# 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

DAVID NEIL WEDLOCK
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.

# TABLE OF CONTENTS

TABLE OF CONTENTS CONT'D

PAGE

# LIST OF FIGURES

# LIST OF TABLES

LIST OF TABLES CONTINUED



APPENDIX TABLES

# LIST OF PLATES

# 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)<br>*Pisum* (garden peas)<br>*Vicia* (vetches)<br>*Lens* (lentils) |
| *Rhizobium trifolii* | nodulates | *Trifolium* (clovers) |
| *Rhizobium phaseoli* | nodulates | *Phaseolus* spp. (beans) |
| *Rhizobium meliloti* | nodulates | *Melilotus* (sweet clover)<br>*Medicago* (lucerne)<br>*Trigonella* (leguminous herbs) |
| *Rhizobium lupini* | nodulates | *Lupinus* (lupines)<br>*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 vicifolis* 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.

# MATERIALS AND METHODS

## 1. MICROBIOLOGICAL METHODS

### 1.1 Bacterial Strains and Maintenance

The bacterial strains used in this study are listed in Table II. The rhizobia were isolated and cultivated on yeast mannitol agar (YMA, Vincent 1970) at 28°C. All strains were maintained on YMA slopes at 4°C and sub-cultured at intervals of 2-4 months. Cultures were freeze-dried for long-term storage.

### 1.2 Media and Cultivation

1.21 Yeast Mannitol Broth (YMB, Vincent 1970) contained the following (g/l): mannitol, 10.0; yeast extract (Difco) 0.5; dipotassium hydrogen phosphate ($K_2HPO_4$), 0.5; magnesium sulphate ($MgSO_4 .7H_2O$), 0.2; sodium chloride (NaCl), 0.1. The pH was adjusted to 6.8 - 7.0 if necessary. Yeast mannitol agar (YMA) was prepared by the addition of 15g of agar (Davis) per litre of medium. Congo red dye (final concentration 0.0025%) was added to YMA to facilitate the detection of contaminant bacteria.

1.22 Brain Heart Infusion Broth (BHI) consisted of 37g/litres of brain heart infusion (Difco) in deionized water. For the solid medium (BHA), 15g of agar (Davis) was added.

1.23 No. 2 Medium (Vincent, 1970) had the following composition (g/l): Ammonium nitrate ($NH_4NO_3$), 1.0; dipotassium hydrogen phosphate ($K_2HPO_4$), 0.3; potassium dihydrogen phosphate ($KH_2PO_4$), 0.3; magnessium sulphate ($MgSO_4 .7H_2O$)), 0.1; Calcium nitrate ($Ca(NO_3)_2$, 0.05; yeast extract, 1.0; glucose, 1.0; trace element solution, 1ml. The pH was adjusted to 6.8 - 7.0. A trace element

TABLE II: THE *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS USED IN THIS STUDY

| Strain | Forms effective nodules on | Source |
|---|---|---|
| *Rhizobium* of *Galega* | | |
| gal 1 | *G. officinalis* | New Zealand |
| gal 3 | *G. officinalis* | New Zealand |
| gal 7 | *G. officinalis* | New Zealand |
| gal 12 | *G. officinalis* | New Zealand |
| gal 14 | *G. officinalis* | New Zealand |
| gal 129 | *G. orientalis* | Finland |
| gal 1261 | *G. orientalis* | Finland |
| gal NW 1 | *G. officinalis* | New Zealand |
| gal NW 2 | *G. officinalis* | New Zealand |
| gal NW 3 | *G. officinalis* | New Zealand |
| 59A2 | *G. officinalis* | U.S.A. |
| Hambi 540 | *G. orientalis* | Finland |
| Hambi 1147 | *G. orientalis* | U.S.S.R. |
| Hambi 1155 | *G. orientalis* | U.S.S.R. |
| Fast-growing soybean nodulating rhizobia | | |
| USDA 191 | *Glycine max* | China |
| USDA 192 | *Glycine max* | China |
| USDA 193 | *Glycine max* | China |
| USDA 194 | *Glycine max* | China |
| USDA 201 | *Glycine max* | China |
| USDA 205 | *Glycine max* | China |
| USDA 206 | *Glycine max* | China |
| USDA 208 | *Glycine max* | China |
| USDA 214 | *Glycine max* | China |
| USDA 217 | *Glycine max* | China |
| USDA 257 | *Glycine max* | China |
| *Rhizobium trifolii* | | |
| NZP 561 = CC 275e = PDD 2163 | *Trifolium repens* | Australia |
| ATCC 10004 | *T. repens* | U.S.A. |
| TA2 | *T. repens* | Australia |
| NZP 549 | *T. subterraneum* | New Zealand |

Cont'd..

TABLE II: Continued

| Strain | Forms effective nodules on | Source |
|---|---|---|
| *Rhizobium meliloti* | | |
| NZP 4009 = SU 47 | *Medicago sativa* | Australia |
| ATCC 9930 | *M. sativa* | U.S.A. |
| RM 1 | *M. sativa* | New Zealand |
| RM 2 | *M. sativa* | New Zealand |
| *Rhizobium loti* and related strains | | |
| NZP 2213 | *Lotus corniculatus* | New Zealand |
| NZP 2037 | *Lotus pedunculatus* | New Zealand |
| NZP 2014 | *L. corniculatus* | New Zealand |
| NZP 2238 = LC 265 Da | *L. corniculatus* | Ireland |
| NZP 5201 = CaWisc | *Caragana arborescens* | U.S.A. |
| NZP 5361 | *Parochetus communis* | New Zealand |
| Rhizobia of *Leucaena* | | |
| NZP 5434 | *Mimosa invisa* | Indonesia |
| NZP 5259 | *Leucaena leucocephala* | Sarawak |
| Other fast-growers | | |
| NZP 5462 | *Coronilla varia* | U.S.A. |
| Slow-growers | | |
| Slow growing *Lotus* rhizobia | | |
| NZP 2257 | *Lotus pedunculatus* | New Zealand |
| NZP 2192 | *L. pedunculatus* | New Zealand |
| NZP 2309 = cc814S | *L. pedunculatus* | Australia |
| NZP 5223 = 3G2c2a | *Onobrychis viciifolia* | U.S.A. |
| *Bradyrhizobium japonicum* | | |
| ATCC 10324 | *Glycine hispida* | U.S.A. |

stock solution was as follows (mg/ml): Boric acid ($H_3BO_3$), 0.1; zinc sulphate ($ZnSO_4 . 7H_2O$). 1.0; copper sulphate ($CuSO_4 . 5H_2O$), 0.5; manganese chloride ($MnCl_2 . 4H_2O$), 0.5; sodium molbydate ($NaMo O_4.2H_2O$), 0.1; Fe EDTA, 1.0.

1.24 Yeast Sucrose Broth (Dilution Broth, Vincent 1970) had the following composition (g/litre): Sucrose, 2.5; dipotassium hydrogen phosphate ($K_2 HPO_4$), 0.5; sodium chloride (NaCl) 0.1; yeast extract, 0.5. This stock solution was diluted 10-fold before use and dispensed in 10ml aliquots.

1.25 Water Agar Plates were prepared by the addition of 15g agar (Davis) to 1 litre of deionized water. The agar was autoclaved at 121°C for 15 min and plates poured.

1.26 Hoagland's Nutrient Solution was added to jars containing pumice (grade B) for the plant tests. The stock solutions required are shown in Table III and the appropriate volume of each sterile solution was added to 5 litres of sterile deionized water.

1.27 Thorton's Nutrient Solution was prepared as shown on Table IV.

1.28 Culture growth and purity.
All stock cultures were streaked on YMA and BHA and checked for purity. Only single well isolated colonies were used to inoculate liquid media.

For the preparation of DNA, YMB (100ml) was inoculated with a single well isolated colony from the YMA plate and incubated at 28°C for 2 days on a gyratory shaker. The culture was then checked for purity by streaking onto both YMA and BHA plates and then used to inoculate 2.4 litres

TABLE III: HOAGLANDS NUTRIENT SOLUTION (1/5 Strength. -N)

| Stock Solutions | g/litre | Volume added to 5 litres deionized water |
|---|---|---|
| A. $K_2HPO_4$ | 17.4 | |
| $KH_2PO_4$ | 122.5 | 1.0 ml |
| B. $MgSO_4.7H_2O$ | 246.5 | |
| NaCl | 14.6 | 2.0 ml |
| C. $K_2SO_4$ | 87.1 | 4.5 ml |
| D. $CaCl_2.6H_2O$ | 219.1 | 4.5 ml |
| E. Micro elements | | |
| $H_3BO_3$ | 2.86 | |
| $MnCl_2.4H_2O$ | 1.81 | |
| $ZnSO_4.H_2O$ | 0.22 | |
| $CuSO_4.5H_2O$ | 0.08 | |
| $CoSO_4.7H_2O$ | 0.095 | |
| $Na_2MoO_4.2H_2O$ | 0.054 | 1.0 ml |

TABLE IV: THORTONS NUTRIENT SOLUTION (-N)

| Stock Solutions | g/500 ml | Amount added to 1 litre deionized water |
|---|---|---|
| A. $KH_2PO_4$ | 27.3 | |
| $K_2HPO_4$ | 15.0 | 2.0 ml |
| B. $MgSO_4.7H_2O$ | 20.0 | |
| NaCl | 10.0 | 5.0 ml |
| C. $FeCl_3$ | 50.0 | 0.1 ml |
| $Ca_3(PO_4)_2$ | | 1.0 g |
| $FePO_4$ | | 1.0 g |
| Agar | | 6.0 g |

of YMB. This was incubated at 28°C for 2 days and the cells harvested by centrifugation.

Cells for the preparation of radioactively labelled DNA were grown in YMB without dipotassium hydrogen phosphate (500ml) which was inoculated with 10% of a 48 hour culture in YMB (100 ml), incubated at 28°C for 3 hour and supplemented with 10 mCi carrier free $^{32}P$ orthophophate in dilute HCL (Amersham, UK). Incubation was continued overnight.

1.29 Isolation of rhizobia from nodules

Three of the strains used in the hybridization experiments, gal NW 1, gal NW 2, gal NW 3 were new isolates obtained from *Galega officinalis*. A total of five isolates RM 1, RM 2, RM 3, RM 4, RM 5 were made from *Medicago sativa*. Nodules were surface sterilized by immersion in a freshly prepared mixture of hydrogen peroxide ($H_2O_2$) and 95% ethanol (50:50) for 5-10 min and rinsing in sterile deionized water. The nodules were squashed and the contents plated onto YMA plates and incubated at 28°C for 4 days. Well isolated colonies were picked and streaked onto fresh YMA plates to obtain pure cultures.

## 2. DNA EXTRACTION AND PURIFICATION

### 2.1 Materials

2.11 Tris-EDTA buffer contained 0.05 M Tris (hydroxy methyl) amino methane and 0.02 M Ethylene diamine tetra acetic acid ($(CH_2.N\ (CH_2COOH)CH_2COONa)_2.2H_2O$), pH 8.0.

2.12 Ribonuclease was prepared at a concentration of 2 mg/ml, heated at 90°C for 10 min to destroy the DNase and stored at -20°C.

2.13 Protease (Sigma, Type IV) was prepared at 10 mg/ml, incubated at 37°C for 1 hr to digest impurities and stored at -20°C.

2.14 Lysozyme (Sigma) was prepared at 10 mg/ml and stored at -20°C.

2.15 Sodium Lauryl Sulphate (SLS) (BDH) was prepared as a stock solution (25%).

2.16 Tris-HCl buffer (1.0 M) was prepared by adding 121.1g of Tris (Sigma 7-9$^{R}$) to 1 litre deionized water and adjusting the pH to 8.0 with hydrochloric acid (6 M).

2.17 Tris-Saturated Phenol was prepared as follows: 500 g of phenol was dissolved in a minimum amount of deionized water (62.5 ml) by warming. A further 500 ml of deionized water and 62.5 ml of 1.0 M Tris-HC buffer was added to the cooled phenol solution.

The mixture was shaken to give an even suspension which was then poured into a separating funnel, The bottom layer containing the Tris-saturated phenol was collected.

2.18 Urea (8 M in 0.14 M phosphate buffer) was prepared as follows. Fertilizer grade urea was purified by column chromatography using a diethylaminoethyl cellulose ion exchanger (Whatman DE 52). The ion exchanger was prepared by suspending it in 0.14 M PB, allowing it to settle and decanting the supernatant. This procedure was repeated until the supernatant had the same pH as the buffer (pH 6.8). The ion exchanger was resuspended in 0.14 M PB (6ml/g of ion exchanger), allowed to settle for 20 min and the supernatant containing the fines decanted. The prepared ion exchanger was used to pack a column (19 cm x 5 cm dimensions).

Fertilizer grade urea (9.2 M) was prepared in deionized water and passed through the column. Urea (8 M) was prepared by adding 869 ml of urea to 100 ml of 0.14 M PB and making the volume up to 1 litre with deionized water.

Following use, the ion exchanger was regenerated by washing with 15 volumes of 0.1 M NaOH and adjusting the pH to 11.0 with constant stirring. After stirring for 30 min, the supernatant was decanted and the gel was resuspended in two successive volumes of deionized water. The ion exchanger was stirred into 15 volumes of 0.1 M HCl and the pH adjusted to 2.0 with constant stirring. After stirring for 30 min, the ion exchanger was washed with two successive volumes of deionized water, titrated to pH 6.8 and resuspended in 0.14 M PB.

2.19 Hydroxyapatite (DNA-grade Bio-Gel HTP, Bio-Rad Laboratories, Richmond, California) was prepared by resuspending 100 g of hydroxyapatite (HA) in 600 ml of 0.0014 M PB. The HA was allowed to settle and the supernatant decanted. This process was repeated twice to remove the fines. The settled HA was finally resuspended in 300 ml of 0.0014 M PB.

2.20 Phosphate Buffer (1.4 M): A stock solution of 1.4 M Phosphate buffer (PB) was prepared by dissolving 198.8 g di-sodium hydrogen phosphate ($Na_2 HPO_4$, BDH, AnalaR) and 218.4 g of sodium di-hydrogen phosphate ($NaH_2 PO_4 .2H_2 O$, BDH, AnalaR) in deionized water and making the volume up to 2 litres in a volumetric flask.

## 2.2 Lysis of Cells (Jarvis *et al* 1980)

The culture (2.4 l) was centrifuged (6,000 g for 15 min at 4°C) and the cells resuspended in 50 ml Tris-EDTA buffer. Sodium chloride (NaCl) was added to a final concentration of 0.1 M. Protease was added to a final concentration of

50 µg/ml, ribonuclease was added to a final concentration of 50 µg/ml and sodium lauryl sulphate (SLS) at 10 mg/ml. The mixture was incubated at 37 C for 2 hours. Protease was then added to a final concentration of 25 µg/ml, ribonuclease was added at 25 µg/ml, SLS at 5 mg/ml and the solution incubated for a further 1 hour at 37 C. This lysis method proved satisfactory for the majority of strains but several strains required alternative methods of lysis in order to extract the DNA.

## 2.3 Alternative Methods of Lysis

2.31 Cells were lysed by a modification of the method of Fischer & Lerman (1979): The cells were resuspended in 50 ml of Tris-EDTA buffer. Sarkosyl (Sigma) was added to a final concentration of 0.1%, protease was added to 50 µg/ml and SLS at 10 mg/ml and the mixture incubated at 50 C overnight. Ribonuclease was added to a final concentration of 50 µg/ml and the solution incubated at 37 C for 1 hour.

2.32 Modification of the method of Zaenen *et al* (1974):

The cells were resuspended in 50 ml of Tris-EDTA buffer. Lysozyme (1 mg/ml) was added and the solution incubated at 37°C for 1 hour. The mixture was made 0.1 M with respect to NaCl; protease (0.5 mg/ml) was added and the mixture incubated for a further 1 hour at 37°C. Finally, SLS (1%) and ribonuclease (50 µg/ml) were added and incubation continued for 30 min at 37°C.

2.33 Freeze-thaw method: After centrifugation the cells were frozen by immersing the centrifuge tube in liquid air. The cells were then thawed at 50°C in a water bath. This procedure was repeated three times and the cells were resuspended in 50 ml of Tris-EDTA buffer and lysed as in method 2.2.

## 2.4 Extraction of DNA

An equal volume of Tris-saturated phenol was added to the lysate and the mixture was shaken vigorously to ensure an uniform suspension. The suspension was then centrifuged (6,000 g for 10 min). The aqueous phase was quantitatively removed and the volume measured. Phosphate buffer (1.4 M) was added to a final concentration of 0.14 M and solid urea (BDH) was added to a final concentration of 8.0 M. The DNA solution was warmed at 37°C to dissolve the urea.

## 2.5 Purification of DNA by the Hydroxyapatite-Urea Method

DNA was purified by a modification of the hydroxyapatite-urea method (Britten *et al* 1970). Hydroxyapatite (60 ml) was washed once with the 8 M urea solution (6,000 g for 2 min at 20 °C) and the supernatant discarded. The crude DNA solution was added to the prepared HA and resuspended with an overhead stirrer (Multispeed, Anderman and Co. Ltd, Central Ave., E. Molsey, Surrey, England) and left to adsorb to the HA for 30 min. The HA was then centrifuged at 6,000 g for 2 min and the supernatant containing protein, ribonucleic acid and polysaccharide discarded. The HA was then washed at least 10 times with 100 ml of 8.0 M urea in 0.14 M PB. Following this treatment, the HA was washed 4 times with 80 ml of 0.014 M PB to remove the urea. On the 5th wash the HA was quantitatively transferred to a capped SS34 centrifuge tube by resuspending the HA in 20 ml of 0.014 M PB. The HA was centrifuged at 6,000 g and the supernatant discarded.

The DNA was eluted from the HA by resuspending in 10 ml of 0.66 M PB and leaving for 10 min. After centrifugation the supernatant was collected in an acid-washed Universal bottle and labelled F4-1. The DNA was further eluted from the HA by resuspending in 10 ml of 0.4 M PB, centrifugation and collecting the supernatant. In this manner, two further

fractions were collected and labelled F4-2 and F4-3. Most of the DNA was contained in the F4-1 and F4-2 fractions. The DNA was stored at 4°C for short periods and at -70°C for long-term storage.

## 2.6 Measurement of DNA Concentration and Purity

The purity of the DNA preparations was determined from the absorbance at wavelengths 230 nm, 258 nm and 280 nm using a PYE-unicam SP 1800 UV spectrophotometer as described by Jarvis *et al* (1980).

DNA concentrations of 1 mg/ml have an extinction of 20.0 for unsheared DNA and 24.0 for sheared DNA (Brenner & Falkow 1971). The absorbance of each DNA sample was read at 258 nm and the DNA concentration calculated as follows:

$$\text{DNA concentration} = \frac{OD_{258} \times \text{dilution factor}}{\text{Factor}} \quad \mu g/ml$$

Factor: 0.020 for unsheared DNA
0.024 for sheared DNA

DNA purity was determined from the spectral ratios 258/230nm and 258/280 nm. DNA with spectral ratios 1.8 or greater was considered satisfactory. A ratio of 258/230 nm which was below 1.8 indicated protein contamination, while a ratio of 258/280 nm below 1.8 indicated the presence of phenol contamination.

The radioactivity of labelled DNA was determined by placing 10 µl of the DNA in a scintillation vial containing 8 ml of water and assaying the amount of radioactivity by Cerenkov counting (Clausen 1968) in a Beckman LS7000 liquid scintillation counter.

## 2.7 Dialysis, Sonication and Dilution of DNA

DNA with satisfactory ratios was placed in dialysis tubing which had been boiled in deionized water for 10 min prior to use. The DNA was dialysed in 2 litres of 0.28 M PB for 2 hours and then overnight in a further 2 litres of 0.28 M PB. Dialysed DNA was stored at -70°C until required.

Unlabelled DNA preparations with a concentration below 200 µg/ml were concentrated to at least this level prior to dialysis. Each DNA solution was placed in dialysis tubing and placed 10 - 20 cm from the fan unit in the cold room at 4°C to evaporate excess water.

