Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

A MOLECULAR AND GENETICAL ANALYSIS OF SYMBIOTIC GENES IN *LOTUS* RHIZOBIA

By

KAW-YAN CHUA

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

October, 1984

ABSTRACT

Relative DNA homologies were determined among 24 strains of fast-growing rhizobia and 26 strains of slow-growing rhizobia with particular reference to those which nodulate *Lotus* species. Two major DNA homology groups were identified which shared less than 10% relative DNA homology. Fast-growing *Lotus* rhizobia were grouped with related fast-growing strains in DNA homology group I, and these strains are designated as *Rhizobium loti*. Slow-growing strains from *Lotus*, (*Bradyrhizobium spp*. (*Lotus*)), *Glycine max* (*Bradyrhizobium japonicum*), *Ornithopus* and *Lupinus* formed DNA homology group II which was further divided into 4 DNA homology subgroups. *Bradyrhizobium spp*. (*Lotus*) were shown to be genetically distinct from *B. japonicum*.

Plasmid profiles were determined for strains of *R. loti* and *Bradyrhizobium spp*. (*Lotus*). All *R. loti* strains contained a single large indigenous plasmid, whereas strains from *Bradyrhizobium spp*. (*Lotus*) carried multiple plasmids of molecular weights ranging from 130-280 MDal. A plasmid-cured derivative of *R. loti* NZP2213 was isolated mDal and found to still form effective nodules on *Lotus tenuis*, suggesting that nodulation and nitrogen fixation genes are not plasmid-borne in this strain. The functions of the indigenous plasmids carried in *Lotus* rhizobia are unknown.

Symbiotic mutants of *R. loti* strain NZP2037 were isolated by random Tn5-mutagenesis. Mutants included strains blocked in root hair curling (Hac), nodule initiation (Noi), bacterial release (Bar) and nitrogen fixation (Cof) on *Lotus pedunculatus*.

The nodulation (*nod*) gene region from *R. loti* strain NZP2037 was isolated from a pLAFR1-NZP2037 gene library using the cloned Tn5 containing *Eco*RI fragment from the Nod$^-$ mutant as a probe. Two cosmids were isolated and were found to complement the NZP2037 Nod$^-$ mutant. Hybridisation and complementation experiments confirmed that a 7.1 kb *Eco*RI fragment present in both *nod* cosmids carried gene sequences involved in nodulation. An *Eco*RI and *Hind* III restriction enzyme map of the *nod* gene region in *R. loti* NZP2037 was constructed using *nod* cosmids pPN305 and pPN306.

Using the cloned *R. loti* NZP2037 *nod* gene region (7.1 kb *Eco*RI fragment from pPN305) as a hybridisation probe, highly conserved DNA sequences from other strains of *R. loti* and *Bradyrhizobium* *spp*. (*Lotus*) were identified. The *nod* gene region from *Bradyrhizobium* *spp*. (*Lotus*) strain NZP2309 was isolated by direct 'in planta' complementation of the *R. loti* Nod$^-$ mutant using a NZP2309 pLAFR1 gene library.

Comparative physical and genetical studies showed that the *R. loti* NZP2037 *nod* gene region isolated shared functional similarities with previously isolated *nod* gene regions from *R. trifolii* and *R. meliloti* despite the fact that only weak DNA homology was observed between the corresponding regions. This suggested that at least some of the *nod* gene sequences carried on the cloned *R. loti* *nod* gene region belong to the highly conserved 'common' *nod* gene sequence category.

ACKNOWLEDGEMENTS

I wish to thank my supervisors Dr B.D.W. Jarvis, Department of Microbiology and Genetics, Massey University, for his constant interest and advice throughout this study and Dr D.B. Scott, Applied Biochemistry Division, Department of Scientific and Industrial Research (D.S.I.R.) for his inspiration and guidance with all aspects of this work and his generosity in sharing his laboratory space and research resources for the past two years.

