from clover was 46% and concluded that *R. phaseoli* should remain as a separate species until examined in more detail.

Crow *et al* (1981) studied DNA homologies between 113 strains of fast-growing acid producing rhizobia and seven reference *Rhizobium* strains. These strains were divided into four homology groups. Group 1 comprised strain of *R. trifolii*, *R. leguminosarum* and *R. phaseoli* all consolidated under the name *R. leguminosarum*. Group 2 comprised *Rhizobium* strains obtained from *Coronilla varia* and some strains from *Onobrychis viciifolis* and *Sophora* spp. Group 3 comprised *R. meliloti* and Group 4 the fast-growing *Lotus* rhizobia which nodulated a variety of hosts including *Lotus corniculatus*, *Lotus tenuis* and *Lupinus densiflorus*. A total of nine fast-growing stains could not be classified within these groups. Low DNA homologies (< 10% homology) were found between the fast-growing and the slow-growing rhizobia supporting the view that these two groups are distinct enough to warrant dividing the root nodule bacteria into two genera.

Jarvis *et al* (1982) found the fast-growing *Lotus* rhizobia were distinct from other rhizobia by DNA homology, plant specificity, phage-typing and soluble protein patterns. They proposed that the fast-growing *Lotus* rhizobia and related strains be recognised as a new species, *Rhizobium loti*.

Jordan (1984) revised the classification of the root nodule bacteria for the ninth edition of Bergey's Manual of Determinative Bacteriology. Under this classification, *Rhizobium trifolii*, *R. phaseoli* and *R. leguminosarum* were combined

as one species, designated *R. leguminosarum* Frank comprising three biovars *trifolii, phaseoli* and *viceae*. *R. meliloti* was retained as a separate species and the fast-growing *Lotus* strain designated *R. loti*. The slow-growing rhizobia were transferred to a separate genus, *Bradyrhizobium*. At present only one species is recognised in this genus, *B. japonicum* which includes strains capable of effectively nodulating lupines and soybeans. The genus *Agrobacterium* was retained despite the proposals that this genus and *Rhizobium* should be amalgamated in part (Herberlein *et al* (1967) and Moffett & Colwell (1968).

4. Identification of Rhizobia

A number of techniques have been used to identify strains of rhizobia. Serological techniques involving agglutination immunodiffusion and immunofluorescence have been used by Bohlool & Schmidt (1970) and Diatloft (1977). Kishinevsky & Gurfel (1980) used the enzyme-linked immunosorbent assay (ELISA) to identify fast and slow-growing rhizobial strains both in culture and within nodules. Both the ELISA method and microagglutination tests have been used to identify strains of *Rhizobium meliloti* in commercial alfalfa inoculants (Olsen *et al* 1982).

Antibiotic resistance markers have been used for strain identification. Josey *et al* (1979) identified strains of *Rhizobium* using intrinsic antibiotic resistance. Schwinghamer & Dudham (1973) evaluated spectinomycin resistance as a marker for ecological studies with *Rhizobium*. Brockwell *et al* (1977) examined the application of both the use of streptomycin resistance and gel immune diffusion for the identification of rhizobia. They concluded that both methods were reliable for identifying strains of *R. trifolii* reisolated from field environments.

Other methods to identify rhizobia include phage-typing (Kowalski *et al* 1974) and two-dimensional polyacrylamide gel electrophoresis (Roberts *et al* 1980).

Hodgson *et al* (1983) used DNA colony hybridization to identify *Rhizobium* strains from nodules and found the method had a high degree of specificity, allowing successful identification of rhizobia.

5. Aims of this investigation

Lindstrom *et al* (1983) studied the relationship between the rhizobia that nodulate *Galega orientalis* and *G. officinalis* and other species of rhizobia using plant nodulation tests, bacteriophage-typing and DNA homology. They concluded that the *Galega* rhizobia formed a specific taxonomic group within the genus *Rhizobium*.

Keyser *et al* (1982) described the isolation of fast-growing soybean nodulating strains of rhizobia from the People's Republic of China. Although sybiotically similar to the slow-growing soybean rhizobia, *Bradyrhizobium japonicum* , they were judged on the basis of their physiology, carbohydrate utilization and biochemistry to be distinct.

The objectives of this study were:

1. To further examine the relationship between the *Galega* rhizobia with the known species and DNA homology groups of *Rhizobium* and with *Bradyrhizobium* spp.

2. To determine the relationship of the fast-growing soybean nodulating rhizobia to other species of *Rhizobium* and *Bradyrhizobium*.

It was planned that this would be done primarily by the use of DNA:DNA hybridization, although bacteriophage-typing and legume nodulation tests would also be performed.

3. To investigate the application of colony hybridization as a means to identifying rhizobia.