All DNA used in the reassociation experiments was sonicated to produce low molecular weight fragments. Unlabelled DNA preparations were diluted with 0.28 M PB to a concentration of 200 µg/ml and 5 ml volumes were sonicated for a total of 75 sec. Each sample was placed on ice and sonicated for intervals of 15 sec, allowing 30 sec between each interval to disspipate heat. Labelled DNA was sonicated for only 56 sec because of the increased fragility of $^{32}$P-DNA and then diluted to a concentration of 2-4 µg/ml. All sonicated and diluted DNA was stored at -70° C for long periods and at 4°C for short periods.

# 3. DNA HOMOLOGY

## 3.1 Reassociation of DNA

The procedure used was that of Jarvis *et al* (1980). Reassociation of DNA was carried out at 65°C in screw-capped Kimax tubes (1 x 10 cm) and at 80°C in Quickfit glass-stoppered tubes (1.5 x 10 cm). Each tube contained 0.75 ml of unlabelled DNA at a concentration of 200 µg/ml and 0.1 ml

of labelled DNA at a concentration of either 2.0 or 4.0 µg/ml. The tubes were made up to a final volume of 1.0 ml by the addition of 0.15 ml of 0.28 M PB. The tubes were placed in a boiling water bath for 10 minutes to produce single-stranded DNA and immediately quenched in ice-water for 10 minutes to prevent renaturation. They were transferred to a water bath at either 65°C or 80°C for 40 hours to reassociate.

Controls were set up with each batch and included a sample of homologous DNA and DNA from *Streptococcus lactis* H1. In addition, a tube containing only reference DNA was used in order to measure the apparent self-association of labelled DNA. After incubation the tubes were quenched in ice-water and diluted to 0.14 M PB by the addition of an equal amount of deionized water (1 ml).

## 3.2 Separation of Single and Double-Stranded DNA on Hydroxyapatite

The procedure was similar to that described by Brenner *et al* (1969a). Tubes containing 7 ml of HA suspension (Methods 2.19) were centrifuged and the HA resuspended in 0.10-0.12 M PB containing 0.4% sodium lauryl sulphate. The tubes were recentrifuged and the supernatant discarded. Diluted reassociated DNA solution (0.5 ml) was added, followed by 8.0 ml of PB. The tubes were stirred with an overhead stirrer, (Multispeed, Anderman and Co Ltd, Central Ave., E. Molsey, Surrey, England) and were raised to the required temperature (65°C or 80°C) in a circulating water bath (Haake Type F, Haake Circulators, Berlin, Germany). They were placed in a Sorvall bench centrifuge fitted with a Type A rotor which was held at 65°C in an incubator and centrifuged at 1,000 g 1 min and stopped quickly by hand. The supernatant from each tube was immediately poured into a scintillation vial.

Further buffer was added and the process was repeated. Four low molarity PB were used to elute the unbound single-stranded DNA washes from the HA. This was followed by four washes with 0.4 M PB to elute the double-stranded DNA.

The radioactivity in each vial was measured in a Beckman LS7000 liquid scintillation counter using program no 10.

The hybridization that occurred in each reassociated DNA solution was calculated as the percentage of double-stranded DNA to total DNA. The percentage relative homology was obtained by subtracting the apparent self-hybridization of reference DNA from the percentage hybridization of reference DNA with each unlabelled DNA. The percentage hybridization of labelled reference DNA with unlabelled homologous DNA was adjusted to 100% and the factor used was then applied to all hybridizations with heterologous DNA. Each reassociated DNA mixture was separated in duplicate. Where the results did not agree to within 5% further separations were carried out.

## 3.3 Statistical Analysis of DNA Homology Data

The 'Student's' t test (Bailey 1981) for comparing the means of two small samples was used to compare the means of each homology group. The 'Student's' t with $n_1 + n_2 - 2$ degrees of freedom and a rejection of 0.001 was applied, using the equation:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{S \frac{1}{n_1} \times \frac{1}{n_2}}}$$

$$S = \sqrt{\frac{(n_1 - 1)\, S_1^2 + (n_2 - 1)\, S_2^2}{n_1 + n_2 - 2}}$$

$\bar{x}_1$ and $\bar{x}_2$ are the number of comparisons in each homology group $s_1$ and $s_2$ are the standard deviations of each homology group.

The means of two homology groups 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.

## 3.4 Thermal Stability of Reassociated Duplexes

The thermal stability of reassociated duplexes was determined by the method of Jarvis *et al* (1980). Tubes containing labelled reference DNA and unlabelled DNA were allowed to reassociate for 40 hours at 65°C. The tubes were diluted with an equal amount of deionized water and the single-strained DNA eluted with a low molarity buffer containing 0.4% SLS. The double-stranded DNA was eluted by resuspending the HA in low molarity PB of the same molarity with out the SLS. The tubes were placed in the circulating water bath and the temperature raised to 70°C. The tubes were immediately centrifuged and the supernatant collected in a scintillation vial. The HA in each tube was resuspended in a further 8.0 ml of PB and the temperature raised to 75°C. In this manner the temperature was raised in 5°C intervals until a temperature of 100°C was reached. The remaining DNA bound to the HA was eluted by washing the HA with 0.4 M PB and the supernatant collected. The radioactivity in each supernatant was measured on a scintillation counter and the temperature at which 50% of the radioactivity associated with the double-stranded DNA was eluted, was calculated. This temperature is the thermal melting point, Tm(e).

The Tm(e) was calculated for both the homologous and heterologous reassociated duplexes and the difference between the two is known as ΔTm(e) (Brenner *et al* 1972). This value provides an index of base sequence divergence in the reassociated DNA fragments.

## 3.5 Regeneration of Hydroxyapatite

The HA used in the hybridization experiments was regenerated by an adaptation of the HA preparation method of Bernadi (1971). A 5 litre flask was used to regenerate 500 ml of wet packed HA. A volume of 2 litres of 0.1 M NaOH was added and the HA was mixed with the sodium hydroxide by gentle swirling for 5 minutes. The HA was allowed to settle for 25 minutes and the supernatant decented and discarded. The process was repeated with a further 2 litres of 0.1M NaOH. Following this, 4 litres of deionized water was added to the HA, mixed and allowed to settle. The supernatent was decanted and discarded. This step was repeated with 4 litres of 0.014 MPB. The HA was mixed with 4 litres of 0.014 M PB, the flask placed in a water bath and the HA brought almost to 100$^{o}$C, stirring constantly with an overhead stirrer. The HA was allowed to settle and the supernatant decanted and discarded. This procedure was repeated and the HA suspension was held at 100$^{o}$C for 5 minutes and then repeated twice while the suspension was held at 100$^{o}$C for 15 minutes. Finally the HA was resuspended in 4 litres of 0.0014 M PB and boiled for 15 minutes, allowed to settle, decanted and then stored in 0.0014 M PB at 4$^{o}$C until required.

# 4. PLANT NODULATION TESTS

## 4.1 Surface Sterilization of Seeds

Legume seeds were scarifyed by briefly rubbing between two sheets of emery paper and then placed in a quickfit flask containing a freshly prepared mixture of hydrogen peroxide ($H_2O_2$) and 95% ethanol (50:50) for a period of 5-10 minutes. The sterilized seeds were then rinsed in 4 successive quantities of sterile deionized water. Following this, 15-20 seeds were placed on the surface of water agar plates. The plates were inverted and placed in the dark for a period of between 2-4 days to allow germination.

## 4.2 Seedling Preparation

The smaller seeded plants; clover, *Lotus* and lucerne were set up in test-tubes (1.5 x 15 cm) containing 10 ml of Thortons nutrient solution with 0.6% agar. The tubes were autoclaved at 121°C for 15 min, sloped and one germinated seedling was transferred aseptically to each tube. The seedlings were placed in the dark for 2 days to encourage further germination and then inoculated with the appropriate strain of rhizobia. Strains were grown in YMB (100 ml) and incubated at 28°C for 2 days. A few drops of this culture was used to inoculate the seedlings. For each species of legume, six plants were left uninoculated as - nitrogen (-N) controls.

The larger seeded plants; *Leucaena*, *Galega* and soybean were sown into jars (600 ml) containing Hoaglands nutrient solution (1/5 strength). The jars were prepared as follows: Each jar was filled to within 2 cm of the top with pumice (grade B) and a watering tube placed in each jar. Each watering tube consisted of a glass tube blocked at one end with a cotton plug. The open end was embedded within the pumice. Aluminium foil was placed over the top of

each jar and over the cotton plug of the watering tube and the jars were sterilized by autoclaving for 1 hour at 121°C.

Three or four seedlings were transferred aseptically to the jars and placed within small indentations of the surface of the pumice and inoculated by adding a few drops of culture. Three or four seedlings were left uninoculated as -N controls. After sowing the seeds, the entire surface of the pumice was covered with sterile plastic beads to a depth of 1 cm to exclude contaminants. Hoaglands nutrient solution (300 ml) was added to each jar through the watering tube and the jars were placed in the dark until germination of the seedling occurred.

After germination, all seedlings were placed in the glass-house for a period of 8-12 weeks. The plants in the pumice jars were watered at periods of 2 weeks by adding sterile deionized water through the watering tubes.

## 4.3 Assessment of Nodulation and Nitrogen-Fixing Ability

All plants were assessed visually for the presence of nodules and for the ability to fix nitrogen in symbiosis with rhizobia. Each rhizobia-legume combination was compared with the uninoculated -N controls. When it proved difficult to differentiate between the effective and ineffective symbioses, dry-weight measurements of the plant tops were determined. The plant tops were harvested and placed in paper envelopes and then dried at 80°C overnight and weighed. The 'Student's' t test was applied to determine if there was any significant difference between the inoculated and the uninoculated plants.

The fast-growing soybean nodulating strains that nodulated *Lotus pedunculatus* were tested for their nitrogen-fixing

ability by the use of gas chromatography. The acetylene/ethylene nitrogenase assay was performed on each plant in the original test-tube. The cotton plug of each tube was replaced with a greased rubber stopper. A 1 $cm^3$ Tuberculin syringe was used to withdraw 1 $cm^3$ of air from each tube and to replace it with 1 $cm^3$ of acetylene at atmospheric pressure. A sample (100 µl) was removed from each tube and injected into the injection port of the gas chromatograph (Varian series 2700 gas chromatograph). Each tube was left for at least 3 hours and a further sample assayed. An effective nitrogen-fixing strain of *Rhizobium* and an uninoculated plant were also assayed as controls.

## 5. PHAGE TYPING

### 5.1 Isolation and Purification of Phages

An enrichment method similar to that used by Patel (1976) was used to isolate a bacteriophage specific for *R. meliloti*. Soil (500 g) was obtained from a paddock in which lucerne had been previously grown. The soil was suspended in 500 ml of tap water and filtered through cheese cloth. An equal volume of yeast sucrose broth was added to the filtrate and the suspension was inoculated with one of the five isolates of *R. meliloti* obtained from lucerne root nodules. After 24 hours incubation at 28°C the enrichment was centrifuged (8,000 g for 20 min). The supernatant was treated with chloroform by the addition of a few drops of chloroform and then assayed for phage activity. The double layered agar-plate method was used as described by Vincent, 1970. Each plate consisted of a bottom layer of No. 2 medium (1.25% agar) overlaid with No. 2 medium semi-soft agar (0.5% agar). The semi-soft agar was distributed in 3 ml aliquots, and maintained in a molten form at 55°C until required. Each tube of semi-

soft agar was inoculated with 0.2 ml of rhizobia culture grown in YMB and 0.5 ml of treated supernatant and poured onto the surface of the bottom layer and allowed to set. The plates were then inverted and incubated at 28$^{o}$C for 48 hours. Single plaques were picked from plates that showed phage activity and re-plated on fresh No 2 plates in order to obtain a pure phage isolate.

After purification each phage isolate was plated undiluted onto 4 No 2 plates. The plaques produced on each plate were harvested by adding 2 ml of yeast sucrose broth and placing the plates on an orbital shaker for 2 hours. The medium, containing phage particles, was removed along with the top semi-soft agar layer and placed in a SS34 centrifuge tube. A few drops of chloroform were added to lyse the bacterial cells and the suspension was centrifuged at 10,000 g for 20 min. The supernatant was then plated with the appropriate strain of *Rhizobium* on No 2 plates to produce confluent lysis. The phage could then be harvested again in the above manner and diluted $10^{-2}$ before re-plating to produce confluent lysis of the bacterial cells.

In this fashion, all phage isolates and phage stocks were built up to a titre of at least $10^{9}$ plaque-forming units (PFU)/ml. Each phage stock suspension was stored at 4$^{o}$C in a universal bottle containing a few drops of chloroform.

## 5.2 Lysis of Rhizobial Strains by Bacteriophage

The phage-typing procedure of Patel (1976) was used. Before use, all phage stocks were filtered through a 22 µm, 25 mm Millipore membrane to remove bacterial cells. Each phage stock was diluted $10^{-6}$ fold to give at least $10^{3}$ PFU/ml. This concentration was known as a routine test dilution (RTD) and produced confluent lysis on the original host strain of *Rhizobium*. The susceptibility of strains to phage was tested by spotting both the undiluted

suspension and the RTD onto plates seeded with the rhizobia. A particular strain was considered to be highly susceptible to phage if lysis occurred in $10^{-6}$ dilution of the phage stock. Strains were less suscetible to phage if lysis occurred only at a phage concentration greater than RTD and not susceptible if no lysis occurred with the undiluted phage.

## 6. COLONY HYBRIDIZATION

### 6.1 Materials

6.11 DNase Buffer consisted of 20 mM Tris-HCl and 10 mM magnesium chloride ($MgCl_2$), pH 7.4.

6.12 *Hae* III Buffer (x10 stock) consisted of 100 mM Tris-HCl, 80 mM $MgCl_2$ and 14 mM B-mercaptoethanol, pH 7.5.

6.13 Standard Saline Citrate (20 x SSC) was 3.0 M sodium chloride and 0.3 M sodium citrate.

6.14 Colony Hybridization Buffer (modified Denhardt's buffer, Jarvis *et al*, 1983) had the following composition: Hepes buffer (1 M), pH 7.0 (N-2-Hydroxyethylpiperazine N-2-ethane sulphonic acid, Sigma H-3375), 25 ml; standard saline citrate (20 x SSC), 75 ml; Herring sperm DNA (degraded free acid, Sigma Type IV), 0.03 g/10 ml, extracted with Tris-saturated phenol before use, 3 ml; *E. coli* transfer RNA (Sigma Type XXI), 10 mg/ml, 2.5 ml; SLS (20%), 2.5 ml; Ficoll (Sigma Type 70), 1 g; bovine serum albumin (BSA, Sigma), 1 g; polyvinyl pyrrolidone (Sigma PVP-10), 1 g. The buffer was made up to 500 ml with deionized water and stored at 4°C. Since the SLS comes out of solution at 4°C, the buffer was warmed in a waterbath at 37°C before use.

## 6.2 Preparation of DNA Random Primers

The method of Jarvis *et al* (1983) was used.
Calf Thymus (Sigma, 1 g) was dissolved in 25 ml of DNase buffer to a final concentration of 40 mg/ml. DNase I (deoxyribonuclease 5'-oligonucleotide hydrolase) was added (2 mg) to cut the DNA into smaller fragments and the mixture incubated at 37°C for 45 min. Following incubation, the DNase I was destroyed by the addition of SLS to a final concentration of 1% and protease (Sigma) at 1 mg/ml and incubation continued for a further 45 min at 37°C. Protein was removed by phenol/chloroform extraction. An equal volume of phenol and chloroform was added to the suspension and the mixture shaken and centrifuged (8,000 g for 10 min). The top aqueous layer was collected and denaturated at 100°C in a boiling water bath for 10 min and then transferred to an ice-water bath. This procedure produced single-stranded DNA fragments.

DNA fragments between 5 and 12 nucleotides in length were collected by separating the mixture on a Whatman DE 52 cellulose column (20 cm x 1 cm dimensions). The column was equalibrated with 5 mM Tris, pH 7.4, 1 mM EDTA, 0.1 M NaCl. The DNA fragment mixture was loaded onto the column and the column washed with this buffer until all $OD_{258}$ absorbing material was washed free. This removed all the fragments of up to 5 nucleotides in length. Fragments between 5 and 12 nucleotides in length were eluted by washing the column with 5 mM Tris, pH 7.4, 1 mM EDTA, 0.3 M NaCl until all $OD_{258}$ absorbing material was washed free. This fraction was ethanol precipitated by the addition of 0.2 mM NaCl, two volumes of 95% ethanol and placing the mixture at -70° C overnight. The precipitated DNA fragments were resuspended in deionized water at a concentration of 50 µg/ml.

## 6.3 Preparation of *in vitro* Labelled DNA

DNA to be used as probes for colony hybridization was labelled *in vitro* by the random primer method (Taylor *et al* 1976). The DNA (1 µg) was incubated with the restriction enzyme *Eco* RI at 37 C for 30 min to digest the DNA and produce small fragments. The DNA was added to 2 µl of 10 x *Hae* III buffer, 12 µl deionized water, 2 µl of *Eco* RI (Biolabs, New England) in an Eppendorf tube. The mixture was made 0.1 M with respect to NaCl. Following incubation, DNA random primers (100 µg) were added and the mixture diluted by the addition of 10 µl of deionized water. The mixture was placed in boiling water for 1 min and then in ice-water to obtain denatured single-stranded DNA.

To the reaction mixture, 1 µl each of 2-deoxyguanosine-5'-triphosphate (Sigma, 20 mM), 2-deoxyadenosine-5'-triphosphate (Sigma, 20 mM) was added. Deoxycytosine-5'-($\alpha$ - $^{32}$P)triphosphate (Amersham, U.K., 30 Ci) was added and 2 µl of 10 x *Hae* III buffer was added to make up for the loss of mercaptoethanol by volatilization. Finally, 1 µl of DNA polymerase I Kleno fragment was added. The mixture was incubated at 37 C for 30 min and the reaction stopped by the addition of 0.75 µM EDTA.

The mixture was extracted with phenol/chloroform by the addition of an equal volume of Tris-saturated phenol (40 µl) and chloroform (40 µl), mixing by vortexing and centrifuging for 1 min in an Eppendorf centrifuge. The aqueous phase was removed with an automatic pipette and the remaining phenol phase washed twice by adding 40 µl deionized water, mixing by vortexing, recentrifuging and removing the aqueous layer. The final volume of the aqueous phase was 120 µl.

The DNA in the aqueous phase was separated from the unreacted label by chromatography on a Sephadex G-50-80 (Sigma) column (7 cm x 2 cm dimensions). The column was equilibrated with eluant buffer (0.1 M NaCl, 1 mM EDTA and 10 mM Tris, pH 8.0). The sample (120 µl) was added in one lot onto the top of the column and 200 µl eluant buffer was added. A peristaltic pump was used to supply eluant to the top of the column and a total of 20 fractions were collected in Eppendorf tubes. Each fraction was comprised of 2 drops of eluant collected from the bottom of the column. The radioactivity of each fraction was estimated by holding each Ependorf tube close to a monitor. The $^{32}P$ labelled DNA was the first to pass through the column and most of this was contained within fractions 7-12. These fractions were then used further, while the remaining fractions which contained the unbound label were discarded. Each tube contained labelled DNA was made 0.3 M with respect to NaCl, *E. coli* transfer-RNA (30 µg) was added and the DNA co-precipitated by adding two volumes of ethanol and placing each tube at -70°C overnight.

Each tube was centrifuged for 3 min in the Eppendorf centrifuge and the supernatant discarded and the precipitate washed by adding 200 µl of cold ethanol (-20° C) and recentrifuging the tubes for 3 min. The supernatant was discarded and the tubes dryed in a vacuum dessicator for 5-20 min to evaporate the ethanol. The precipitate from all the tubes was pooled by taking up each precipitate in a total 100 µl of deionized water. The specific activity of the labelled DNA was measured in a Beckman LS7000 liquid scintillation counter. Each labelled DNA preparation was stored at 4°C until required.

## 6.4 Preparation and Hybridization of Membranes

The method of Grunstein *et al* (1975) was used. Sheets of nitrocellulose (BA-85, Schleicher and Schull GmbH, Dassel West Germany) were cut into circles of diameter 8 cm to fit into a standard petri dish. The filters were sterilized by autoclaving at 115°C for 10 min and were placed onto the surface of YMA plates. A small volume of YMB medium was added to each petri dish and the air bubbles removed with a sterile glass spreader and the residual liquid removed.