I also wish to thank Dr C.E. Pankhurst of Applied Biochemistry Division, D.S.I.R., for the advice and assistance with the light and electron microscopy studies and the construction of *Rhizobium loti* genomic library, as well as for his continued interest in this work.

Special thanks are also due to the following members of D.S.I.R., Palmerston North: Dr J.J. Patel, for his assistance with the bacteriophage typing technique; Dr W.T. Jones, for carrying out the tandem crossed-immunoelectrophoresis experiment; Mr D.H. Hopcroft, for his assistance with the electron microscopy studies and Dr B. Terzaghi for her advice with the transposon mutagenesis experiment.

I would also like to thank the staff members of *Rhizobium* laboratory, D.S.I.R., for their cooperation and encouragement over the past two years; Mr C.B. Court for his assistance in thesis figures preparation and Mrs G. Shaw for typing the thesis. To all the members of my family, I would like to show my greatest gratitude, for their love and support over the years.

During the period of my Ph.D. programme I have been supported by a study grant from D.S.I.R. which I gratefully acknowledge.

CONTENTS

PAGE

ABSTRACT

ACKNOWLEDGEMENTS

CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

ABBREVIATIONS

CHAPTER ONE. GENERAL INTRODUCTION 1

1.1 SIGNIFICANCE OF BIOLOGICAL NITROGEN FIXATION 1

1.2 SIGNIFICANCE OF *LOTUS SPP*. IN NEW ZEALAND AGRICULTURE 1

1.3 CHARACTERISTICS OF *LOTUS* RHIZOBIA 2

1.4 CLASSIFICATION OF ROOT NODULE BACTERIA 3

1.5 NODULE DEVELOPMENT 5

1.6 NODULE FORMATION IN *LOTUS* 7

1.7 GENETICS OF NITROGEN FIXATION 7

1.8 AIMS OF THIS STUDY 13

CHAPTER TWO. DNA HOMOLOGY STUDIES OF LOTUS RHIZOBIA 14

2.1 INTRODUCTION 14

2.2 METHODS AND MATERIALS 14
2.2.1 Bacterial strains 14
2.2.2 Preparation of culture media 14
2.2.2.1 Liquid media 14
2.2.2.2 Solid media 15
2.2.3 Maintenance of cultures 15
2.2.4 Growth of bacteria 15
2.2.5 Preparation of unlabelled DNA 15
2.2.6 Preparation of [^{32}p]-labelled DNA 18 ^{32}P
2.2.7 DNA reassociation 18
2.2.8 Separation of single and double stranded DNA on hydroxyapatite (HA) 18
2.2.9 Thermal stability study of reassociated duplexes 19

2.3 RESULTS 20
2.3.1 DNA-DNA reassociation at 65^{o}C among rhizobia from *Lotus spp*. and strains from other plants 20

2.3.2 Thermal stability of reassociated DNA duplexes 21

2.4 DISCUSSION 22

2.5 SUMMARY 26

CHAPTER THREE. CHARACTERISATION OF PLASMIDS IN LOTUS RHIZOBIA 27

3.1 INTRODUCTION 27

3.2 METHODS AND MATERIALS 28
3.2.1 Bacterial strains and plasmids 28
3.2.2 Preparation of media 28
3.2.2.1 Liquid media 28
3.2.2.2 Solid media 29
3.2.3 Maintenance of cultures 29
3.2.4 Growth of bacteria 29
3.2.5 Ultraviolet light mutagenesis of *Rhizobium loti* 30
3.2.6 Plant test 30
3.2.7 Isolation of bacteria from nodules 31
3.2.8 Heat curing experiment 31
3.2.9 Bacteriophage susceptibility tests 31
3.2.10 Tandem-crossed immunoelectrophoresis 32
3.2.11 Plasmid isolation by Eckhardt method 32
3.2.12 Isolation of plasmids by the Tait procedure 33
3.2.13 Preparation of *R. loti* plasmid DNA 35
3.2.14 Isolation of genomic DNA 37
3.2.15 Digestion of DNA with restriction enzymes 38
3.2.16 Purification of DNA preparation 38
3.2.16.1 Purification by phenol chloroform extraction 38
3.2.16.2 Desalting with Sephadex G-50 column 39
3.2.17 Horizontal agarose gel electrophoresis of DNA 39
3.2.18 Preparation of [^{32}P]-labelled DNA probes 40
3.2.19 DNA transfer and hybridisation 41

3.3 RESULTS 42
3.3.1 Physical characterisation of plasmids in *Lotus* rhizobia 42
3.3.2 DNA homology between pRlo2213a and pRlo2037a 42
3.3.3 Attempt to isolate Nod$^-$ derivatives of NZP2213 by heat curing 43
3.3.4 Attempt to isolate a Nod$^-$ derivative of NZP2213 by UV mutagenesis 43
3.3.5 Hybridisation of pRlo2213a to total DNA of *R. loti* strains NZP2213 and PN225 44
3.3.6 Identification of strain PN225 as derivative of *R. loti* NZP2213 44
3.3.6.1 Strain identification by phage typing 44
3.3.6.2 Strain identification by tandem crossed-immunoelectrophoresis 44
3.3.6.3 Strain identification by restriction enzyme digest patterns 45
3.3.7 DNA homology between the *nod* gene region of *R. loti* NZP2037 and the plasmid-cured derivative strain PN225 45

3.4 DISCUSSION 45

3.5 SUMMARY 49

CHAPTER FOUR. ISOLATION AND CHARACTERISATION OF TN5-INDUCED SYMBIOTIC MUTANTS OF RHIZOBIUM LOTI 50

4.1 INTRODUCTION 50

4.2 METHODS AND MATERIALS 52
- 4.2.1 Bacteria strains and plasmids 52
- 4.2.2 Preparation of culture media 52
  - 4.2.2.1 Liquid media 52
  - 4.2.2.2 Solid media 52
- 4.2.3 Maintenance of cultures 52
- 4.2.4 Growth of bacteria 53
- 4.2.5 Conjugation 53
- 4.2.6 Screening method for symbiotic mutants 53
- 4.2.7 Plasmid analysis by the Eckhardt procedure 54
- 4.2.8 Bacteriophage susceptibility tests 54
- 4.2.9 Preparative isolation of plasmid DNA from *E. coli* by clear lysate method 54
- 4.2.10 Isolation of genomic DNA 56
- 4.2.11 Digestion of DNA with restriction enzymes 56
- 4.2.12 Purification of DNA 56
- 4.2.13 Agarose gel electrophoresis of DNA 56
- 4.2.14 Preparation of $[^{32}P]$-labelled DNA-probes 56
- 4.2.15 DNA transfer and hybridisation 56
- 4.2.16 Determination of molecular weights 56
- 4.2.17 Light and electron microscopy of nodules 56
- 4.2.18 Examination of root hairs by light microscopy 57

4.3 RESULTS 57
- 4.3.1 Transposon mutagenesis of *Rhizobium loti* 57
- 4.3.2 Physical analysis of presumptive Tn5-induced symbiotic mutants by Southern hybridisation 58
- 4.3.3 Hybridisation of total DNA from *R. loti* Tn5-mutants with $[^{32}P]$-labelled *nif* HDK probe 59
- 4.3.4 Light microscopic examination of *Lotus pedunculatus* root hairs inoculated with Nod⁻ mutant of *R. loti* 59
- 4.3.5 Light and electron microscopic examination of nodules induced by the Fix⁻ mutants 60
  - 4.3.5.1 Morphology of nodules induced by the wild-type *R. loti* strain NZP2037 60
  - 4.3.5.2 Morphology of tumour-like nodules 61
  - 4.3.5.3 Morphology of ineffective nodules 61