All the bacterial strains used in the study were firstly inoculated onto YMA plates and incubated at 28°C for 4 days to obtain single colonies. From these single colonies a small amount of culture was aseptically inoculated onto the surface of the membrane with a sterile toothpick. For each membrane, either 7 or 12 strains were inoculated in a grid pattern. The colonies were allowed to grow for 48 hours at 28°C.

After the colonies had grown, the membrane filters were blotted by placing, colony side up, on 2 sheets of Whatman 3 MM paper (14 cm x 23 cm) containing 30 ml of lysing buffer (0.5 M NaOH, 1.5 M NaCl) for 5 min. The filters were then transferred to dry 3 MM paper, colony side up, to remove excess liquid and then placed on 2 sheets of 3 MM paper (14 cm x 23 cm) containing 30 ml of 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5 for 5 min. Following this, the filters were placed colony side up on a buchner funnel and washed under suction with 2 x 25 ml quantities of 95% ethanol. The filters were placed between filter paper and dryed under vacuum at 80°C for 2 hours. All membranes were stored at room temperature until required for hybridization.

For hybridization, each filter was placed in a small heat sealed plastic bag and 15 ml of hybridization buffer (Method 6.14) was added. The membrane was allowed to wet evenly, air was excluded and the bag sealed. This bag was then placed within another bag, which was sealed to avoid the possibility of leakage. The bag was placed in a water bath at 65°C for 2 hours, during which time all non-specific binding sites were loaded. Following this, a corner was cut off the interior bag and about 10 ml of hybridization buffer was removed. The *in vitro* labelled probe DNA (10-15 µl) was placed in an Eppendorf tube and denatured in boiling water for 1 min and then placed on ice. The DNA was then added to the hybridization buffer in the bag, and gently mixed. Air was excluded, the bag resealed and returned to the waterbath at 65°C for 16-20 hours.

The filter was removed, placed on a dish containing 250 ml of 2 x SSC and washed for 10-15 min. The liquid was removed and the process repeated three times to remove unhybridized label. The filter was blotted dry, placed on a piece of Whatman 3 MM paper and secured with cellotape. The membrane filter and backing paper was covered with a sheet of Glad-wrap and placed in a X-ray cassette (Ilford RR cassette for 18 cm x 24 cm film) containing an intensifying screen ("Cronex" Du Pont lightning-plus). The X-ray film (Agfa-Gevaert, 18 cm x 24 cm) was added to the cassette in the darkroom and the cassette wrapped in brown paper and placed in a freezer at -70° C for 2-4 days. After the desired exposure time the film was removed in the darkroom and developed using a Kodak RP-X-OMAT processor.

## 7. DETERMINATION OF CONTAMINANTS IN LABELLED DNA PREPARATIONS

### 7.1 Colourimetric Determination of Protein Concentration

The method of Bradford (1976) was used to determine protein concentration. The dye-binding reagent was prepared by adding 85% (w/v) phosphoric acid ($H_3PO_4$) (100 ml) to Coomassie Brilliant Blue G-250 which was dissolved in 95% ethanol (50 ml). Deionized water was added to a final volume of one litre. A bovine serum albumin protein standard was prepared gravimetrically and adjusted to 1 mg/ml to give an absorbance of the solution at 280 nm of 0.66 (Kirschenbaum 1970). Five ml of the dye-binding reagent was added to 0.1 ml samples containing 10-100 µg of protein and the contents mixed by vortexing. The absorbance at 595 nm was measured after two minutes and before one hour against a reagent blank. For the determination of protein in the DNA preparations, 1.0 ml of undiluted DNA solution was used in the determinations.

### 7.2 Colourimetric Determination of Polysaccharide Concentration

The method of Bailey (1967) was used to determine polysaccharide concentration. Concentrated sulphuric acid ($H_2SO_4$, 70 ml) was added to deionized water (30 ml) and the mixture was cooled. Anthrone (BDH, 0.1g) was added to the dilute acid (100 ml), allowed to age for two hours at $0^{\circ}C$ and discarded after 24 hours. A standard solution containing 10 mg/ml of glucose was prepared. To a one ml sample containing either DNA or 10-100 µg of glucose, in a cold water bath, anthrone reagent was added slowly (5 ml). The tubes were mixed by inversion, transferred to a vigorously boiling water-bath for 7 minutes and then placed in an ice-water bath in the dark for 30 minutes. The absorbance was determined at 625 nm against a reagent blank.

## 7.3 Qualitative Determination of RNA

7.31 Tris-Acetate Buffer consisted of 40 mM Tris, 5 mM sodium acetate and 1 mM EDTA, pH 7.8.

7.32 Gel Electrophoresis: The presence of RNA in labelled DNA was determined by agarose gel electrophoresis in a flat bed gel apparatus. Tris-acetate buffer (100 ml) containing 0.8% agarose (Sigma) was heated to dissolve the agarose. The buffer was allowed to cool and was poured into a horizontal gel apparatus to produce a gel 12 cm x 10 cm and the walls formed with a teflon comb. After the gel had set, the DNA samples were mixed with 50% (w/v) sucrose in $H_2O$ and 0.5% bromophenol blue and volumes of 50 µl were loaded into the wells with an autopipette. Tris-acetate electrophoresis buffer was added to the gel apparatus and a voltage of 10 V/cm was applied. Each gel was run for 1.5 hrs and stained in 0.5 µg/mlethidium bromide for 30 min and visualized on an ultraviolet transilluminator (UV Products, California) at 265 nm. The gels were photographed on Kodak Tri-X 12.2 cm x 10.7 cm film with a Linhoff Technika lens and Kodak Wratten 23A filter with 5 min exposure. The film was developed in Kodak D-19 developer for 5 min, fixed in Ilford Hypon fixer (5 min), washed under running water (10 min) and dryed at room temperature.

# RESULTS

## 1. EXTRACTION AND PURIFICATION OF DNA

All strains used in the hybridization experiments except NZP 4009, ATCC 9930, 59A2, NZP 5462, NZP 5434 and USDA 257 were lysed using the method described in Method 2.2. Yields of DNA varied from 173 µg/ml to 538 µg/ml for the F4-1 fractions. Appendix Table AI shows a representative sample of DNA yields and spectral ratios. Spectral ratios which were 1.8 or greater were considered satisfactory. Only DNA from the F4-1 fractions was used in the hybridizations.

Strains ATCC 9930, 59A2, NZP 4009, NZP 5462 and USDA 257 were found to produce a greater yield of DNA with improved spectral ratios, when lysed by an alternative method as in Method 2.31 (Fischer & Lerman 1979).

Strain NZP 5434 proved the most difficult to lyse and was resistant to lysis by the above methods and the method of Zaenen *et al* 1974 (Method 2.32). When the cells were subjected to freeze-thaw conditions (Method 2.33) they could then be lysed by the method of Jarvis *et al* (1980) (Method 2.2). Table V shows the yield and the spectral ratios of DNA when cells of strain NZP 5434 were lysed by the four methods. The greatest yield of DNA was obtained by lysing the cells by Method 2.33. The purity of the DNA was considered acceptable.

## 2. CROSS-NODULATION OF LEGUME SPECIES

The results of the nodulation tests using six species of legume showed there was no cross-nodulation between rhizobia that nodulate *Galega officinalis* and the other legume species (Table VI)

TABLE V: A COMPARISON OF YIELDS AND PURITY OF DNA FROM STRAIN NZP 5434 OBTAINED BY FOUR DIFFERENT METHODS OF CELL LYSIS

| Methods of Lysis | Fraction Number | DNA concentration (µg/ml) | Special Ratios 258/230 | 258/280 |
|---|---|---|---|---|
| Method 2.2 | F4 - 1 | 64 | 1.4 | 1.2 |
| Method 2.3 1 | F4 - 1 | 90 | 1.3 | 1.5 |
| | F4 - 2 | 43 | 1.2 | 1.3 |
| | F4 - 3 | 26 | 1.1 | 1.2 |
| Method 2.3 2 | F4 - 1 | 55 | 1.2 | 1.3 |
| Method 2.3 3 | F4 - 1 | 138 | 1.8 | 1.7 |

All of the 11 *Galega* rhizobia nodulated *G. officinalis*, 9 of these forming effective nodules. Plate 1 shows the effective nodulation of *Galega officinalis* by *Galega* rhizobia None of the six fast-growing soybean nodulating rhizobia tested, formed nodules on *G. officinalis*.

All the fast-growing soybean rhizobia formed ineffective nodules on *Glycine max* cv. Matara. Strain USDA 191 formed nodules approximately 5 mm in diameter, whereas the other strains formed smaller nodules approximately 1-2 mm in diameter. The slow-growing soybean nodulating strain, *Bradyrhizobium japonicum* ATCC 10324 formed effective nodules on this cultivar of *Glycine max* (Plate 2).

No cross-nodulation was found between the fast-growing soybean nodulating rhizobia and four of the other legume species clover, lucerne, *Galega officinalis* and *Leucaena leucocephala*. These strains were found to be able to form ineffective nodules on *Lotus pedunculatus* Grassland's Maku. All of the seven strains tested formed such nodules, which were either white or pink in colour and varied in size, depending on the strain. No viable rhizobia could be isolated from these nodules. There was no detectable acetylene reduction by any of the *Lotus pedunculatus* plants when tested by gas chromatography. By comparison, strain NZP 2037 formed effective nitrogen-fixing nodules on *Lotus pedunculatus* (Plate 3) as determined by dry-weight measurements of the plant tops and by gas chromatography.

## 3. PHAGE-TYPING

The phage-typing patterns of the *Galega* rhizobia and the fast-growing soybean nodulating rhizobia are shown in Table VII. The *Galega* rhizobia formed a distinct phage-typing group with all of the six strains tested being susceptible to the wide host-range type *Galega* phage Øgal R/1. Four

TABLE VI: NODULATION OF LEGUME SPECIES BY RHIZOBIA

| Legumes | Rhizobial Strains* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Fast-growing soybean | *Bradyrhizobium japonicum* | *Galega* strains | *R. leguminosarium* | *R. meliloti* | *R. loti* | *Leucaena* strains |
| *Galega officinalis* | 0/6 | | 11/11 | | | | |
| *Glycine max* | 11/11 | 1/1 | 0/3 | | | | |
| *Trifolium repens* | 0/8 | | 0/8 | 2/2 | | | |
| *Medicaco sativa* | 0/8 | | 0/10 | | 3/3 | | |
| *Lotus pedunculatus* | 7/7 | | 0/10 | | | 3/3 | |
| *Leucaena leucocophala* | 0/6 | | 0/3 | | | | 2/2 |

* This indicates the number of strains that nodulate a particular legume species.

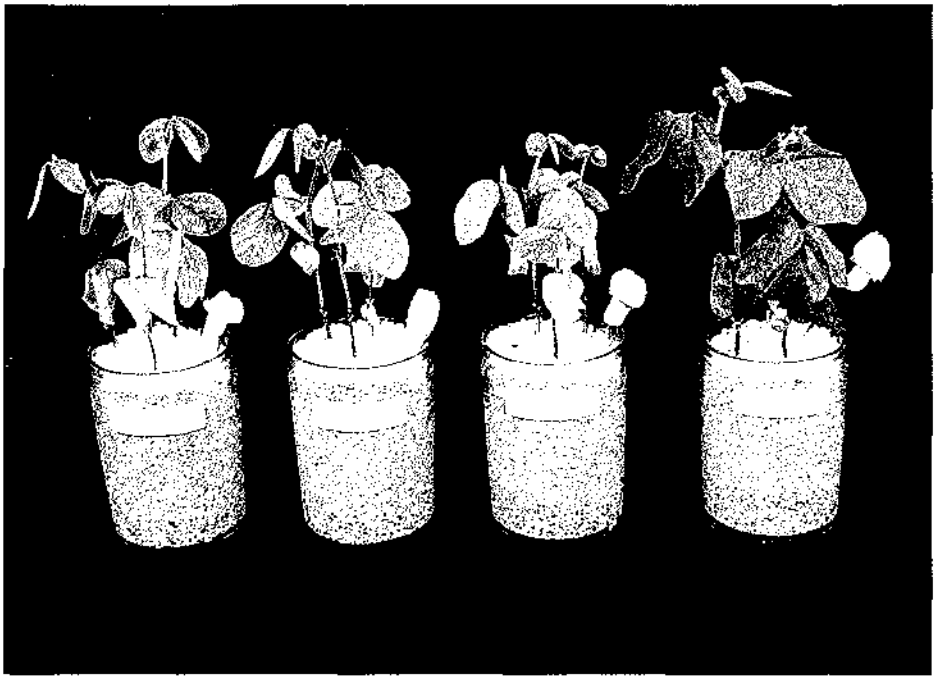
PLATE 1: Nodulation of *Galega officinalis* by *Galega* rhizobia.
From left to right: -N control, *Galega* strains gal NW 1 and 59A2.

1

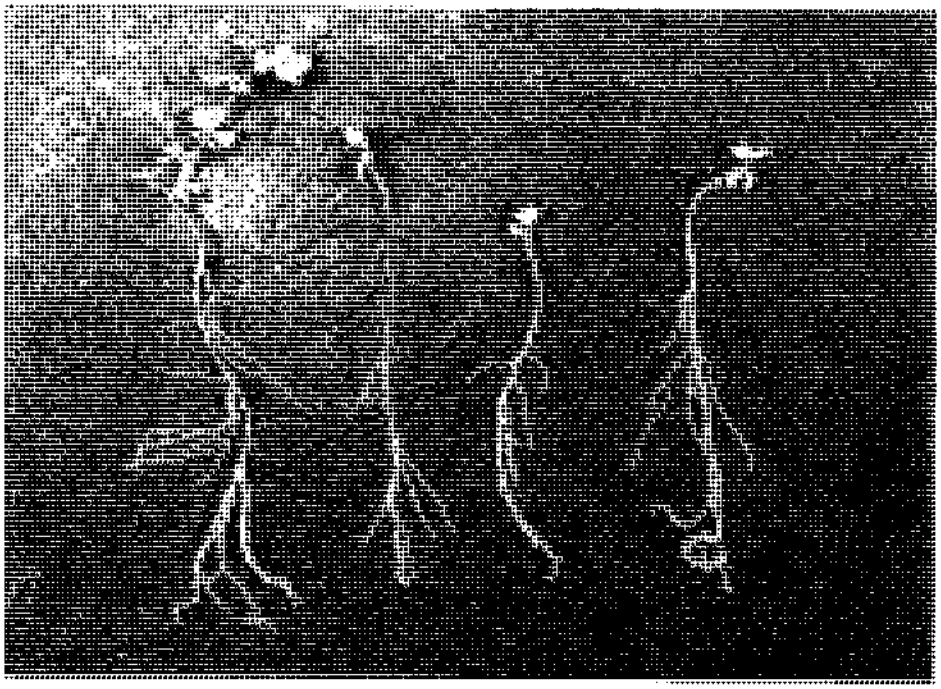
1

PLATE 2: Nodulation of *Glycine max* by fast and slow-growing soybean strains.
From left to right: -N control, fast-growing soybean strains USDA 192, USDA 205 and *Bradyrhizobium japonicum* ATCC 10324.

PLATE 3: Nodulation of *Lotus pedunculatus* by *R. loti* and fast-growing soybean rhizobia.
From left to right: *R. loti* NZP 2037, fast-growing soybean strains USDA 208, USDA 217 and -N control.



2



3

2

3

TABLE VII: SUSCEPTIBILITY OF *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS TO RHIZOBIAL PHAGES

| Phage | Rhizobial Strains* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Fast-growing soybean rhizobia | *Bradyrhizobium japonicum* | *Galega* rhizobia | *R. leguminosarum* | *Cononilla varia* rhizobia | *R. meliloti* | *R. loti* | Slow-growing *Lotus* rhizobia | *Leucaena* rhizobia |
| Clover ϕD2 | 0/11 | 0/1 | 0/2 | 3/4 | 0/1 | 0/4 | 0/3 | 0/2 | 0/2 |
| Lucerne ϕRM 1 | 0/11 | 0/1 | 0/2 | 0/2 | 0/1 | 2/4 | 0/3 | 0/2 | 0/2 |
| *Lotus* ϕ2037/1 | 0/11 | 0/1 | 0/2 | 0/2 | 0/1 | 0/4 | 4/5 | 0/2 | 0/2 |
| *Galega* ϕgal 1/R | 0/11 | 0/1 | 6/6 | 0/2 | 0/1 | 0/4 | 0/3 | 0/2 | 0/2 |

* This indicates the number of strains susceptible to lysis by the bacteriophages.

of these were highly susceptible, while the remaining two were less susceptible. This phage did not lyse any of the 26 other rhizobia tested. These rhizobia which included the fast-growing soybean strains, represented the major taxonomic groups. None of the *Galega* rhizobia tested were found to be susceptible to any of the other phages. Phage ØD2 has a wide host-range range and can lyse *Rhizobium trifolii* , *R. leguminosarum* and *R. phaseoli* strains, the *Lotus* phage Ø2037/1 is specific for fast-growing *Lotus* rhizobia (*R. loti*) and related organisms and the lucerne phage ØRM 1 is specific for strains of *R. meliloti*. In this study, phage ØD2 lysed 3/4 *R. trifolii* strains, phage ØRM1 lysed 2/4 *R. meliloti* strains and phage Ø2037/1 lysed 4/5 *R. loti* strains.

None of these bacteriophages lysed any of the 11 fast-growing soybean nodulating rhizobia. Since it was not possible to isolate a phage capable of lysing this group of rhizobia, these strains could not be shown to form a distinct phage-typing group, as did the *Galega* rhizobia.

## 4. DNA HOMOLOGY BETWEEN *GALEGA* RHIZOBIA, FAST-GROWING SOYBEAN NODULATING RHIZOBIA AND OTHER SPECIES OF *RHIZOBIUM* AND *BRADYRHIZOBIUM*

### 4.1 Labelled DNA Preparations

A total of six labelled reference strains were prepared. These were: *Galega* strains, gal 1 and gal NW 3; fast-growing soybean strains, USDA 191 and USDA 208; *R. leguminosarum* strain ATCC 10004 and *R. meliloti* strain NZP 4009.

4.11 Specific Activities: The specific activities of labelled DNA preparations were as follows (counts/min/µg DNA): gal 1, 70,053; gal NW 3, 53,112; USDA 191, 47,800: USDA 208, 77,105; ATCC 10004; 40,936 and NZP 4009, 40,650.

When the labelled DNA was diluted to a concentration of 2 µg/ml, a total of 2,000 - 4,000 counts per hybridization comparison resulted. During the period of use of a particular DNA preparation, if the counts dropped below 2,000, the concentration of labelled DNA was increased to 4 µg/ml.

### 4.12 DNA Hybridization

The reassociation of labelled DNA with homologous DNA at 65°C was as follows: gal 1, 71.2%; gal NW 3, 91.1%; USDA 191, 92.0%; USDA 208, 66.4%; ATCC 10004, 87.7% and NZP 4009, 75.9%. At 80°C the corresponding value for USDA 208 was 49.7%.

The reassociation of labelled DNA fragments with each other in the absence of unlabelled DNA at 65°C was as follows: gal 1, 22.5%; gal NW 3, 6.8%; USDA 191, 7.7%; USDA 208, 2.8%; ATCC 10004, 14.5% and NZP 4009, 23.4%. At 80°C the corresponding value for USDA 208 was 1.4%.