4.4 DISCUSSION 64

4.5 SUMMARY 67

CHAPTER FIVE. MOLECULAR CLONING AND GENETICAL ANALYSIS OF NODULATION GENES IN RHIZOBIUM LOTI 69

5.1 INTRODUCTION 69

5.2 METHODS AND MATERIALS 71
5.2.1 Bacterial strains and plasmids 71
5.2.2 Preparation of culture media 71
5.2.2.1 Liquid media 71
5.2.2.2 Solid media 71
5.2.3 Maintenance of cultures 71
5.2.4 Growth of bacteria 71
5.2.5 Isolation of genomic DNA 71
5.2.6 Isolation of plasmid DNA 71
5.2.6.1 Cleared lysate method 71
5.2.6.2 Alkaline lysis method 71
5.2.6.3 Rapid boiling method 72
5.2.7 Electroelution of DNA from agarose 73
5.2.8 DNA ligation 74
5.2.9 Transformation of *E.coli* 74
5.2.10 Colony hybridisation 75
5.2.11 Preparation of [^{32}P]-labelled DNA probe 76
5.2.12 DNA transfer and hybridisation 76
5.2.13 Conjugation 76
5.2.14 Plant test 76
5.2.15 Isolation of bacteria from nodules 76
5.2.16 Light and electron microscopy of nodules 76
5.2.17 Construction of a pLAFR1 gene bank to *Bradyrhizobium spp (Lotus)* strain NZP2309 (CC814s) 76
5.2.18 'In planta' complementation of *Rhizobium loti* Nod$^-$ mutant 78

5.3 RESULTS 78
5.3.1 Cloning of Tn5::*nod* region of NZP2037 78
5.3.2 Isolation of wide type nodulation genes by colony hybridisation 79
5.3.3 Construction of a physical map in the nodulation gene region of *R. loti* NZP2037 79
5.3.4 Complementation of Nod$^-$ mutant of *R. loti* strain NZP2037 by cosmids carrying presumptive wild type *nod* DNA of NZP2037 81
5.3.5 Light and electron microscopy of nodules formed on *Lotus pedunculatus* by complemented derivatives of the Nod$^-$ mutant strain PN233 82
5.3.6 DNA homology between the *nod* gene region of *R.loti* NZP2037 and other *Rhizobium* strains 83
5.3.7 '*In planta*' direct complementation of *R.loti* NZP2037 Nod$^-$ mutant with a gene library of *Bradyrhizobium spp* (*Lotus*) strain NZP2309 84
5.3.8 Complementation of *Rhizobium* nodulation function using heterologous *nod* regions 86

5.4. DISCUSSION 86

5.5 SUMMARY 93

CHAPTER SIX: GENERAL DISCUSSION 94

REFERENCES 99

INDEX OF TABLES

Table Number	Title
1.1	Proposed steps in *Rhizobium*-legume symbiosis.
2.1	Strains of fast and slow-growing rhizobia included in this study.
2.2	DNA-DNA reassociation at 65°C among rhizobia from *Lotus spp.* and strains from other plants.
2.3	Thermal stability of rhizobial DNA duplexes formed at 65°C.
3.1	Bacterial strains and plasmids.
3.2	Plasmids in *Lotus* rhizobia.
4.1	Bacterial strains and plasmids.
4.2	Characterisation of Tn5-induced mutants of *R. loti* NZP2037.
5.1	Bacterial strains and plasmids.
5.2	*Eco*RI restriction fragments from *Rhizobium loti*, *R. trifolii* and *R. meliloti* total DNA homologous to *R. loti nod* probe and/or *R. trifolii nod* probe.