## 4.2 DNA Homology

### 4.21 DNA homology between *Galega* strains and other rhizobia

The percentage relative homology of 11 strains of *Galega* rhizobia with gal 1 reference DNA at 65°C ranged from 58% to 106% (Table VIII). The mean relative homology of this group was 77 ± 14% (mean ± group standard deviation). DNA from the gal 1 reference strain showed a significantly ('Student's' t test, $P<0.001$) lower degree of homology with other groups of rhizobia. The mean relative homology of gal 1 DNA to the fast-growing soybean strains was 19 ± 10%; *Rhizobium leguminosarum*, 22%; *R. meliloti*, 8%; *R. loti*, 10%; NZP 5462 (Homology group 2-*Coronilla*), 14%; *Leucaena* rhizobia, 10% and the slow-growing soybean strain *Bradyrhizobium japonicum* ATCC 10324, 2%. The fast-growing

TABLE VIII: RELATIVE HOMOLOGY OF DNA FROM *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS WITH REFERENCE DNA FROM STRAIN GAL 1 OF *GALEGA OFFICINALIS*

| Strain | DNA Homology (%) 65°C | ΔTm(e) (°C) |
|---|---|---|
| *Rhizobium of Galega* | | |
| gal 1 | 100 | 0 |
| gal 3 | 77 | |
| gal 7 | 77 | |
| gal 12 | 67 | 2.7 |
| gal 14 | 58 | 1.8 |
| gal 129 | 73 | |
| gal 1261 | 84 | |
| gal NW 1 | 76 | |
| gal NW 2 | 83 | 0.7 |
| gal NW 3 | 106 | 0.1 |
| 59A2 | 70 | 2.0 |
| Group mean | 79 ± 14 | |
| Fast-growing soybean nodulating rhizobia | | |
| USDA 191 | 16 | |
| USDA 192 | 19 | |
| USDA 193 | 18 | |
| USDA 194 | 25 | |
| USDA 201 | 10 | |
| USDA 205 | 16 | |
| USDA 206 | 14 | |
| USDA 208 | 14 | |
| USDA 214 | 9 | |
| USDA 217 | 20 | |
| USDA 257 | 47 | |
| Group mean | 19 ± 10 | |
| Group 1* clover-pea | | |
| *R. leguminosarum* | | |
| ATCC 10004 | 27 | |
| NZP 561 | 17 | |
| Group mean | 22 | |

* DNA homology groups (Crow *et al* 1981)

Cont'd..

TABLE VIII: CONTINUED

| Strain | DNA Homology (%) 65°C |
|---|---|
| Group 2* *Coronilla* | |
| NZP 5462 | 14 |
| Group 3* lucerne | |
| *R. meliloti* | |
| NZP 4009 | 12 |
| ATCC 9930 | 3 |
| Group mean | 8 |
| Group 4* *Lotus* | |
| *R. loti* | |
| NZP 2037 | 10 |
| *Rhizobium* of *Leucaena* | |
| NZP 5434 | 11 |
| NZP 5259 | 8 |
| Group mean | 10 |
| Slow-growing soybean nodulating rhizobia | |
| *Bradyrhizobium japonicum* | |
| ATCC 10324 | 2 |
| *Streptococcus lactis* 1 | 0 |

* DNA homology groups (Crow *et al* 1981)

soybean strain USDA 257 exhibited a relative homology of 47% with gal 1 reference DNA, which was greater than the mean for the group.

When gal NW 3 was used as a reference strain, the relative homology at 65° C of the *Galega* rhizobia ranged from 71% to 98%, with a mean of 85 ± 8%. The relative homology of gal 1 reference DNA to the other groups of rhizobia was significantly lower. The fast-growing soybean rhizobia gave a mean relative homology of 38 ± 12%; *R. leguminosarum*, 44%; *R. meliloti*, 22%; *R. loti*, 16 ± 2%; NZP 5462 (Homology group 2- *Coronilla*), 25% and the *Leucaena*rhizobia, 24%. The relative homology with the slow-growing rhizobia was 16% with *Bradyrhizobium japonicum* ATCC 10324 and a mean of 11 ± 5% for the *Lotus* rhizobia.

In general, the relative homologies of DNA to the gal NW 3 reference strain were higher than the corresponding relative homologies to the gal 1 reference strain.

The relationship of the *Galega* rhizobia to other homology groups was further studied by hybridization with a *R. leguminosarum* and *R. meliloti* reference strain. The mean relative homology at 65 C of 14 strains of *Galega* with *R. leguminosarum* strain ATCC 10004 was 26 ± 7% and 20 ± 5% with *R. meliloti* strain NZP 4009. By comparison, *R. leguminosarum* strain NZP 561 shared a relative homology of 73% with ATCC 10004. The *R. meliloti* strain ATCC 9930 strain shared 92% homology with the NZP 4009 reference strain.

## 4.22 DNA homology between fast-growing soybean nodulating strains and other rhizobia

The percentage relative homology at 65° C and 80° C of the fast-growing soybean nodulating rhizobia with other fast-growing and the slow-growing rhizobia is given in Table IX. The relative homology of 11 strains of fast-growing soybean nodulating rhizobia with DNA from USDA 208 at 65° C ranged

TABLE IX: RELATIVE HOMOLOGY OF DNA FROM *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS WITH REFERENCE DNA FROM THE FAST-GROWING SOYBEAN NODULATING STRAIN, USDA 208

| Strain | 65°C | 80°C | TBI | ΔTm(e)(°C) |
|---|---|---|---|---|
| Fast-growing soybean nodulating rhizobia | | | | |
| USDA 208 | 100 | 100 | 1.00 | 0 |
| USDA 191 | 84 | 76 | 0.91 | 0.7 |
| USDA 192 | 90 | 90 | 1.00 | 0.5 |
| USDA 193 | 88 | 94 | 1.07 | |
| USDA 194 | 52 | 26 | 0.49 | 6.3 |
| USDA 201 | 51 | 29 | 0.56 | 6.5 |
| USDA 205 | 92 | 91 | 0.99 | |
| USDA 206 | 92 | 92 | 1.00 | |
| USDA 214 | 92 | 84 | 0.91 | |
| USDA 217 | 85 | 85 | 1.00 | 0.3 |
| USDA 257 | 56 | 35 | 0.62 | |
| Group Mean | 80 ± 18 | 73 ± 28 | 0.87 | |
| *Galega* rhizobia | | | | |
| gal 1 | 10 | 1 | 0.08 | 14.0 |
| gal 3 | 10 | 2 | 0.22 | |
| gal 7 | 10 | 3 | 0.32 | |
| gal 12 | 8 | 3 | 0.40 | |
| gal 14 | 10 | 3 | 0.24 | |
| gal 129 | 10 | 2 | 0.22 | |
| gal 1261 | 10 | 1 | 0.08 | |
| gal NW 1 | 13 | 3 | 0.23 | |
| gal NW 2 | 13 | 4 | 0.28 | |
| gal NW 3 | 10 | 2 | 0.17 | 14.7 |
| 59A2 | 9 | 2 | 0.26 | |
| NZP 5562 | 18 | 7 | 0.38 | |
| NZP 5563 | 19 | 11 | 0.56 | |
| NZP 5564 | 23 | 12 | 0.53 | |
| Mean | 12 ± 5 | 4 ± 3 | 0.28 | |

Cont'd...

TABLE IX: Continued

| Strain | 65°C | 80°C | TBI | ΔTm(e)(°C) |
|---|---|---|---|---|
| Group 1 clover-pea - *R. leguminosarum* | | | | |
| ATCC 10004 | 12 | 7 | 0.55 | 14.0 |
| NZP 561 | 11 | 3 | 0.24 | |
| Group 2 *Coronilla* | 12 | 5 | | |
| NZP 5462 | 12 | | | |
| Group 3 Lucerne - *R. meliloti* | | | | |
| NZP 4009 | 26 | 5 | 0.20 | 11.3 |
| ATCC 9930 | 22 | 6 | 0.26 | |
| | 24 | 6 | | |
| Group 4 *Lotus* | | | | |
| NZP 5201 | 10 | | | |
| NZP 2213 | 7 | 3 | 0.36 | 14.0 |
| NZP 2037 | 6 | 3 | 0.52 | |
| Mean | 8 ± 2 | 3 | 0.44 | |
| *Leucaena* rhizobia | | | | |
| NZP 5434 | 12 | | | |
| NZP 5259 | 9 | 1 | 0.09 | |
| | 11 | | | |
| Slow-growing rhizobia | | | | |
| Slow-growing *Lotus* rhizobia | | | | |
| NZP 2309 | 6 | 2 | 0.36 | |
| NZP 2257 | 3 | 0 | 0.00 | |
| Group mean | 5 | 1 | | |
| *Bradyrhizobium japonicum* | | | | |
| ATCC 10324 | 4 | 2 | 0.64 | 15.5 |
| *Streptococcus lactis* H1 | 0 | 0 | | |

from 51% to 92%. The mean for this group was 80 ± 18%. The mean relative homology of USDA 208 DNA to DNA from other fast-growing strains was significantly lower. The mean relative homology of USDA 208 DNA to the *Galega* rhizobia was 12 ± 5%; *R. leguminosarum*, 12%; NZP 5462 (Group 2-*Coronilla*), 12%; *R. meliloti*, 24%; *R. loti*, 8 ± 2%, *Leucaena* rhizobia, 11%. The relative homology with the slow-growing rhizobia was 5 ± 2% with the slow-growing *Lotus* rhizobia and 4% with the slow-growing soybean strain *Bradyrhizobium* ATCC 10324.

Three of the fast-growing soybean nodulating rhizobia shared less homology with the USDA 208 reference strain than the soybean group as a whole. Strains USDA 194, USDA 201, and USDA 257 give relative homologies of 52%, 51% and 56% respectively.

The mean relative homology of the fast-growing soybean nodulating rhizobia with USDA 208 at 80 C was 73 ± 28%. The mean relative homology at 80°C of USDA 208 DNA with the *Galega* rhizobia was 4 ± 3%; *R. leguminosarum*, 5%; *R. meliloti*, 6%; *R. loti*, 3% and the *Leucaena* rhizobia, 1%. The relative homology with the slow-growing rhizobia was 1% for the slow-growing *Lotus* rhizobia and 2% with ATCC 10324.

The fast-growing soybean nodulating rhizobia formed a distinct homology group when the hybridizations were performed at both 65°C and 80°C. The relative homology obtained at 65°C, divided by the relative homology at 80°C, is known as the thermal binding index (TBI) and is a measure of the base sequence similarity between the two DNA samples. When the TBI is close to a value of 1.00, this indicates considerable base sequence similarity between the labelled reference DNA and the unlabelled heterologous DNA. Conversely, a low TBI value indicates a low degree of base sequence similarity. The mean TBI of the fast-growing soybean rhizobia with USDA 208 was 0.87 compared to 0.28 for the

*Galega* group of rhizobia; *R. leguminosarum*, 0.40; *R. meliloti* 0.23; *R. loti* , 0.44; *Leucaena* rhizobia, 0.09. The TBI values for the slow-growing rhizobia was 0.18 for the *Lotus* rhizobia and 0.64 for ATCC 10324.

When USDA 191 was used as a reference strain, the relative homology at 65 °C of the fast-growing soybean nodulating rhizobia ranged from 66% to 92%, with a mean of 86 ± 10%. The other groups of rhizobia showed significantly lower homology with this reference strain. The *Galega* rhizobia gave a mean relative homology of 28 ± 10%; *R. leguminosarum*, 32%; NZP 5462 (Homology group 2 - *Coronilla* ), 22%; *R . meliloti* , 44%; *R. loti*, 20 ± 5%; *Leucaena* rhizobia, 24%. The mean relative homology with the slow-growing rhizobia was 9 ± 2% with the *Lotus* rhizobia and 21% with ATCC 10324. In general, the relative homologies of DNA to the USDA 191 reference strain were higher than the corresponding relative homologies to USDA 208.

The *Galega* strain gal 1261 appeared to share greater homology with USDA 191 than to USDA 208, with relative homologies at 65°C of 52% and 10% respectively.

The fast-growing soybean nodulating rhizobia gave a mean relative homology at 65°C with the *R. leguminosarum*reference strain ATCC 10004 of 30 ± 7% and 41 ± 2% with the *R. meliloti* reference strain NZP 4009.

The relative homology of DNA from the *Rhizobium* and *Bradyrhizobium* strains used in the hybridization experiments, with each of the six reference strains, is shown in Appendix Tables AII and AIII.

## 4.3 Thermal Stability of Reassociated DNA Duplexes

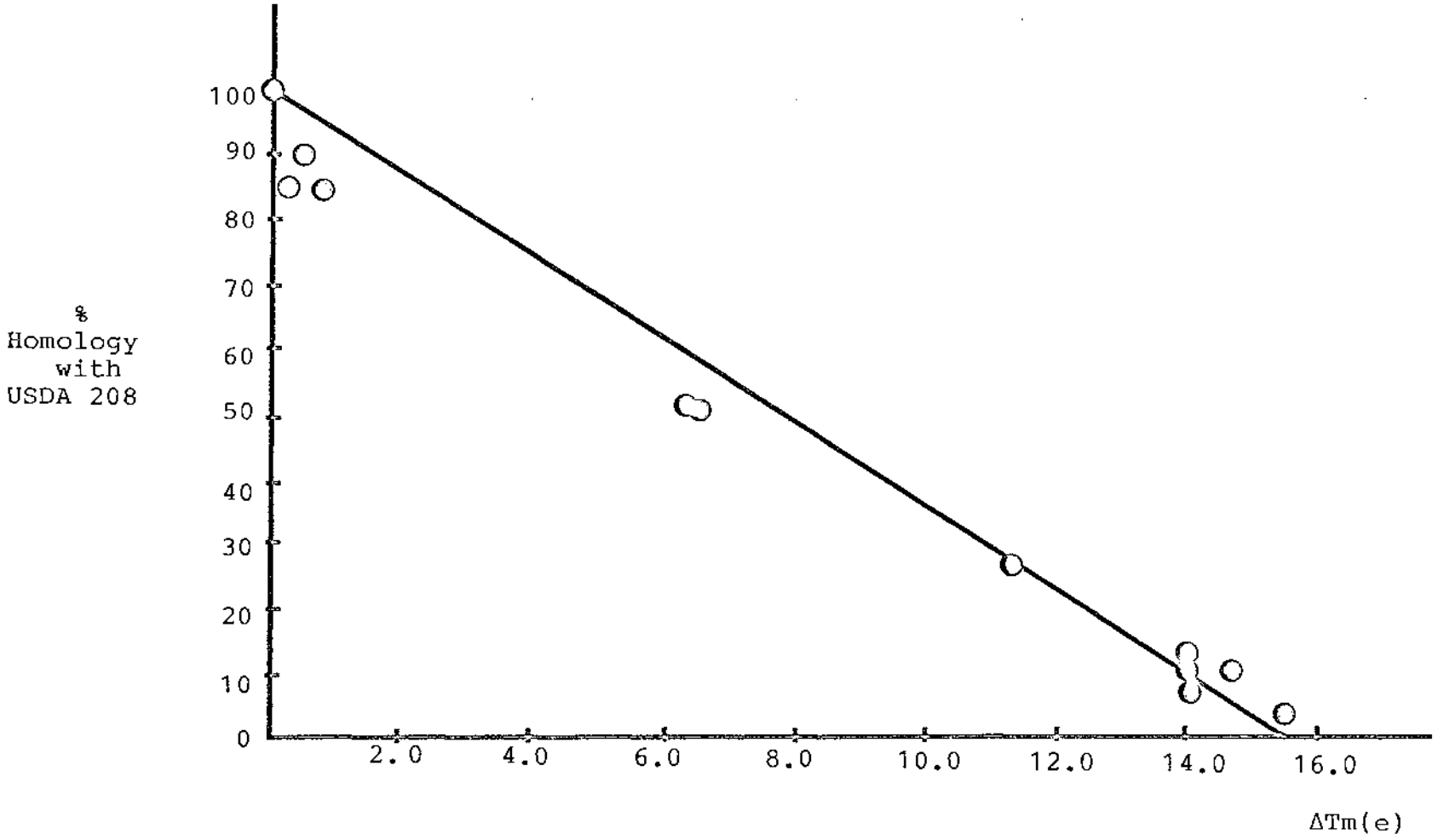
When the gal 1 reference DNA was hybridized with homologous DNA the Tm(e) was 92.0 C. The $\Delta$Tm(e) values for the *Galega* rhizobia with gal 1 as reference strain ranged from 0 to 2.7°C as shown in Table VIII, with corresponding relative homologies at 65°C ranging from 100% to 67%. When the USDA 208 reference DNA was hybridized with homologous DNA the Tm(e) was 91.8 C. The $\Delta$Tm(e) values for 12 reassociated DNA duplexes are given in Table IX. Strains that showed a high degree of homology with the USDA 208 reference strain gave a low $\Delta$Tm(e) value, whereas strains showing a lower degree of homology had a correspondingly larger $\Delta$Tm(e) value. The $\Delta$Tm(e) values of the fast-growing soybean strains ranged from 0 to 6.5°C. Strains USDA 194 and USDA 201 showed the largest $\Delta$Tm(e) values corresponding with their lower relative homologies with USDA 208.

A linear relationship was found between the percentage relative homology of heterologous DNAs with a particular reference DNA and the corresponding $\Delta$Tm(e) values. Figure 1 shows the relative homology at 65°C of 12 strains of rhizobia with reference strain USDA 208, plotted versus their corresponding $\Delta$Tm(e) values.

The $\Delta$Tm(e) values of six *Galega* rhizobia with gal NW 3 as reference strain ranged from 0 to 4.4°C with corresponding relative homologies at 65°C of 100% and 71% respectively. By comparison, the slow-growing *Lotus* strain NZP 2192 which had a DNA homology of 9% at 65°C had a $\Delta$Tm(e) value of 16.3°C and *R. loti* NZP 2213 with 19% homology showed a $\Delta$Tm(e) value of 14.0°C.

The $\Delta$Tm(e) values of DNA duplexes formed between five of the fast-growing soybean rhizobia and reference strain USDA 191 ranged from 0 to 1.3°C with corresponding relative homologies of 100% and 90% respectively. The USDA 257 strain

FIGURE 1: DNA homology versus ΔTm(e)



which gave a relative homology of 70% with USDA 191 had a ΔTm(e) value of 6.2 °C. The *Galega* strain gal 1261 which showed a relative homology with USDA 191 of 52% had a ΔTm(e) value of 5.6, whereas the *Galega* strain 59A2 with a relative homology of 21% gave ΔTm(e) value of 14.5 °C. The slow-growing *Lotus* strain NZP 2257 gave a ΔTm(e) value of 16.0°C corresponding with a low DNA homology of 8%.

The three *Galega* strains tested; gal 1, gal 14 and gal NW 3 gave a mean ΔTm(e) value of 11.5°C with *R. leguminosarum* ATCC 10004 as reference strain and 9.7°C with *R. meliloti* NZP 4009. The three fast-growing soybean nodulating strains; USDA 191, USDA 192 and USDA 194 gave a mean ΔTm(e) value of 8.9°C with ATCC 10004 and a mean ΔTm(e) value of 8.7°C with NZP 4009. In the reciprocal arrangement, when NZP 4009 was hybridized with USDA 191 as reference strain, the ΔTm(e) of the DNA duplex was 11.0°C. The ΔTm(e) value of the DNA duplex formed between *R. leguminosarum* NZP 561 and the ATCC 10004 reference strain was 3.2°C, while *R. meliloti* ATCC 9930 gave a ΔTm(e) value of 0.0 with NZP 4009.

## 4.4 Relationship between the *Galega* Rhizobia, the Fast-Growing Soybean Nodulating Rhizobia and other Groups of Rhizobia

The percentage relative homology of *Rhizobium* strains with gal NW 3 at 65°C was plotted versus the percentage relative homology with gal 1 (Figure 2). The *Galega* rhizobia clustered together to form a distinct DNA homology group which was distinguishable from other groups of rhizobia. The fast-growing soybean strain USDA 257 was the closest strain to this group.

The percentage relative homology of *Rhizobium* and *Bradyrhizobium* strains with USDA 191 at 65°C was plotted versus the corresponding homology with USDA 208 (Figure 3). Eight

FIGURE 2: Homology of *Rhizobium* strains with gal NW 3 and gal 1 reference strains at 65°C

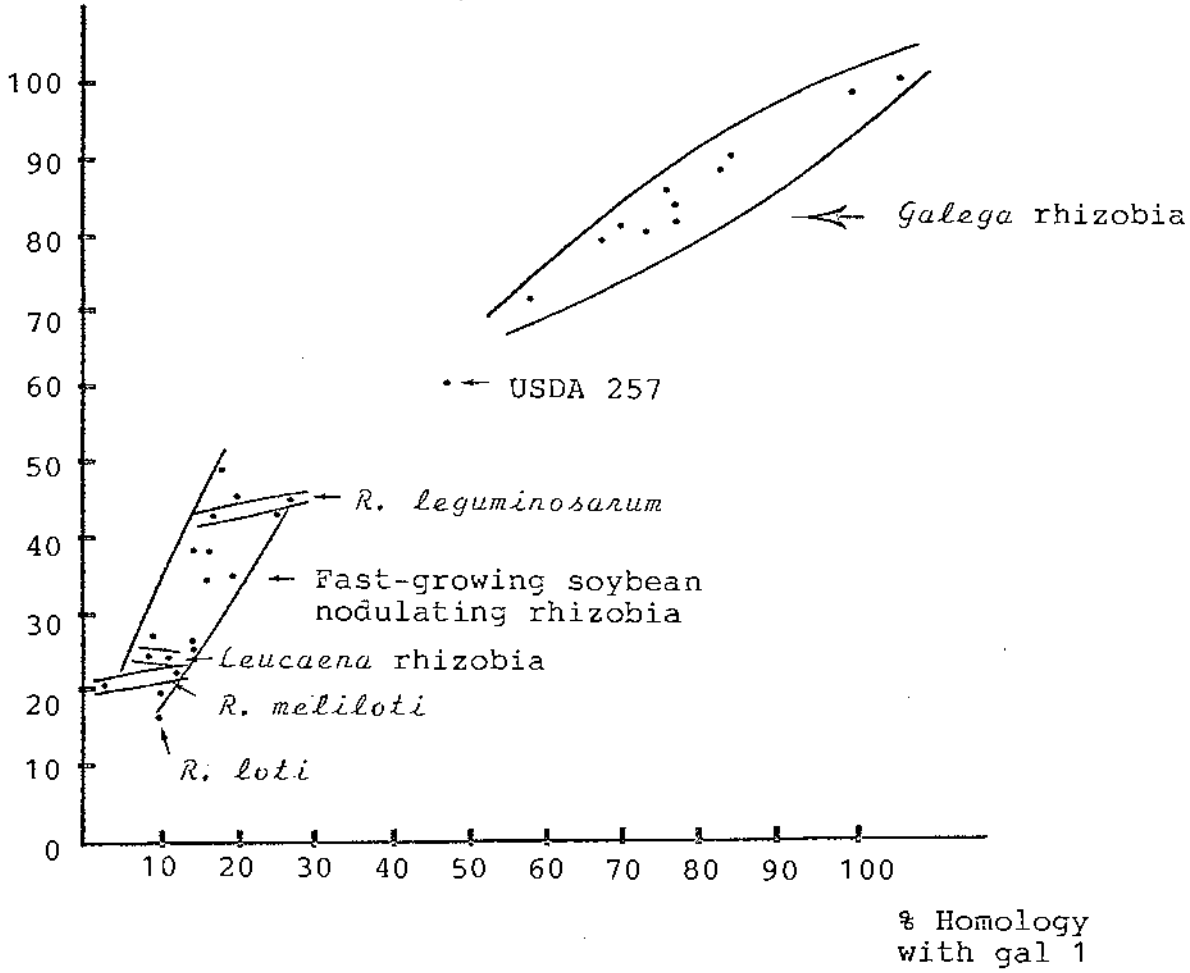
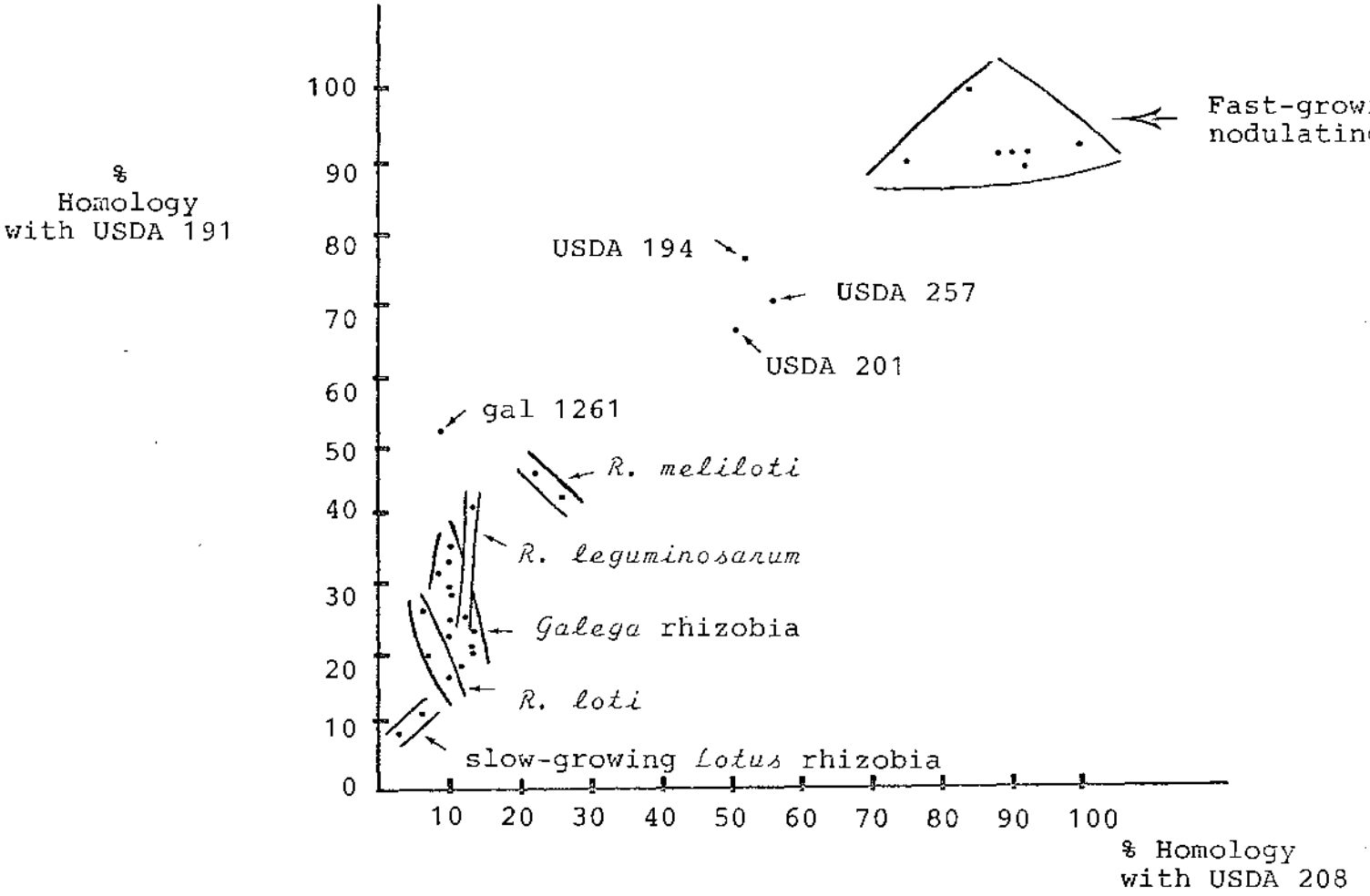


FIGURE 3: DNA homology of *Rhizobium* and *Bradyrhizobium* strains with USDA 191 and USDA 208 reference strains at 65°C



of the eleven fast-growing soybean nodulating strains formed a distinct cluster which was quite separate from other groups of rhizobia. Three of the fast-growing soybean strains; USDA 194, USDA 201 and USDA 257 appeared to form a separate cluster and represent a subgroup within the main DNA homology group.

Figure 4 shows the percentage relative homology at 65° C of *Rhizobium* strains with gal 1 versus the homology with USDA 208. This indicated that the *Galega* rhizobia and the fast-growing soybean nodulating rhizobia were distinct from each other and both groups were unrelated to other species of rhizobia.

## 5. DIFFICULTIES ENCOUNTERED DURING DNA HYBRIDIZATION

### 5.1 Hybridizations at 80°C

The relative homology of heterologous DNA with a given reference DNA at 80°C, should be less than the relative homology obtained at 65°C. Only when closely related DNA are hybridized, will the relative homology at 80°C be similar to that at 65°C. During the hybridization experiments an interesting and perplexing problem arose. Some of the hybridizations, when repeated at the more stringent temperature of 80° C, produced relative homology values greater than the corresponding 65° C values. This problem occurred with four of the six labelled DNA preparations. Table X shows a representative sample of homology values illustrating this problem. Since the relative homology values at 80° C were greater than those obtained at 65° C, the TBI values were greater than 1.00. Clearly this was anomalous since TBI values are expected to range between 0.00 and 1.00.

FIGURE 4: DNA homology of *Rhizobium* strains with gal 1 and USDA 208 reference strains at 65°C

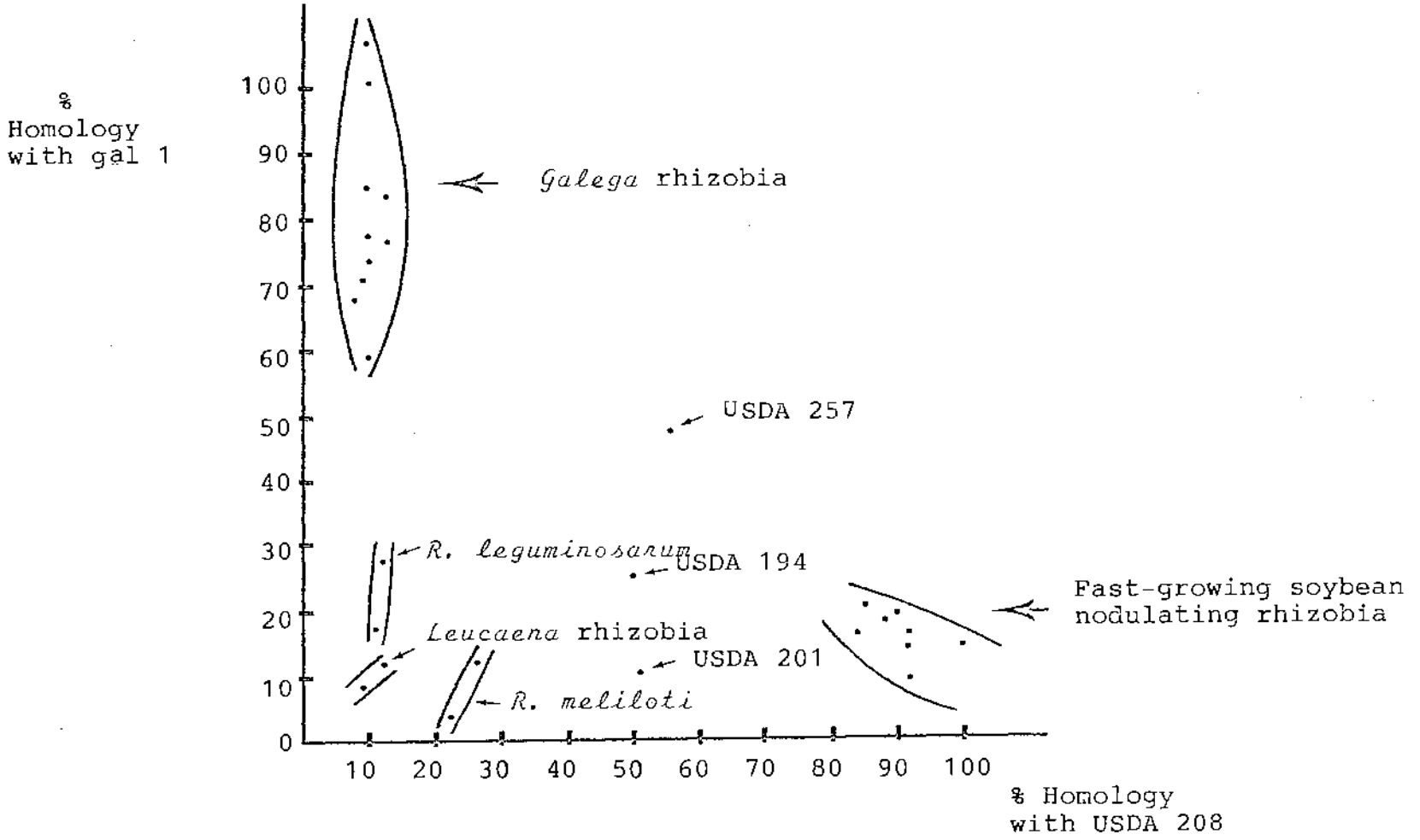


TABLE X: RELATIVE HOMOLOGY AT 65°C AND 80°C OF DNA FROM STRAINS OF *GALEGA* RHIZOBIA, FAST-GROWING SOYBEAN RHIZOBIA AND *R. LEGUMINOSARUM* WITH REFERENCE DNA FROM GAL 1

| *Rhizobium* Strain | Relative Homology (%) 65°C | 80°C | TBI |
|---|---|---|---|
| Rhizobia of *Galega* | | | |
| gal 1 | 100 | 100 | 1.00 |
| gal 3 | 77 | 63 | 0.82 |
| gal 14 | 58 | 40 | 0.59 |
| Fast-growing soybean nodulating rhizobia | | | |
| USDA 191 | 12 | 21 | 1.31 |
| USDA 192 | 19 | 25 | 1.32 |
| USDA 205 | 16 | 29 | 1.81 |
| USDA 208 | 14 | 39 | 2.79 |
| *R. leguminosarum* | | | |
| ATCC 10004 | 27 | 19 | 0.70 |
| NZP 561 | 17 | 19 | 1.12 |

## 5.2 Attempts to Resolve the Problem

When a particular labelled DNA was hybridized with heterologous DNA, only some of the relative homology values obtained at 80° C were greater than the values obtained at 65° C. In general, when the DNA from the *Galega* rhizobia was hybridized with the labelled *Galega* strains gal 1 and gal NW 3, the 80° C relative homology values were either similar or less than the corresponding 65°C values. A similar situation occurred with the fast-growing soybean nodulating strains hybridized with the USDA 191 reference strain. Thus it appeared that the problem didn't occur when closely related DNAs were hybridized together. The problem occurred when the heterologous DNA was more distantly related to the reference DNA.

A number of possible explanations were proposed to explain the anomalous results. When the *Galega* strain gal NW 3 was used as a reference strain, a number of the homology values at 80°C were higher than the corresponding 65°C values. One possible explanation was, that prior to hybridizing the unlabelled DNA with the labelled DNA, the two strands of the double-stranded labelled DNA failed to separate during the thermal denaturation process. The hybridization at 80° C were carried out in Quickfit tubes and normally about 15 tubes containing hybridization mixtures, were placed in a beaker of boiling water and boiled for 10 min to denature the double-stranded DNA. These Quickfit tubes were thicker walled than the Kimax tubes used for the hybridizations at 65° C. Therefore, it was possible that some of the tubes were not being heated sufficiently to melt the DNA completely and this might account for the apparent high degree of hybridization at 80° C. To test for this, six hybridization mixtures were set up at 80° C. These included a homologous and a label only control. The six tubes were placed in a beaker of boiling water and boiled for 10 min. Particular attention was given to the denaturing process and the water was maintained vigorously boiling

for the entire 10 min period. The hybridization mixtures were separated following the normal procedure (Method 3.2). The relative homology of heterologous DNA to the gal NW 3 reference DNA were found to be similar to that previously found, with TBI value greater than 1.00.

All the hybridizations at 65°C using the gal NW 3 reference strain were performed using a labelled DNA concentration of 2 µg/ml, whereas a label concentration of 4 µg/ml was used for the 80°C hybridizations. The label concentration was increased in order to allow for the decay of the label and to maintain an acceptable level of radioactivity in each hybridization mixture. Since this was the only hybridization factor that had been altered, the effect of label concentration on the relative homologies at 80° C needed to be investigated. A total of six hybridization mixtures were set up at 80°C using a label concentration of 2 µg/ml, allowed to hybridize and then separated. A comparison of the relative homologies at 80°C showed no significant differences between the two different concentrations of label used.

The problem of unacceptable table high 80°C homology values also occurred when USDA 191 was used as a reference strain. It was proposed that the Quickfit tubes used for the hybridizations at 80° C were too large. Since a large proportion of the tube remained above the water level in the water bath during incubation, distillation of water within the tube may have occured, with the collection of pure water droplets along the sides of the tube and on the stopper. Consequently, the ionic strength of the phosphate buffer and the concentration of the DNA would be altered and DNA renaturation could then proceed under conditions which differed from those imposed during the hybridizations at 65°C. To investigate this a total of six hybridization mixtures were set up at 80°C using smaller Quickfit tubes (1.5 x 7 cm) from those that were previously used. The percentage relative homology values were found to be similar to those previously obtained.

A number of reassociated DNA mixtures were separated on fresh HA, in order to determine if the regenerated HA was responsible for the problem. When six reassociated DNA mixtures were separated on fresh HA, the homology results were similar to those obtained when the same mixtures were separated on regenerated HA.

Since it appeared likely that the labelled reference DNA was the source of the problem, the USDA 191 labelled DNA was phenol/chloroform extracted and ethanol precipitated to remove any possible contaminants. This label was used in further hybridizations using homologous USDA 191 DNA, a label only control and DNA from strains NZP 2037, NZP 5259, gal 14 and ATCC 10324. The apparent self-hybridization of the label decreased from 9.7% to 5.8%. The relative homology of the heterologous DNAs were found to have decreased. This decrease however, was only significant with gal 14 DNA, which decreased from 23% to 9%. The relative homology of gal 14 with USDA 191 at 65$^{o}$C was 18% and the revised TBI value was 0.53 compared to 1.31. These results suggested that some contaminant had been removed from the DNA which was responsible for the high 80$^{o}$C homology values. DNA from strains gal 1, gal 3, gal 12 and gal 1261 were hybridized with the phenol/chloroform extracted USDA 191 labelled DNA. The relative homologies of gal 12 and gal 3 with the reference DNA were similar to that previously obtained with the original label. The relative homology of both gal 1 and gal 1261 with the reference DNA decreased giving TBI values of 0.98 and 0.89 compared to 1.06 and 1.60 previously obtained. These results again suggested that a contaminant in the label was responsible for the high 80$^{o}$C values.

The problem of TBI values being greater than 1.00 also occurred with the ATCC 10004 label. An experiment was conducted to determine if the problem was due to non-specific binding of single-stranded DNA to the HA during the separation of

single from double-stranded DNA. The occurrence of significant amounts of non-specific binding to HA could account for the apparent high amounts of hybridization occurring at 80° C. To test for this possibility, a total of six hybridization mixtures, including a homologous and a label only control were re-separated on HA using 0.8% sodium lauryl sulphate in the phosphate buffer, instead of the customary 0.4%, to elute the single-stranded DNA from the HA. No differences in the percentage relative homology values were found.

## 5.3 Reduction of Non-specific Binding of Reference DNA to Hydroxyapatite

An attempt to reduce any non-specific binding of the labelled DNA to the HA was made and the ATCC 10004 label was treated as follows: Undiluted, sonicated ATCC 10004 labelled DNA in 0.28 M PB was incubated at 80° C for 16 hr, denatured at 100° C diluted with an equal amount of deionized water and separated on HA. The single-stranded DNA was eluted from the HA with 0.14 M PB containing 0.4% sodium lauryl sulphate. Any DNA that remained bound to the HA was eluted with 0.4 M PB. The first fraction accounted for 95% of the total radioactive counts. The DNA concentration and specific activity of this fraction was determined and the DNA was diluted to 4 µg/ml and used to set up four hybridization mixtures using homologous ATCC 10004 DNA, a label only control and DNA from USDA 192 and USDA 194.

The label-label reassociation decreased from 8.9% to 6.3% and neither the USDA 192 nor the USDA 194 DNA showed any homology with the ATCC 10004 label at 80° C. These strains had previously shown a relative homology with ATCC 10004 of 41% and 39% respectively with TBI values of 2.15 and 1.02.

The above procedure was applied during the preparation of the next labelled reference strain, the fast-growing soybean nodulating strain USDA 208. Using this label, the homology results at 80°C and the corresponding TBI values were more meaningful. Table XI shows a representative sample of the results obtained using this label. None of the TBI values were found to be greater than 1.00.