INDEX OF FIGURES

Figure Number	Title
2.1	Thermal elution profiles of reassociated DNA duplexes formed at 65°C between *B. japonicum* ATCC10324 reference DNA and DNA from other selected strains.
3.1	A Sephadex G-50 column used for DNA purification.
3.2	Apparatus for DNA transfer by Southern blotting.
3.3	DNA homology between pRlo2213a and pRlo2037a.
3.4	Agarose gel of plasmids in *R. loti* strains.
3.5	Hybridisation of pRlo2213a to *Eco*RI enzyme digests of *R. loti* total DNA.
3.6	Identification of *R. loti* strains by phage typing.
3.7	Tandem crossed-immunoelectrophoresis patterns of *R. loti* strains.
3.8	*Eco*RI restriction enzyme digest patterns of total DNA from *R. loti* strains.
3.9	Hybridisation of the *nod* gene region of NZP2037 to *Eco*RI digests of DNA from strains NZP2213, PN225 and NZP2037.
4.1	The vector plasmid pSUP1011 used for Tn5 mutagenesis.
4.2	*Lotus pedunculatus* plants inoculated with *R. loti* wild type and Tn5-induced symbiotic mutant strains.
4.3	Physical analysis of presumptive Tn5 mutants by Southern hybridisation.
4.4	Hybridisation of Tn5 and *nif* HDK sequences to total DNA from *R.loti* Tn5-induced mutants.
4.5	Light micrographs of root hairs of *Lotus pedunculatus* plants.
4.6	Light and electron micrographs showing the structure of root nodules formed by *R. loti* strain NZP2037 on *L. pedunculatus*.
4.7	Light and electron micrographs showing the morphology of the tumour-like nodule formed by the Noi mutants PN234, PN236, PN237, PN238 and PN244 on *L. pedunculatus*.
4.8	Light and electron micrographs showing the structure of the nodules formed by the Bar mutant PN239 on *L. pedunculatus*.
4.9	Light and electron micrographs showing the structure of the nodules formed by the Cof mutants PN235 on *L. pedunculatus*.

4.10 Light and electron micrographs showing the structure of nodules formed by Cof mutant PN246 on *L. pedunculatus*.

5.1 Agarose gel of an *Eco*RI digest of pPN301.

5.2 Hybridisation of Tn5::*nod* region from pPN301 to cosmids carrying *R. loti* wild type DNA sequences.

5.3 *Hind* III (H) and *Eco*RI (E) restriction enzyme map of the *nod* gene region of *R. loti* NZP2037.

5.4 Complementation of the Tn5-induced Nod$^-$ mutant with *R. loti* NZP2037 wild type gene sequences.

5.5 Light and electron micrographs showing the structure of ineffective nodules and tumour-like nodules formed on *Lotus pedunculatus* by a derivative of the Nod$^-$ mutant PN233 carrying pPN305.

5.6 Hybridisation of the cloned *nod* gene region of NZP2037 to *R. loti* and *Bradyrhizobium spp. (Lotus)* total DNA.

5.7 Hybridisation of the cloned *nod* gene region of NZP2037 to *R. loti*, *R. trifolii* and *R. meliloti* total DNA.

5.8 Hybridisation of cloned *R. trifolii nod* DNA to *R. loti*, *R. trifolii* and *R. meliloti* total DNA.

5.9 Hybridisation of *R. trifolii nod* DNA to cloned *nod* gene sequences of *R. loti* NZP2037.

5.10 Light and electron micrographs showing the structure of root nodules formed on *Lotus pedunculatus* by a derivative of the Nod$^-$ mutant PN233 containing pPN329.

ABBREVIATIONS

Nm	neomycin
Km	kanamycin
Cb	carbenicillin
Tc	tetracycline
Ap	ampicillin
Sp	spectinomycin
Cm	chloramphenicol
Str	streptomycin
Kb	kilobases
MES	2-(N-morpholino)ethanesulfonic acid
SDS	sodium dodecyl sulphate
Nod^+Fix^+	*Rhizobium* phenotype characterised by the ability to induce visible nodules on plant roots which are capable of nitrogen fixation.
Nod^+Fix^-	*Rhizobium* phenotype characterised by the ability to induce visible nodules on plant roots which are not capable of nitrogen fixation.