### 5.4 Determination of Contaminants in Labelled DNA Preparations

The presence of protein, ribonucleic acid and polysaccharide was assayed in several of the labelled DNA preparations. Undiluted sonicated or un-sonicated DNA in 0.28 M PB was used in the assays. Table XII shows the results obtained. Neither protein or ribonucleic acid was shown to be present in significant quantities in the DNA preparations tested. The amount of polysaccharide present was greater and ranged from 8 to 27 µg/100 µg of DNA. The smallest amounts of polysaccharide was found in the USDA 208 and the NZP 4009 labels, neither which exhibited high 80° C hybridization values. The USDA 208 label was assayed for polysaccharide before being treated to reduce non-specific binding to HA. The method used for polysaccharide determination proved unreliable for assaying the treated DNA.

## 6. COLONY HYBRIDIZATION

### 6.1 Preparation of *in vitro* Labelled DNA

DNA was labelled with $^{32}P$ by the random primer method of *in vitro* labelling. A total of five labelled strains were used in hybridizations in order to study the applicability of this method to the identification of *Rhizobium* strains.

TABLE XI: RELATIVE HOMOLOGY AT 65°C AND 80°C of DNA FROM STRAINS OF *GALEGA* RHIZOBIA, FAST-GROWING SOYBEAN NODULATING RHIZOBIA AND *R. LEGUMINOSARUM* WITH REFERENCE DNA FROM USDA 208

| *Rhizobium* strain | Relative Homology (%) 65°C | 80°C | TBI |
|---|---|---|---|
| Rhizobia of *Galega* | | | |
| gal 1 | 10 | 1 | 0.08 |
| gal 3 | 10 | 2 | 0.22 |
| gal | 10 | 3 | 0.32 |
| Fast-growing soybean nodulating rhizobia | | | |
| USDA 191 | 84 | 76 | 0.91 |
| USDA 192 | 90 | 90 | 1.00 |
| USDA 205 | 92 | 91 | 0.99 |
| USDA 208 | 100 | 100 | 1.00 |
| *R. leguminosarum* | | | |
| ATCC 10004 | 12 | 7 | 0.55 |
| NZP 561 | 11 | 3 | 0.24 |

TABLE XII: CONTAMINANTS IN LABELLED DNA PREPARATIONS

| Labelled DNA (Strain) | Protein (µg/100 µg DNA) | Polysaccharide | Ribonucleic Acid* |
|---|---|---|---|
| gal NW 3 | ND | 25 | ND |
| USDA 191 | ND | 21 | ND |
| USDA 208 | ND | 11 | ND |
| ATCC 10004 | 3 | 27 | - |
| NZP 4009 | 5 | 8 | - |

* denotes qualitative presence of RNA, as determined by agarose gel electrophoresis.

ND Not determined

- no ribonucleic acid detected

Two fast-growing soybean nodulating strains, USDA 191 and USDA 208; the *Galega* strains, gal 1 and 59A2 and *R. leguminosarum* strain ATCC 10004 were used. The gal 1 labelled probe was *in vitro* labelled and had been previously used in the hybridization experiments (Results Section 4). The specific activity of the *in vitro* labelled probes ranged from 2.4 - 3.0 x $10^7$ counts per min/µg DNA. Between 10-15 µl of probe was added to each membrane filter to give a total of 3 x $10^5$ counts per filter.

## 6.2 Identification of Rhizobia

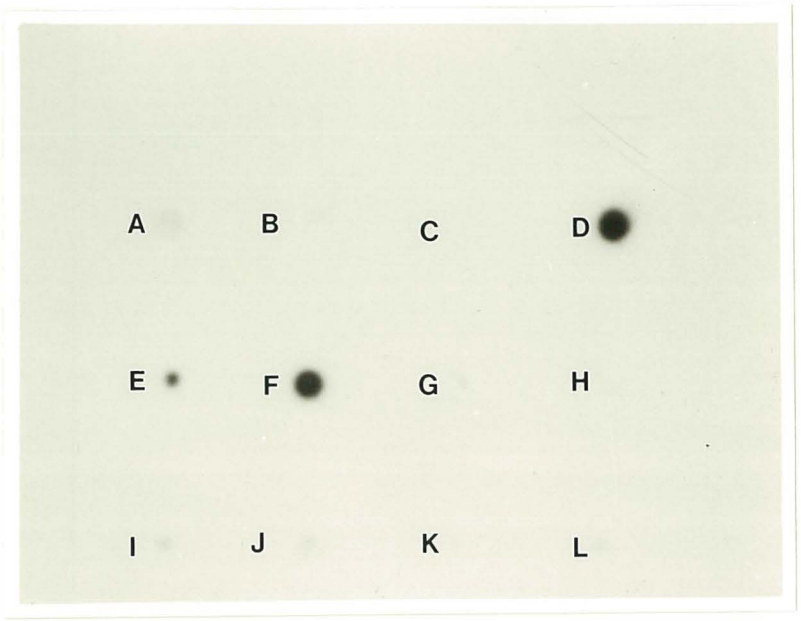
The ability of colony hybridization to distinguish between different species and strains of *Rhizobium* was investigated using the above five probes. The USDA 191 and gal 1 probes were hybridized with membranes containing DNA from 7 colonies. The other three probes were hybridized with membranes containing DNA from 12 colonies, representing strains of *Rhizobium* from each of the main DNA homology groups. All membranes were initially washed with 2 x SSC following hybridization and then washed with 0.1 x SSC to increase the stringency of labelling. This resulted in greater differentiation between those strains that were genetically related to the probe and strains that were more distantly related. Plate 4 shows a typical autoradiogram produced after washing the hybridized membranes with 0.1 x SSC and exposing to X-ray film for 2 days. Only colonies which had DNA with a high degree of base sequence similarity to the probe DNA hybridized and therefore became labelled. The results indicate that strains from the same homology group as the probe became the most intensely labelled. These results are summarised in Table XIII. The relative amount of labelling is indicated as follows: +++ intense, ++ definite, + weak, $\pm$ just detectable, - no labelling. Under the hybridization conditions used, strains that were distantly related to the reference strain were only labelled weakly or were not labelled.

PLATE 4: Colony hybridization of DNA from *Rhizobium* strains with *Galega* strain, 59A2

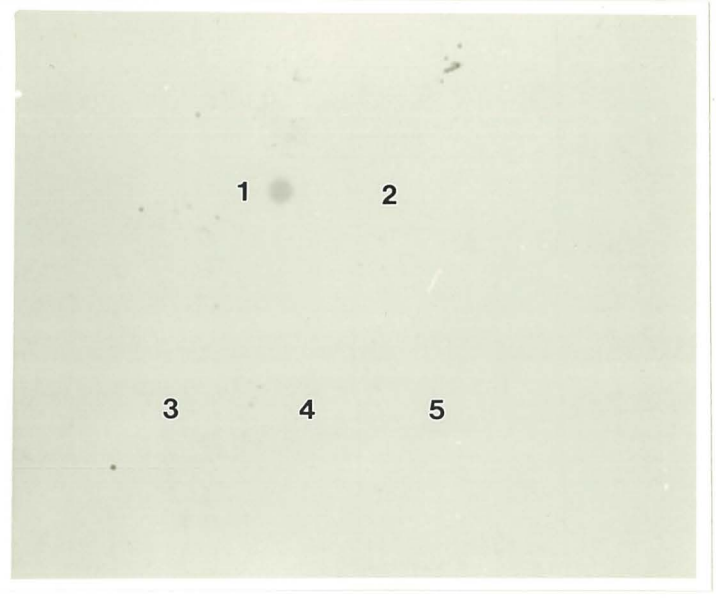
Fast-growing soybean rhizobia: A. USDA 191; B. USDA 206; C. USDA 208; *Galega* rhizobia: D. gal 1; E. gal NW 3; F. 59A2; *R. leguminosarum*: G. ATCC 10004; H. NZP 561; *R. meliloti*: I. NZP 4009; J. ATCC 9930; *R. loti*: K. NZP 2213; L. NZP 2037

PLATE 5: Colony hybridization of nodule contents with the fast-growing soybean nodulating strain, USDA 208 Nodule from *Lotus pedunculatus* inoculated with:

1. *R. loti* NZP 2037 (effective nodulation);
2. *R. loti* NZP 2213 (ineffective); Fast-growing soybean rhizobia (ineffective): 3. USDA 257; 4. USDA 192; 5. USDA 201.




4




5

TABLE XIII: SPECIFICITY OF LABELLING OF *RHIZOBIUM* STRAINS WITH LABELLED REFERENCE DNAs

| Strain | Homology Group | Labelled DNA Reference Probes | | | | |
|---|---|---|---|---|---|---|
| | | USDA 191 | USDA 208 | gal 1 | 59A2 | ATCC 10004 |
| USDA 191 | Fast-growing | +++ | ++ | | ± | - |
| USDA 192 | Soybean nodulating | +++ | | | | |
| USDA 193 | rhizobia | +++ | | | | |
| USDA 206 | | +++ | +++ | | ± | - |
| USDA 208 | | | +++ | | - | - |
| gal 1 | *Galega* rhizobia | | ± | ++ | +++ | + |
| gal 3 | | | | +++ | | - |
| gal 14 | | | | ++ | | |
| gal NW 3 | | + | - | | +++ | - |
| 59A2 | | | ± | | +++ | - |
| ATCC 10004 | Group 1 clover-pea | | ± | ± | ± | +++ |
| NZP 561 | | | ± | | ± | +++ |
| NZP 5462 | Group 2 | | | - | | |
| NZP 4009 | Group 3 Lucerne | | + | - | + | - |
| ATCC 9930 | | + | ± | - | ± | - |
| NZP 2213 | Group 4 | | - | | - | - |
| NZP 2037 | | - | ± | | ± | - |

The relative amount of labelling of each colony is indicated as follows: +++ intense, ++ definite, + weak, ± just detectable, - no labelling

One of the membranes contained a mixed colony consisting of gal 1 and USDA 208 strains. The DNA from this colony was found to hybridize to both the gal 1 and the USDA 208 probes, indicating the applicability of this method to distinguish between individual strains in mixed cultures of *Rhizobium*.

The method of colony hybridization was used to determine whether the nodules formed by the fast-growing soybean rhizobia on *Lotus pedunculatus* (Results Section 2) contained rhizobial DNA. Four such nodules were surface sterilised, squashed and the contents placed on a nitrocellulose filter which was then treated as in Method 6.4. The membrane was hybridized with a fast-growing soybean nodulating reference strain, USDA 208, washed with 2 x SSC and X-ray film exposed for 4 days. The results are shown in Plate 5. The contents of an effective nigrogen fixing nodule produced by *R. loti* strain NZP 2037 was used as a control and hybridized slightly with the probe as indicated by the weak labelling. None of the four fast-growing soybean nodule squashes hybridized with the probe, suggesting that little or no rhizobial DNA was present within the nodules.

# DISCUSSION

## 1. THE RELATIONSHIP OF THE *GALEGA* RHIZOBIA TO OTHER SPECIES OF *RHIZOBIUM* AND *BRADYRHIZOBIUM*

The results obtained in this study establish that the rhizobia nodulating *Galega officinalis* and *G. orientalis* are taxonomically distinct from other fast-growing species of *Rhizobium* and from the slow-growing rhizobia. The DNA homology, cross-nodulation and phage-typing results support this view.

The classification of the *Galega* rhizobia has been confused. Hauke-Pacewiczowa (1952) suggested that the *G. officinalis* rhizobia should be included in *R. leguminosarum*, since *Rhizobium* strains from *Vicia* spp. nodulated *G. officinalis*. Proctor (1963) characterized organisms from *G. officinalis* as belonging to the "fast-growing lupin-cowpea complex". This was based on plant nodulation data, biochemical and serological tests. He concluded that the organisms from *G. officinalis* were related to strains that nodulate other members of the *Galegeae* family. Wilson (1939) found that rhizobia from a number of host plants including *Lotus*, *Medicago*, *Phaseolus*, *Trifolim* and *Vicia* nodulated *G. officinalis* and *G. officinalis* var. *hartlandii*. Lindstrom *et al* (1983) found no cross-nodulation relationships between the *Galega* rhizobia and strains from the major recognized taxonomic groups. In their studies they used 23 species of legumes and included members of the Galegeae family such as *Lupinus*, *Caragana arborescens* and *Robinia pseudoacacia*, none of which were nodulated by the *Galega* rhizobia. The results obtained in this study, agree with those of Lindstrom *et al*, since the *Galega* rhizobia were found to nodulate only *G. officinalis* and none of the other legume species (Table VI).

Lindstrom *et al* (1983) concluded that the *Galega* rhizobia formed a distinct phage-typing group, suggesting a lack of relationship between the *Galega* rhizobia and rhizobia from other taxonomic groups. The clover, lucerne and *Lotus* phages did not lyse the *Galega* rhizobia and the wide host-range type *Galega* phage Øgal R/1 lysed all 12 strains used in their study. The phage-typing results in the present study (Table VII), using the same phages, agreed with these conclusions.

The genetic relationships between the *Galega* rhizobia and other strains of rhizobia were studied by the use of DNA:DNA hybridization. The *Galega* rhizobia showed high homology with DNA from the *G. officinalis* strains: mean relative homologies at 65°C of 79 ± 14% with DNA from gal 1 and 85 ± 8% with DNA from gal NW 3 (Results Section 4.21). Significantly lower homologies were observed between these reference strains and DNA from strains of *R. leguminosarum*, *R. loti* and the slow-growing rhizobia. The 11 *Galega* strains gave a mean relative homology at 65°C with reference strain *R. leguminosarum* ATCC 10004 of 26 ± 7%. Three of *Galega* strains gave a mean ΔTm(e) value of 11.5°C when hybridized with ATCC 10004, indicating considerable base sequence divergence between the *Galega* rhizobia and *R. leguminosarum*.

In addition, the DNA hybridization values indicated that there was little relationship between the *Galega* rhizobia and *R. meliloti* , *Leucaena* nodulating rhizobia and strain NZP 5462 from DNA homology group 2 (Results Section 4.21). The mean relative homology at 65°C of *Galega* rhizobia with the reference strain *R. meliloti* NZP 4009 was 20 ± 5%, indicating little base sequence similarity between *R. meliloti* and the *Galega* rhizobia. When three of the *Galega* rhizobia were hybridized with NZP 4009, the mean ΔTm(e) value was 9.7° C, again indicating little relationship between these two groups of rhizobia.

The fast-growing soybean strain, USDA 257, showed a relative homology of 47% with gal 1 and 60% with gal NW 3. Since the mean relative homology for the fast-growing soybean group was 19 ± 10% and 38 ± 12% with gal 1 and gal NW 3 respectively, USDA 257 appeared to be more closely related to the *Galega* rhizobia than the fast-growing soybean rhizobia as a whole. However, the plant nodulation tests, phage-typing and DNA hybridizations with other reference strains, clearly placed USDA 257 within the fast-growing soybean group of rhizobia.

From the DNA homology results, it was concluded that the *Galega* rhizobia formed a distinct genetic group within the genus *Rhizobium* . This conclusion was also supported by the thermal stabilities of DNA duplexes. The *Galega* rhizobia gave ΔTm(e) values ranging from 0 to 2.7°C with gal 1 and between 0 and 4.4°C with gal NW 3, further indicating the genetic uniqueness of this group of rhizobia. This is summarised in Figures 2 and 4.

Studies using synthetic polynucleotides and chemically altered base sequences indicate that the presence of 1% unpaired bases in a reassociated DNA duplex will lower the thermal stability by approximately 1% (Laird *et al* 1969, Ullman & McCarthy 1973). Hence the base sequence divergence of DNA within the *Galega* group was no greater than 4%. By comparison, *R. loti* NZP 2213, when hybridized to gal NW 3 gave a ΔTm(e) value of 14.0°C (Results Section 4.3), representing a base sequence divergence of 14%.

Lindstrom *et al* (1983) found a high degree of DNA homology between the *Galega* rhizobia and reference DNA from the *G. orientalis* strain HAMBI 540. Using the same hybridization techniques as were used in the present study, they found 77 ± 9% mean relative homology at 65°C, between the *Galega* rhizobia and HAMBI 540. A total of 20 strains from *R. leguminosarum* , *R. loti* and the slow-growing rhizobia showed a mean relative homology of 19 ± 6% with this refer-

ence strain. They concluded, that since the *Galega* rhizobia cannot be classified with the known species of *Rhizobium*, they may represent a new species of *Rhizobium*. The results in this study also suggest the necessity to form a new species for the *Galega* rhizobia.

## 2. THE RELATIONSHIP OF THE FAST-GROWING SOYBEAN NODULATING RHIZOBIA TO OTHER SPECIES OF *RHIZOBIUM* AND *BRADYRHIZOBIUM*

The fast-growing soybean nodulating strains of rhizobia were found to be taxonomically distinct from other fast-growing species of *Rhizobium* and from the slow-growing root nodule bacteria. This conclusion was based on the results of the DNA:DNA hybridization values, phage-typing and plant nodulation tests.

On the basis of legume host preference, these strains would be classified with the slow-growing *Bradyrhizobium japonicum*. Keyser *et al* (1982) on the basis of physiological and biochemical tests concluded that these strains were different from the typical *Bradyrhizobium japonicum*. The fast-growing soybean rhizobia were found to form effective nitrogen fixing nodules only on the Peking cultivar (a black seeded, genetically unimproved line from China) of *Glycine max* (soybean) and with the wild progenitor species, *Glycine soja* Sieb. and Zucc. Largely ineffective nodules were formed on commercial cultivars of soybeans. Several other species of legume; *Leucaena leucocephala*, *Medicago sativa*, *Trifolium repens*, *Trifolium pratense* and *Astragalus sinicus* were not nodulated.

Stowers & Eaglesham (1984) found that the fast-growing soybean strains nodulated pigeon pea, mung beans and cowpea to varying extents. Effective nitrogen fixing symbioses occurred with pigeon pea, cowpea, Asian type soybeans and

the Peking cultivar of soybean. Jansen Van Rensburg *et al* (1983) reported the effective nodulation of soybean cultivars Geduld and Usutu. Both cultivars were of South African origin which were derived from a genetic line imported from China. Only ineffective nodules were formed on five American cultivars and on one other South African cultivar.

The presence of large plasmids ( > 100 Md) has been demonstrated in all of the fast-growing soybean strains (Sadowsky & Bohlool 1983, Masterton *et al* 1982). The curing of one of these plasmids resulted in the loss of nodulation ability (Sadowsky & Bohlool 1983) suggesting that megaplasmids are involved in nodulation of soybeans. Masterton *et al* (1983) reported the location of the structural nitrogen fixation genes (*nif* D, *nif* H and *nif* K) on these plasmids.

In the present study, the 11 strains of fast-growing soybean nodulating rhizobia formed ineffective nodules on the Matara cultivar of Glycine max (Results Section 2). However, the slow-growing soybean strain, *Bradyrhizobium japonicum* ATCC 10324 formed effective nodules on this cultivar (Plate 1). The Matara cultivar was a commercially available variety of soybean.

Four of the other five legume species used in the nodulation tests were not nodulated. No nodules were formed on *Leucaena leucocephala*, *Medicago sativa* and *Trifolium repens*, in agreement with the results of Keyser *et al* (1982). *Galega officinalis* also was not nodulated but all the 7 strains tested for their ability to nodulate *Lotus pedunculatus* formed ineffective nodules (Results Section 2). The ability of these strains to nodulate *Lotus pedunculatus* was unexpected, particularly since the DNA hybridization results indicated very little genetic similarity between the fast-growing soybean rhizobia and those that nodulate *Lotus* spp. These latter rhizobia are classified as *R. loti* (Jarvis

*et al* 1982) and shared a mean relative homology of 20 ± 5% with the fast-growing soybean reference strain, USDA 191 and a mean relative homology of 8 ± 2% with USDA 208 (Results Section 4.22). However, nodulation genes only represent a small proportion of the genome and their presence or absence in a particular strain may not significantly alter the extent of homology with a reference strain (Jarvis *et al* 1980). Thus it is possible that the fast-growing soybean strains could harbour at least some of the symbiotic genes necessary to nodulate *Lotus pedunculatus* or to induce a plant response leading to the formation of nodules. Further study on the plasmids of these strains may lead to a better understanding of this ability.

Phage-typing of the fast-growing soybean nodulating strains indicated that this group of rhizobia was distinct from other species of *Rhizobium*. The clover, lucerne, *Lotus* and the *Galega* phages did not lyse the soybean rhizobia (Table VII). To complete this work however, it would be necessary to isolate a bacteriophage or bacteriphages specific for the fast-growing soybean nodulating group. These rhizobia may then be shown to form a distinct phage-typing group in a similar manner to the *Galega* rhizobia. This is likely, since the phage-typing of the *Galega* strains indicated similar relationships to those elucidated by DNA hybridization. J.J. Patel (unpublished results) phage-typed over 400 strains of *Rhizobium*. The resultant phage-typing groups corresponded with the DNA homology groups of Crow *et al* (1981).

The genetic relationships between the fast-growing soybean nodulating rhizobia and other *Rhizobium* and *Bradyrhizobium* strains was studied by DNA:DNA hybridization. A high degree of homology existed between the fast-growing soybean rhizobia and the reference strains USDA 191 and USDA 208 which nodulate soybeans. Significantly lower homologies were found between these reference strains and strains of *R. leguminosarum*, *R. meliloti*, *R. loti*, *Leucaena* rhizobia,

NZP 5462 of homology group 2 and the slow-growing rhizobia.

The fast-growing soybean strains gave a mean relative homology at 65°C with DNA from USDA 191 of 86 ± 10% and 80 ± 18% with USDA 208 (Results Section 4.22). The mean relative homology at 80°C with USDA 208 was 73 ± 28% with a corresponding TBI of 0.87. The mean relative homology of other groups of rhizobia with USDA 208 was 24% or less at 65°C and 8% or less at 80°C (Table IX).

The ΔTm(e) values for DNA duplexes formed between the fast-growing soybean rhizobia and USDA 191 ranged from 0 to 6.2°C (Results Section 4.3). The ΔTm(e) values for DNA duplexes formed between the fast-growing soybean rhizobia and USDA 208 ranged from 0 to 6.5°C (Table IX). The ΔTm(e) values for DNA duplexes formed between other rhizobia and these reference strains were larger, ranging from 11.0 to 16.0°C with USDA 191 and from 11.3 to 15.5°C with USDA 208, indicating greater base sequences divergence between the DNA. A linear relationship was found between these ΔTm(e) values and the corresponding relative homology values and is illustrated in Figure 1.

The *Galega* strain, gal 1261, was unusual since it shared 52% homology with USDA 191, which was greater than the mean value for the *Galega* group. The corresponding ΔTm(e) value was 5.6°C which lies within the range for the fast-growing soybean group, 0 - 6.2°C. This strain however, only shared 10% homology with USDA 208. The apparent closeness of the relationship between gal 1261 and the fast-growing soybean group must therefore be held in question.

Scholla *et al* (1984) determined the DNA homology between five fast-growing soybean nodulating strains and other strains of *Rhizobium* and *Bradyrhizobium*. The five fast-growing soybean strains had a mean relative homology of 17% with *R. meliloti* ATCC 9930 and 21% with *R. leguminosarum* ATCC 10004. These hybridizations were carried out at 70°C

using a spectrophotometric technique. In the present study, the fast-growing soybean strains shared a mean relative homology at 65° C with reference strains *R. meliloti* NZP 4009 and *R. leguminosarum* ATCC 10004 of 41 ± 2% and 30 ± 7% respectively. In the reciprocal hybridizations, two strains each of *R. meliloti* and *R. leguminosarum* gave a mean relative homology at 65° C with USDA 191 of 44% and 32% respectively and a mean relative homology of 24% and 12% respectively with USDA 208. Hence, the fast-growing soybean nodulating rhizobia, although appearing to be more closely related to *R. meliloti* than to *R. leguminosarum*, shared little relationship to either of these two species of *Rhizobium*. This conclusion is supported by the ΔTm(e) values. The DNA duplexes formed between three of the fast-growing soybean strains, USDA 191, USDA 192 and USDA 194 gave a mean ΔTm(e) value of 8.7°C with NZP 4009 and 8.9°C with ATCC 10004 (Results Section 4.3). NZP 4009, when hybridized with USDA 191 as reference strain, gave a ΔTm(e) value of 11.0° C. These values confirm the differences in base sequence between the fast-growing soybean rhizobia and the other species of *Rhizobium*. The genetic distinctness of the fast-growing soybean nodulating rhizobia is illustrated in Figures 3 and 4.

The DNA hybridization results suggest that the fast-growing soybean rhizobia form two distinct subgroups. Strains USDA 194, USDA 201 and USDA 257 gave relative homologies at 65°C with USDA 208 of 52%, 51% and 56% respectively; these values being lower than the fast-growing soybean group as a whole, which had a mean relative homology of 80% (Table IX). DNA duplexes formed between USDA 191, USDA 201 and reference strain USDA 208 had ΔTm(e) values of 6.3°C and 6.5°C respectively. The DNA duplexes formed between USDA 257 and the reference strain USDA 191 gave a ΔTm(e) of 6.2°C (Results Section 4.3). By comparison, five other fast-growing soybean strains, when hybridized with USDA 191, gave lower ΔTm(e) values ranging from 0 to 1.3°C.

Scholla *et al* (1984) found that the five fast-growing soybean strains formed two distinct DNA homology groups. The first contained USDA 191 and USDA 201 which were closely related to each other, while the second group contained USDA 193, USDA 205 and USDA 206. There is good agreement between their results and those found in this study.

Scholla *et al* (1984) found low homology between the fast and slow-growing soybean rhizobia. The fast-growing strains showed a mean relative homology with *Bradyrhizobium japonicum* ATCC 10324 of 26%. In the present study strain ATCC 10324 shared 21% homology at 65° C with USDA 191; 4% homology with USDA 208 and $\Delta$Tm(e) of 15.5° C. Yelton *et al* (1983) found a low homology of 15% between the slow-growing soybean strain USDA 110 and USDA 191. Thus the fast-growing and the slow-growing soybean strains are distinct and can be differentiated on the basis of DNA homology, although both groups have similar symbiotic host specificities.

Sadowsky *et al* (1983) concluded that the fast-growing soybean rhizobia are biochemically distinct from the slow-growers. The fast-growersare similar to other fast-growing species of *Rhizobium* since they tolerate 2% NaCl, are capable of growth at pH 9.5, can utilize a wide range of carbohydrate and produce serum zones in litmus milk. They produce appreciable levels of ß-galactosidase and nicotinamide adenine dinucleotide phosphate-linked 6-phosphogluconate dehydrogenase but had no detectable hydrogenase activity.

Yelton *et al* (1983) found that USDA 191 was more salt-tolerant than the slow-growing *Bradyrhizobium japonicum* strain USDA 110 and was capable of growth at 0.4 M NaCl. This strain utilized a wider range of carbohydrates than USDA 110 and possessed both glucose-6-phosphate and 6-phosphogluconate dehydrogenase. They concluded that USDA 191 more closely resembled *R. meliloti* than the slow-growing soybean strains which lacked 6-phosphogluconate dehydrogenase.

Little similarity between the two-dimensional protein patterns on polyacrylamide gel was found between USDA 191 and USDA 110. On the other hand, some similarity was found between USDA 191 and *R. meliloti*102 F28.

Stowers & Eaglesham (1984) found that the fast-growing soybean strains utilized a wide range of carbon sources in agreement with other fast-growing rhizobia. All but one of the strains tested were tolerant to 1% NaCl, whereas the slow-growing soybean strains were severely inhibited in their growth on 1% NaCl.

On the basis of the above evidence, Scholla & Elkan (1984) proposed a new species name for the fast-growing soybean nodulating rhizobia; *Rhizobium fredii* sp. nov (M.L. gen. noun fredii of E.B. Fred). Within this species, they concluded there were two distinct subgroups which can be differentiated on the basis of DNA homology, acid production on yeast mannitol broth, kanamycin resistance and serology. Two new chemovars were proposed; *R. fredii* chemovar *fredii* and *R. fredii* chemovar *siensis* . *R. fredii* chemovar *fredii* showed 91-96% relative homology at 70°C with USDA 205 and 24-63% with USDA 201. The strain USDA 193 and USDA 206 were included along with the type strain USDA 205 in this subgroup. *Rhizobium fredii* chemovar *siensis* showed 95% homology with USDA 201 and 24-53% homology with USDA 205. The two strains, USDA 194 and USDA 201 were included in this subspecies.

Only five of the eleven fast-growing soybean strains used in this study were included in the description of the new species, *R. fredii* (Scholla & Elkan 1984). On the basis of DNA homology, it appears that USDA 257 should be included in *R. fredii* chemovar *siensis* and USDA 191, USDA 192, USDA 208, USDA 214 and USDA 217 should be included in *R. fredii* chemovar *fredii*. However, serotyping indicates that other serogroups exist (B. Bohlool, personal communication) and therefore more hybridizations, using other reference strains may be required to fully resolve this point.

## 3. DIFFICULTIES ENCOUNTERED DURING DNA HYBRIDIZATION

The majority of the strains used in the hybridizations were easily lysed by the method of Jarvis *et al* (1980) (Method 2.2). This method failed to lyse six strains effectively: insufficient DNA of poor quality was produced. Four of these strains yielded adequate DNA when lysed by the method of Fischer & Lerman (1979) (Method 2.31).

Neither of the above methods of cell lysis, nor the method of Zaenen *et al* (1974) (Method 2.32), sufficiently lysed cells of strain NZP 5434 to extract DNA. When the cells were subjected to rapid freezing and thawing (Method 2.33), this strain was adequately lysed by the method of Jarvis *et al* (1980). Presumably this preliminary treatment destabilized the outer membrane and cell wall, facilitating cell lysis.

During the hybridization experiments, some of the hybridizations yielded higher relative homology values at 80°C than the corresponding values at 65°C (Table X). This problem occurred with hybridizations using four of the six reference strains. A number of experiments were performed in an attempt to clarify and resolve the problem (Results Section 5.2). The results indicated that the problem was due to some inherent feature of the labelled DNA, rather than the unlabelled DNA or some condition imposed during renaturation or the separation of single from double-stranded DNA. It was concluded that some contaminant within the DNA was responsible for non-specific binding of single-stranded DNA to the hydroxyapatite.

When the label was phenol extracted and ethanol precipitated, the problem was alleviated to some extent. After the label was treated to reduce non-specific binding (Results Section 5.3) the relative homology results obtained at 80°C were perfectly acceptable (Table XII).

Jarvis *et al* (1980) used the method of Brenner *et al* (1969b) to reduce non-specific binding of labelled DNA to hydroxyapatite. They eluted the labelled DNA from a hydroxyapatite column at 60°C with 0.14 M phosphate buffer. Their hybridizations were conducted at two temperatures, 65°C and 80°C. TBI values ranged from 0.0 to 0.5 for heterologous DNA duplexes and 1.0 for the homologous DNA duplex.

This step was discontinued since it didn't appear strictly necessary. The hybridization values obtained at 65°C were found to be independent of this step. The hybridization results in the present study indicate that this step is required when hybridizations are performed at temperatures greater than the optimum for renaturation. Thus when hybridizations are carried out at 80°C, it is necessary to reduce non-specific binding of DNA to the hydroxyapatite. The procedure outlined in Results Section 5.3 to reduce non-specific binding was developed somewhat arbitrarily; the method of Brenner *et al* (1969b) offers advantages, since it is quicker and is potentially less damaging to the DNA.

Experiments to discover the nature of the contamination of the labelled DNA ruled out the possibility of either protein or ribonucleic acid being responsible for the non-specific binding. Both these macromolecules were present in negligible quantities (Table XII).

There was however, some evidence to support the view that polysaccharide might have caused non-specific binding of the labelled DNA to the HA (Results Section 5.4). The polysaccharide within the label could have cross-linked the single-stranded DNA molecules enabling them to adhere to the hydroxyapatite crystals. In such an event, low molarity phosphate buffer (0.10 - 0.12 M) would have been insufficient to elute the bound DNA which would have been eluted by the 0.4 M phosphate buffer leading to an apparently greater amount of hybridization. Interactions between heat-

denatured DNA and polysaccharide polymers, involving hydrogen bonding, have been reported by Graves (1968).

Many of the homology values at 65°C obtained using the fast-growing soybean strain USDA 208 were lower than the corresponding results using USDA 191 (Results Section 4.22). For example, the *Galega* rhizobia showed a mean relative homology at 65°C of 10% with USDA 208, compared to 28% with USDA 191. Either: the lower homologies obtained with USDA 208 were a result of the treatment of the label to reduce non-specific binding, or alternatively, some other factor was involved. Differences in genome size between reference DNAs and the hybridizing heterologous DNA can produce variations in the relative amount of base sequence homology that occurs (De Ley 1969, Brenner *et al* 1972). Brenner *et al* (1972), using DNA from two closely related organisms, *Escherichia coli* K-12 and *E. coli* BB, found the relative amount of reassociation, at 60° C, between various strains of *E. coli* , *Shigella* spp, the *Alkalescens* - Dispar group and BB DNA as reference, was lower than the corresponding reassociation between these strains and K 12 DNA. These differences in relative homology were as high as 14% and corresponded with a 9% difference in genome size between the reference strains. Hence, if USDA 191 and USDA 208 differ markedly in genome size this could explain the variations in the DNA homology with other strains. Differences in genome size could also explain the variation in homology results obtained between the *Galega* reference strains, gal 1 and gal NW 3 (Results Section 4.21).

## 4. IDENTIFICATION OF RHIZOBIA BY COLONY HYBRIDIZATION

The results obtained in this study indicate that colony hybridization can be applied to the identification of *Rhizobium* strains. Strains that were genetically related

to the reference strain were readily distinguishable from those that were more distantly related (Table XIII). Thus it was possible to distinguish *Galega* rhizobia (Plate 4), or strains of *R. trifolii*, from other species of *Rhizobium*, using the appropriate reference strain. These hybridization were performed at 65°C and it may be possible to increase the specificity of labelling by hybridizing at a higher more stringent temperature such as 80°C, or alternatively, washing the membrane filters in 0.1 x SSC at this temperature after hybridization.

Hodgson *et al* (1983) conducted colony hybridizations at a low stringency temperature of 57°C. Where greater stringency was required to improve the amount of differentiation between strains, membrane filters were held at 70°C in 0.1 x SSC and 0.5% sodium dodecyl sulphate for 1 hr following hybridization to reduce non-specific labelling.

The results in Section 6.2 suggest that colony hybridization can be used to distinguish rhizobia in mixed populations. When the membrane filter was inoculated simultaneously with the *Galega* strain, gal 1 and the fast-growing soybean strain, USDA 208, the DNA of this mixed colony hybridized with reference DNA from both gal 1 and USDA 208. Hodgson *et al* (1983) found the method of colony hybridization could be used to identify specific *Rhizobium* strains in the presence of other rhizobia and also directly within root nodules.

The results in the present study suggest that it is possible to identify rhizobia within nodules simply by hybridizing the contents of nodules with the appropriate labelled reference strain DNA. Although the fast-growing soybean labelled reference strain, USDA 208, did not hybridize with the contents of the nodules formed by the fast-growing soybean strains on *Lotus pedunculatus* (Plate 5), some hybridization was found between the *R. loti* strain NZP 2037 and this reference strain. Only a small amount of hybridization was

expected since NZP 2037 shared only 6% homology with USDA 208 (Table IX) and therefore was unrelated. Since this small amount of hybridization was easily detected on the autoradiogram, the method of colony hybridization appears to be very sensitive in detecting the presence of DNA within root nodules.

Other techniques available to identify rhizobia within nodules suffer from a number of limitations. The immunological techniques show cross-reaction and non-specific binding (Bohlool & Schmidt 1980). Resistance markers are limited in number, may be lost through genetic exchange with natural populations of rhizobia and effect the symbiotic properties of the strain (Schwinghamer & Dudman 1973, Josey *et al* 1979).

Colony hybridization as a method to identify rhizobia offers advantages over other methods such as simplicity and a high degree of specificity and reliability. Using suitable labelled reference strains, the contents of nodules could be probed directly without the possibility of interfering with the symbiotic properties of the rhizobia.

# APPENDIX

TABLE AI: ANALYSIS OF UNLABELLED DNA PREPARATIONS OBTAINED BY THE HYDROXYAPATITIE-UREA METHOD (Method 2.5)

| Strain | Fraction Number | DNA concentration (µg/ml) | Spectral ratios 258/230 | 258/280 |
|---|---|---|---|---|
| USDA 191 | F4-1 | 369 | 2.0 | 1.9 |
| | F4-2 | 240 | 2.1 | 1.7 |
| | F4-3 | 180 | 2.6 | 2.1 |
| USDA 192 | F4-1 | 419 | 2.0 | 2.0 |
| | F4-2 | 244 | 1.6 | 1.6 |
| | F4-3 | 153 | 1.5 | 1.8 |
| ATCC 10004 | F4-1 | 506 | 2.1 | 1.9 |
| | F4-2 | 140 | 1.6 | 1.8 |
| | F4-3 | 136 | 1.8 | 1.8 |
| gal 14 | F4-1 | 215 | 2.0 | 1.9 |
| | F4-2 | 115 | 2.4 | 1.5 |
| | F4-3 | 46 | 1.2 | 1.0 |
| gal NW 3 | F4-1 | 500 | 2.6 | 2.1 |
| | F4-2 | 300 | 1.2 | 1.4 |
| | F4-3 | 160 | 1.2 | 0.7 |
| NZP 2037 | F4-1 | 363 | 2.0 | 1.9 |
| | F4-2 | 252 | 2.2 | 1.8 |
| | F4-3 | 136 | 2.0 | 1.7 |
| ATCC 10324 | F4-1 | 325 | 2.2 | 2.1 |
| | F4-2 | 194 | 2.3 | 1.8 |
| | F4-3 | 103 | 2.7 | 1.9 |

TABLE AII: RELATIVE HOMOLOGY OF DNA FROM *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS WITH REFERENCE DNA FROM *GALEGA* RHIZOBIA AND THE FAST-GROWING SOYBEAN NODULATING RHIZOBIA

| Strain | DNA homology with reference strains: gal 1 65°C (%) | gal 1 Tm(e) (°C) | gal NW 3 65°C (%) | gal NW 3 Tm(e) (°C) | USDA 191 65°C (%) | USDA 191 Tm(e) (°C) |
|---|---|---|---|---|---|---|
| *Rhizobium* of *Galega* | | | | | | |
| gal 1 | 100 | 0 | 98 | | 28 | |
| gal 3 | 77 | | 81 | | 29 | |
| gal 7 | 77 | | 83 | | 33 | |
| gal 12 | 67 | 2.7 | 79 | 2.7 | 31 | |
| gal 14 | 58 | 1.8 | 71 | 4.4 | 18 | |
| gal 129 | 73 | | 80 | | 23 | |
| gal 1261 | 84 | | 90 | 1.4 | 52 | 5.6 |
| gal NW 1 | 76 | | 85 | | 20 | |
| gal NW 2 | 83 | 0.7 | 87 | 2.4 | 23 | |
| gal NW 3 | 106 | 0.1 | 100 | 0 | 35 | |
| 59A2 | 70 | 2.0 | 81 | 2.1 | 21 | 14.5 |
| Group mean | 79 | | 85 | | 28 | |
| Fast-growing soybean nodulating rhizobia | | | | | | |
| USDA 191 | 16 | | 38 | | 100 | 0 |
| USDA 192 | 19 | | 35 | | 91 | |
| USDA 193 | 18 | | 49 | | 91 | |
| USDA 194 | 25 | | 43 | | 76 | |
| USDA 201 | 10 | | 19 | | 66 | |
| USDA 205 | 16 | | 34 | | 89 | |
| USDA 206 | 14 | | 26 | | 91 | 0.5 |
| USDA 208 | 14 | | 38 | | 92 | 0.7 |
| USDA 214 | 9 | | 27 | | 89 | 0.9 |
| USDA 217 | 20 | | 45 | | 90 | 1.3 |
| USDA 257 | 47 | | 60 | | 70 | 6.2 |
| Group mean | 19 | | 38 | | 86 | |

Cont'd..