Abbreviations not defined in this list are "accepted" abbreviations (Biochemical Journal (1983) **209**, 1-27).

CHAPTER ONE

GENERAL INTRODUCTION

1.1 SIGNIFICANCE OF BIOLOGICAL NITROGEN FIXATION

Biological nitrogen fixation is a very important process that increases the availability of fixed nitrogen for plant growth. It is estimated that about two-thirds of the annual turn over of nitrogen in the biosphere comes from biological sources (Burns and Hardy, 1975; Burris, 1980). One of the most significant biological nitrogen fixing systems is the symbiotic association between leguminous plants and the root nodule bacteria. This microbiological process accounts for 50-70% of world biological nitrogen fixation (Burris, 1977). It also serves as an important, low cost alternative to nitrogenous fertilizers in agriculture. For example, in countries such as Australia and New Zealand, symbiotic nitrogen fixation is the most important source of fixed nitrogen in pasture cultivation. In addition the extension of agricultural practices involving symbiotic nitrogen fixation to under-developed countries would be fruitful in that relatively simple technologies would substantially improve soil fertility and animal or human nutrition. Therefore symbiotic nitrogen fixation has great practical value from both the biosphere and human points of view.

Recently there has been an increased research interest in symbiotic nitrogen fixation, with much of the research effort focussed on the elucidation of the mechanism by which symbiotic nitrogen fixation occurs and is controlled.

1.2 SIGNIFICANCE OF LOTUS SPP. IN NEW ZEALAND AGRICULTURE

The root nodule bacteria studied in this thesis are associated with

Lotus (trefoil) species. There are two agriculturally important Lotus species in New Zealand: Lotus pedunculatus Cav. (big trefoil) and Lotus corniculatus L. (bird's foot-trefoil). "Grassland Maku" is the most commonly sown cultivar of Lotus pedunculatus. On acid hill-country of low fertility (Levy, 1918; Nordmeyer and Davis, 1977) and tussock grasslands (Lowther, 1977; Scott, 1979) Lotus pedunculatus (Maku) gives better yields than white clover (Brock, 1973; Gibson et al., 1975; Hart et al., 1981), and shows a better response to phosphate fertilizers because of its higher tolerance to aluminium toxicity (Davis, 1981a,b and c). In addition, Maku Lotus has a higher resistance to insect damage than white clover in tussock grasslands (Barratt and Johnstone, 1984). Lotus pedunculatus is also widely used by the Forest Service with new tree plantings and by the Ministry of Works in soil conservation and highway construction programmes (Lancashire et al., 1980). Lotus corniculatus is not widely used in New Zealand, but has been recommended for revegetation of eroded hill country (Nordmeyer and Davis, 1977). Recently, Lotus corniculatus has gained in significance in intensive farming situations because of its non-bloating properties (Jones et al., 1970) and grass grub resistance (Kain and Atkinson, 1975).

1.3 CHARACTERISTICS OF LOTUS RHIZOBIA

Two distinct groups of Lotus rhizobia have been identified on the basis of a range of microbiological, biochemical and physiological tests (reviewed by Jarvis et al., 1982). Based on the growth rate and acid production on yeast extract mannitol medium (Norris, 1965; Abdel-Chaffar and Jensen, 1966; Brockwell and Hely, 1966), Lotus rhizobia can be divided into fast-growing acid-producers and slow-growing non-acid-producers. The fast-growers normally form effective nodules on Lotus corniculatus and Lotus tenuis, whereas the slow-growers normally nodulate *Lotus pedunculatus*. Some fast-growing strains such as NZP2037 are also able to nodulate *Lotus pedunculatus* as effectively as their slow-growing counterparts (Pankhurst, 1977). Recent DNA:DNA hybridisation studies have shown that the fast-growing *Lotus rhizobia* are genetically related to a wide range of other rhizobia strains isolated from *Caragana arborescens*, *Cicer arietinum*, *Leucaena leucocephala*, *Lupinus densiflorus*, *Onobrychis viciifolia*, *Parochetus communis*, *Psoralea eriatha*, *Robinia pseudoacacia*, *Sophora microphylla* and *Trifolium lupinaster* (Crow *et al.*, 1981). It has also been found that the slow-growing *Lotus* rhizobia can nodulate plants from the genera *Lupinus*, *Ornithopus* and *Anthyllis* (Jensen, 1967; Jensen and Hansen, 1968).