TABLE AII: CONTINUED

| Strain | DNA homology with reference strains: gal 1 65°C (%) | gal 1 Tm(e) (°C) | gal NW 3 65°C (%) | gal NW 3 Tm(e) (°C) | USDA 191 65°C (%) | USDA 191 Tm(e) (°C) |
|---|---|---|---|---|---|---|
| Group 1 clover-pea *R. leguminosarum* | | | | | | |
| ATCC 10004 | 27 | | 45 | | 24 | |
| NZP 561 | 17 | | 43 | | 40 | |
| Group Mean | 22 | | 44 | | 32 | |
| Group 2 *Coronilla* | | | | | | |
| NZP 5462 | 14 | | 25 | | 22 | |
| Group 3 lucerne *R. meliloti* | | | | | | |
| NZP 4009 | 12 | | 22 | | 42 | 11.0 |
| ATCC 9930 | 3 | | 21 | | 46 | 11.8 |
| Group mean | 8 | | 22 | | 44 | |
| Group 4 *Lotus* *R. loti* | | | | | | |
| NZP 5201 | | | 15 | | 16 | |
| NZP 2213 | | | 19 | 14.0 | 18 | |
| NZP 2037 | 10 | | 17 | 4.4 | 26 | |
| NZP 5361 | | | 14 | | | |
| NZP 2238 | | | 15 | | | |
| NZP 2014 | | | 16 | | | |
| Group mean | | | 16 | | 20 | |
| *Rhizobium* of *Leucaena* | | | | | | |
| NZP 5434 | 11 | | 24 | | 20 | |
| NZP 5259 | 8 | | 24 | | 28 | |
| Group mean | 10 | | 24 | | 24 | |
| Slow-growing rhizobia | | | | | | |
| Slow-growing *Lotus* Rhizobia | | | | | | |
| NZP 2309 | | | 15 | | 11 | |
| NZP 2257 | | | 5 | | 8 | 16.0 |
| NZP 5223 | | | 14 | | | |
| NZP 2192 | | | 9 | | | |
| Group mean | | | 11 | | 10 | |
| *Bradyrhizobium japonicum* ATCC 10324 | 2 | | 16 | | 21 | 7.2 |

TABLE AIII: RELATIVE HOMOLOGY OF DNA FROM *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS WITH REFERENCE DNA FROM THE FAST-GROWING SOYBEAN NODULATING RHIZOBIA, *R. LEGUMINOSARUM* AND *R. MELILOTI*

| Strain | DNA homology with reference strains: USDA 208 65°C (%) | USDA 208 ΔTm(e) (°C) | ATCC 10004 65°C (%) | ATCC 10004 ΔTm(e) (°C) | NZP 4009 65°C (%) | NZP 4009 ΔTm(e) |
|---|---|---|---|---|---|---|
| *Rhizobium* of *Galega* | | | | | | |
| gal 1 | 10 | 14.0 | 24 | 11.9 | 16 | 9.7 |
| gal 3 | 10 | | 22 | | 15 | |
| gal 7 | 10 | | 33 | | 25 | |
| gal 12 | 8 | | 31 | | 20 | |
| gal 14 | 10 | | 16 | 12.4 | 19 | 9.2 |
| gal 129 | 10 | | 23 | | 16 | |
| gal 1261 | 10 | | 45 | | 29 | |
| gal NW 1 | 13 | | 21 | | 15 | |
| gal NW 2 | 13 | | 20 | | 17 | |
| gal NW 3 | 10 | 14.7 | 33 | 10.1 | 23 | 10.3 |
| 59A2 | 9 | | 22 | | 18 | |
| NZP 5562 | 18 | | 23 | | 23 | |
| NZP 5563 | 19 | | 26 | | 25 | |
| NZP 5564 | 23 | | 27 | | 29 | |
| Group mean | 12 | | 26 | | 20 | |
| Fast-growing soybean nodulating rhizobia | | | | | | |
| USDA 191 | 84 | 0.7 | 33 | 7.5 | 42 | 8.0 |
| USDA 192 | 90 | 0.5 | 19 | 10.2 | 43 | 9.4 |
| USDA 193 | 88 | | 39 | | 44 | |
| USDA 194 | 52 | 6.3 | 39 | 9.0 | 43 | 8.6 |
| USDA 201 | 51 | 6.5 | 22 | | 36 | |
| USDA 205 | 92 | | 28 | | 43 | |
| USDA 206 | 92 | | 22 | | 41 | |
| USDA 208 | 100 | 0.0 | 36 | | 42 | |
| USDA 214 | 92 | | 29 | | 40 | |
| USDA 217 | 85 | 0.3 | 34 | | 40 | |
| USDA 257 | 56 | | 25 | | 42 | |
| Group mean | 80 | | 30 | | 41 | |

Cont'd..

TABLE AIII: CONTINUED

| Strain | DNA homology with reference strains: USDA 208 65°C (%) | USDA 208 ΔTm(e) (°C) | ATCC 10004 65°C (%) | ATCC 10004 ΔTm(e) (°C) | NZP 4009 65°C (%) | NZP 4009 ΔTm(e) (°C) |
|---|---|---|---|---|---|---|
| Group 1 clover-pea | | | | | | |
| *R. leguminosarum* | | | | | | |
| ATCC 10004 | 12 | 14.0 | 100 | 0 | | |
| NZP 561 | 11 | | 73 | 3.2 | | |
| Group mean | 12 | | | | | |
| Group 2 *Coronilla* | | | | | | |
| NZP 5462 | 12 | | | | | |
| Group 3 lucerne | | | | | | |
| *R. meliloti* | | | | | | |
| NZP 4009 | 26 | 11.3 | | | 100 | 0 |
| ATCC 9930 | 22 | | | | 92 | 0.0 |
| Group mean | 24 | | | | | |
| Group 4 *Lotus* | | | | | | |
| *R. loti* | | | | | | |
| NZP 5201 | 10 | | | | | |
| NZP 2213 | 7 | 14.0 | | 12.7 | | 10.1 |
| NZP 2037 | 6 | | | | | |
| Group mean | 8 | | | | | |
| *Rhizobium* of *Leucaena* | | | | | | |
| NZP 5434 | 12 | | | | | |
| NZP 5259 | 9 | | | | | |
| Group mean | 10 | | | | | |
| Slow-growing rhizobia | | | | | | |
| Slow-growing *Lotus* rhizobia | | | | | | |
| NZP 2309 | 6 | | | | | |
| NZP 2257 | 3 | | | | | |
| Group mean | 5 | | | | | |
| *Bradyrhizobium japonicum* | | | | | | |
| ATCC 10324 | 4 | 15.5 | | | | |

# BIBLIOGRAPHY

ALLEN, O.N. and E.K. Allen. 1940. Response of the peanut plant to inoculation with rhizobia, with special preference to morphological development of the nodules. Bot. Gazett. 102: 121-142.

BAILEY, N.T.J. 1981. Statistical methods in biology. 2nd ed. Hodder and Stoughton, Great Britain.

BAILEY, R.W. 1967. Loss of ingested plant carbohydrates from the reticulorumen. N.Z. J. Agric. Res. 10: 15-32.

BERNARDI, G. 1971. Chromatography of nucleic acids on hydroxyapatite columns. Methods Enzymol. 21D: 95-139.

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

BOHLOOL, B.B. and Schmidt, E.L. 1970. Immunofluorescent detection of *Rhizobium japonicum* in soils. Soil Sci. 110: 229.

BOHLOOL, B.B. and E.L. Schmidt. 1980. The immunofluorescence approach in microbial ecology. Microbial Ecol. 4: 203-241.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72: 248-254.

BRENNER, D.J., G.R. Fanning, A.V. Rake and K.E. Johnson. 1969a. Batch procedure for thermal elution of DNA from hydroxyapatite. Anal. Biochem. 28: 447-459.

BRENNER, D.J., G.R. Fanning, K.E. Johnson, R.V. Citarella and S. Falkow. 1969b. Polynucleotide sequence relationships among members of *Enterobacteriaceae*. J. Bacteriol. 98: 637-650.

BRENNER, D.J. and C. Falkow. 1971. Molecular relationships among members of the *Enterobacteriaceae*. Adv. Gen. 16: 81-118.

BRENNER, D.J., G.R. Fanning, F.J. Skerman and S. Falkow. 1972. Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. J. Bacteriol. 109: 953-965.

BREWIN, N.J., J.E. Beringer and A.W.B. Johnston. 1980. Plasmid-mediated transfer of host-range specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120: 413-420.

BRITTEN, R.J., M. Pavich and J.A. Smith. 1970. A new method for DNA purification. Carnegie Inst. Washington Yearbook. 68: 400-402.

BROCKWELL, J., E.A. Schwinghamer and R.R. Gault. 1977. Ecological studies of root-nodule bacteria introduced into field environments. V. A critical examination of the stability of antigenic and streptomycin-resistance markers for identification of strains of *Rhizobium trifolii*. Soil Biol. Biochem. 9: 19-24.

BUCHANAN-WOLLASTON, A.V., J.E. Beringer, N.J. Brewin, P.R. Hirsch and A.W.B. Johnston. 1980. Isolation of symbiotically defective mutants of *Rhizobium leguminosenum* by insertion of the transposon Tn5 into a transmissible plasmid. Molec. Gen. Gent. 178: 185-190.

CLAUSEN, T. 1968. Measurement of $^{32}P$ activity in a liquid scintillation counter without the use of scintillator. Anal. Biochem. 22: 70-73.

CROW, V.L., B.D.W. Jarvis and R.M. Greenwood. 1981. Deoxyribonucleic acid homologies among acid-producing strains of *Rhizobium*. Int. J. Syst. Bacteriol. 31: 152-172.

DE LEY, J. and A. Rassel. 1965. DNA base composition, flagellation and taxonomy of the genus *Rhizobium*. J. Gen. Microbiol. 41: 85-91.

DE LEY, J. 1969. Compositional nucleotide distribution and the theoretical prediction of homology in bacterial DNA. J. Theoret. Biol. 22: 89-116.

DIATLOFF, A. 1977. Ecological studies of root-nodule bacteria introduced into field environments: 6 antigenic and symbiotic stability in *Lotononis* rhizobia over a 12-year period. Soil Biol. Biochem. 9: 85-88.

DIXON, R.O.D. 1969. Rhizobia with particular reference to relationships with host plants. Ann. Rev. Microbiol. 23: 137-158.

DUNICAN, L.K. and F.C. Cannon. 1971. The genetic control of symbiotic properties in *Rhizobium*: evidence for plasmid control. Plant and Soil, Special Vol. 73-79.

FISCHER, S.C. and L.S. Lerman. 1979. Length-independent separation of DNA restriction fragments in two dimension gel electrophoresis. Cell 16: 191-200.

FRED, E.B., I.L. Baldwin, and E. McCoy. 1932. Root nodule bacteria and leguminous plants. University of Wisconsin, Studies of Science, No. 5, Madison, Wisconsin.

GIBBINS, A.M. and K.F. Gregory. 1972. Relatedness among *Rhizobium* and *Agrobacterium* species determined by three methods of nucleic acid hybridization. J. Bacteriol. 111: 129-141.

GRAVES, I.L. 1968. Interaction of heat-denatured HeLa cell DNA with synthetic and natural polysaccharides. Biopolymers 6: 1573-1578.

GRUNSTEIN, M. and D.S. Hogness. 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Nat. Acad. Sci. U.S.A. 72: 3961-3965.

HAUKE-PACEWICZOWA, T. 1952. Szczepionki dla *Galega officinalis*. Acta Microbiol. Pol. 1: 37-39.

HEBERLEIN, G.Y., J. De Ley and R. Tijtgat. 1967. Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. J. Bacteriol. 94: 116-124.

HIGASHI, S. 1967. Transfer of clover infectivity of *Rhizobium trifolii* to *Rhizobium phaseoli* as mediated by an episomic factor. J. Gen. Appl. Microbiol. 13: 391-403.

HODGSON, A.L.M. and W.P. Roberts. 1983. DNA colony hybridization to identify *Rhizobium* strains. J. Gen. Microbiol. 129: 207-212.

JANSEN VAN RENSBURG, H., B.W. Strijdom and C.J. Otto. 1983. Effective nodulation of soybeans by fast-growing strains of *Rhizobium japonicum*. S. Afr. J. Sci. 79: 215-222.

JARVIS, B.D.W., K.F. Scott, J.E. Hughes, M. Djordjevic, B.G. Rolfe and J. Shine. 1983. Conservation of genetic information between different *Rhizobium* species. Can. J. Microbiol. 29: 200-209.

JARVIS, B.D.W., A.G. Dick and R.M. Greenwood. 1980. Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. Int. J. Syst. Bacteriol. 30: 42-52.

JARVIS, B.D.W., C.E. Pankhurst and J.J. Patel. 1982. *Rhizobium loti*, a new species of legume root nodule bacteria. Int. J. Syst. Bacteriol. 32: 378-380.

JOHNSTON, A.W.B., J.L. Beynon, A.V. Buchanan-Wollaston, S.M. Setchell, P.R. Hirsch and J.E. Beringer. 1978. High frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature 276: 634-636.

JORDAN, D.C. and D.N. Allen. 1974. Family III *Rhizobiaceae* Conn 1938. In R.E. Buchanan and N.E. Gibbons (ed.), Bergery's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

JORDAN, D.C. 1984. Family III. *Rhizobiaceae* Conn. 1938. In N.R. Kreig and J.G. Holt (ed.), Bergey's Manual of Systematic Bacteriology, 9th ed., Vol. 1. The Williams and Wilkins Co., Baltimore.

JOSEY, D.P., J.L. Beynon, A.W.B. Johnston and J.E. Beringer 1979. Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J. Appl. Bacteriol. 46: 343-350.

KEYSER, H.H., B.B. Bohlool, T.S. Hu and D.F. Weber. 1981. Fast-growing rhizobia isolated from root nodules of soybean. Science. 215: 1631-1632.

KIRSCHENBAUM, D.M. 1970. In H. Sober (ed.) Handbook of Biochemistry, Selected Data for Molecular Biology, 2nd ed., Chemical Rubber Company, Cleveland, Ohio.

KISHINEVSKY, B. and D. Gurfel. 1980. Evaluation of enzyme-linked immunosorbent assay (ELISA) for serological identification of different *Rhizobium* strains. J. Appl. Bacteriol. 49: 517-526.

KOWALSKI, M., G.E. Ham, L.R. Frederick and I.C. Anderson. 1974. Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. Soil Sci. 118: 221-228.

LABANDERA, C.A. and J.M. Vincent. 1975. Loss of symbiotic capacity in commercially useful strains of *Rhizobium trifolii*. J. Appl. Bacteriol. 39: 209-211.

LAIRD, C.D., B.L. McConaughy and B.J. McCarthy. 1968. On the rate of fixation of nucleotide substitutions in evolution. Nature (London) 224: 149-154.

LINDSTROM, K., B.D.W. Jarvis, P.E. Lindstrom and J.J. Patel. 1983. DNA homology, phage-typing, and cross-nodulation studies of rhizobia infecting *Galega* species. Can. J. Microbiol. 29: 781-789.

MACKINON, P.A., J.G. Robertson, D.J. Scott and C.N. Hale. 1975. Legume inoculant usage in New Zealand. N.Z. J. Exp. Agric. 5: 35-39.

t'MANNETJE, L. 1967. A re-examination of the taxonomy of the genus *Rhizobium* and related genera using numerical analysis. Antonie van Leeuwenhoek J. Serol. and Microbiol. 33: 477-491.

MARTINEZ-DE DRETS, G. and A. Arias. 1972. Enzymatic basis for differentiation of *Rhizobium* into fast- and slow-growing groups. J. Bacteriol. 109: 467-470.

MARTINEZ-DE DRETS, G., A. Arias and M. Rovira de Cutinella. 1974. Fast- and slow-growing rhizobia: differences in sucrose utilization and invertase activity. Can. J. Microbiol. 20: 605-609.

MASTERTON, R.V., P.R. Russell and A.G. Atherly. 1982. Nitrogen fixation (nif) genes and large plasmids of *Rhizobium japonicum*. J. Bacteriol. 152: 928-931.

MOFFETT, M.L. and R.R. Colwell. 1968. Adansonian analysis of the Rhizobiaceae. J. Gen. Microbiol. 51: 245-266.

NORRIS, D.O. 1956. Legumes and *Rhizobium* symbiosis. Empire J. Exp. Agric. 24: 247-270.

NORRIS, D.O. 1965. Acid production by *Rhizobium* a unifying concept. Plant & Soil. 22: 143-166.

NUTI, M.P., A.M. Ledeboer, A.A. Lepidi and R.A. Schilperoort. 1977. Large plasmids in different *Rhizobium* species. J. Gen. Microbiol. 100: 241-248.

OLSEN, P.E., W.A. Rice, G.W. Stemke and W.J. Page. 1983. Strain-specific serological techniques for the identification of *Rhizobium meliloti* in commercial alfalfa inoculants. Can. J. Microbiol. 29: 225-230.

PATEL, J.J. 1976. Morphology and host range of virulent phages of lotus rhizobia. Can. J. Microbiol. 22: 204-212.

PROCTOR, M.H. 1963. Cross-inoculation group relationships of *Galega officinalis* . N.Z. J. Bot. 1: 419-425.

QUISPEL, A. 1974. In A. Quispel (ed.) The Biology of Nitrogen Fixation. North-Holland Publishing Company Amsterdam, Netherlands.

ROBERTS, G.P., W.T. Leps, L.E. Silver and W.J. Brill. 1980. Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. Appl. Env. Microbiol. 39: 414-422.

SADOWSKY, M.J. and B.B. Bohlool. 1983. Possible involvement of a megaplasmid in nodulation of soybeans by fast-growing rhizobia from China. Appl. Environ. Microbiol. 46: 906-911.

SADOWSKY, M.J., H.H. Keyser and B.B. Bohlool. 1983. Biochemical characterization of fast- and slow- growing rhizobia that nodulate soybeans. Int. J. Syst. Bacteriol. 33: 716-722.

SCHOLLA, M.H. and G.H. Elkan. 1984. *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. Int. J. Syst. Bacteriol. 34: XXX-XXX (In press, Oct. issue).

SCHOLLA, M.H., J.A. Moorefield and G.H. Elkan. 1984. Deoxyribonucleic acid homology between fast-growing soybean-nodulating bacteria and other rhizobia. Int. J. Syst. Bacteriol. 34: 283-286.

SCHWINGHAMER, E.A. and W.F. Dudman. 1973. Evaluation of spectinomycin resistance as a marker for ecological studies with *Rhizobium* spp. J. Appl. Bacteriol. 36: 263-272.

SCOTT, D.B., and C.W. Ronson. 1982. Identification and mobilisation by cointegrate formation of a nodulation plasmid in *Rhizobium trifolii*. J. Bacteriol. 151: 36-43.

STANIEWSKI, R. 1968. Typing of *Rhizobium* by phages. Can. J. Microbiol. 16: 1003-1009.

STOWERS, M.D. and A.R.J. Eaglesham. 1984. Physiological and symbiotic characteristics of fast-growing *Rhizobium japonicum*. Plant and Soil 77: 3-14.

TAYLOR, J.M., R. Illemensee and J. Summers. 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta. 442: 324-330.

ULLMAN, J.S. and B.J. McCarthy. 1973. The relationship between mismatched base pairs and the thermal stability of DNA duplexes effects of depurination and chain scission. Biochim. Biophys. Acta. 294: 405-415.

VINCENT, J.M. 1970: International Biological Programme Handbook No. 15. A manual for the practical study of root-nodule bacteria. Blackwell Scientific Publications, Oxford.

VINCENT, J.M. and B. Humphrey. 1970. Taxonomically significant group antigens in *Rhizobium*. J. Gen. Microbiol. 63: 379-382.

WHITE, L.O. 1972. The taxonomy of the crown-gall organism *Agrobacterium tumefaciens* and its relationship to rhizobia and other agrobacteria. J. Gen. Microbiol. 72: 565-574.

WILSON, J.K. 1939. Leguminous plants and their associated organisms. Mem. N.Y. Agric. Exp. Stn. (Ithaca) 221.

WILSON, J.K. 1944. Over five hundred reasons for abandoning the cross-inoculation groups of the legumes. Soil Sci. 58: 61-69.

YELTON, M.M., S.S. Yang, S.A. Edie and S.T. Lim. 1983. Characterization of an effective salt-tolerant, fast-growing strain of *Rhizobium japonicum*. J. Gen. Microbiol. 129: 1537-1547.

ZAENEN, I., N. van Larebeke, H. Teuchy, M. van Montagu and J. Schell. 1974. Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J. Mol. Biol. 86: 109-127.