Fast-growing *Lotus* rhizobia differ from other fast-growing rhizobia because they have subpolar rather than lateral flagella (Abdel-Ghaffar and Jensen, 1966) and do not ultilise dulcitol (Vincent, 1974). Although they share less than 5% DNA homology with *R. trifolii*, *R. leguminosarum* and *R. meliloti*, their growth rates and pH changes on yeast extract mannitol medium are comparable to other fast-growers.

1.4 CLASSIFICATION OF ROOT NODULE BACTERIA

The various aspects of the taxonomy of *Rhizobium* have recently been reviewed by Elkan (1981). The genus *Rhizobium* has traditionally been divided into fast- and slow-growing groups (Lohnis and Hansen, 1921). Fred *et al.* (1932) introduced the concept of cross-inoculation groups which were defined as "groups of plants within which the root nodule organisms are mutually interchangeable". On the basis of this concept, six species of *Rhizobium* related to the agronomically important legume groups were designated (Allen, 1957). This classification system proved to be unsatisfactory for several reasons: (1) The cross-inoculation groups are not mutually exclusive, each of the species has been shown to be cross-infective to some degree (Wilson, 1944; Lange, 1961; Dixon, 1969). (2) The plant species included in the six formally accepted cross-inoculation groups represent only a tiny fraction of the 14,000 or so known species of legumes (Jordan and Allen, 1974). (3) In a number of fast-growing species the plant specificity determinants are located on large indigenous plasmids (Denarie *et al.*, 1981).

Alternative methods such as numerical taxonomy (Graham, 1964; 'tMannetje, 1967; Moffett and Colwell, 1968; White, 1972), percent G+C and type of flagellation (De Ley and Rassel, 1965) and DNA homology (Herberlein *et al.*, 1967; Elkan and Usanis, 1971; Gibbins and Gregory, 1972) have been used to improve the classification of the genus *Rhizobium*. Some of these properties were considered in the revised classification of the genus *Rhizobium* in the eighth edition of Bergey's Manual (Jordan and Allen, 1974). However the cross inoculation group concept remained as the distinguishing feature in the description of each species. An additional limitation of this classification was that insufficient biochemical data were was included, hence making it difficult to determine the taxonomic significance of the biochemical differences mentioned. However, the major separation of the root nodule bacteria into slow-growers and fast-growers as originally described by Lohnis and Hansen (1921) seems to carry real taxonomic significance (Elkan, 1981).

The revised classification of root nodule bacteria in the ninth edition of Bergey's Manual (Jordan, 1984) takes into account recent publications concerning DNA homology (Jarvis *et al.*, 1980; Crow *et al.*, 1981; Hollis *et al.*, 1981), two-dimensional polyacrylamide gel electrophoresis of cellular proteins (Roberts *et al.*, 1980) and genetic evidence on the transfer of infectivity via plasmids (Denarie *et al.*, 1981). The present classification shows the following changes: (1) the fast-growing species *Rhizobium trifolii*, *Rhizobium phaseoli* and *Rhizobium leguminosarum* are combined as one species, designated *R. leguminosarum* (Frank) Frank, comprising the three biovars *trifolii*, *phaseoli* and *viceae*. (2) *Rhizobium meliloti* is retained as a separate species. (3) The fast-growing *Lotus* rhizobia and related strains are designated as a separate species *R. loti* based on a variety of evidence reviewed by Jarvis *et al.* (1982). (4) The slow-growing rhizobia have been transferred to a separate genus *Bradyrhizobium* (Jordan, 1982), which consists of only one designated species, *Bradyrhizobium japonicum* (Buchanan, 1980) comb. nov. The slow-growing *Lotus* rhizobia possess most of the characteristics of the genus *Bradyrhizobium*, but there is insufficient information at present to classify them as a new species. In this work, they are referred to as *Bradyrhizobium spp.* (*Lotus*).

1.5 NODULE DEVELOPMENT

The formation of a nitrogen-fixing leguminous root nodule is the result of a sequence of coordinated symbiotic interactions between the host plant and the invading rhizobia. This process has been extensively reviewed (Libbenga and Bogers, 1974; Dart, 1977; Goodchild, 1977; Broughton, 1978; Robertson and Farnden, 1980; Vincent, 1980; Meijer, 1982; Newcomb, 1981; Bauer, 1981; Dazzo and Hubbell, 1982). At least seven individual steps can be identified during the formation of a root nodule by way of root hair entry. These are: (1) Multiplication of bacterial cells in the rhizophere. (2) Adsorption of the bacteria on the surface of the cell wall of the root hair. (3) Curling of the plant root hairs. (4) Infection of the curled root hair with development of an infection thread. (5) Growth of the infection thread down the root hair and across the root cortex. (6) Release of the rhizobia into polyploid root cortical cells. (7) Development of the rhizobia into nitrogen fixing bacteroids.

The early preinfection events of nodule development include mutual host-symbiont recognition, adsorption of the bacteria to root hairs, root hair branching and curling. Various suggestions have been put forward to explain the specificity between the *Rhizobium* and the legume. The most cited one proposes that plant lectins are involved in recognizing surface antigens on the *Rhizobium* and several recent reports discuss this controversial hypothesis (Bauer, 1977; Broughton, 1978; Dazzo, 1980a and b; Napoli and Albersheim, 1980; Dazzo and Hubbell, 1982). Root hair branching and curling are caused by specific factors produced only by rhizobia capable of infecting the root hair (Yao and Vincent, 1968; Dart, 1977; Broughton, 1978). Infection is thought to occur by invagination of the root hair cell wall (at the point of curling) which results in the formation of an infection thread which grows down the root hairs and into the root cortex. At this early stage of infection, the development of polyploid meristem tissue commences within the root cortex. Rhizobia grow and divide within the infection thread as it advances across the host cortex. The thread branches many times resulting in the infection of many cells by the same infection thread. Shortly after the penetration of the cortical cells by the infection thread, disintegration of the thread wall takes place. Rhizobia are released into the plant cells and become enclosed in peribacteroid membranes (Robertson *et al*., 1978). Within the peribacteroid membrane, rhizobia enlarge and differentiate into bacteroids.

These morphological changes are accompanied by profound biochemical changes in the bacteroid (Sutton *et al.*, 1981) and plant cells (Sutton, 1982).

1.6 NODULE FORMATION IN LOTUS

The nitrogen-fixing root nodules formed on *Lotus pedunculatus* by *R. loti* are spherical and first appear on the root 10-12 days after *Rhizobium* inoculation (Pankhurst *et al.*, 1979). The nodules contain a central zone of infected plant cells which are filled with small, rod-shaped bacteroids (Craig and Williamson, 1972) surrounded by a cortex 8-10 cells thick containing vascular traces. *Lotus* rhizobia enter host plant cells via an infection thread, and become enclosed by a peribacteroid membrane. Within the membrane the rhizobia divide once or twice before enlarging into bacteroids (Pankhurst *et al.*, 1979). The nodule cortical cells frequently contain electron-dense osmiophilic flavolans (Parham and Kaustinen, 1977).

Some *R. loti* strains including strain NZP2213 induce ineffective (non-nitrogen-fixing), tumour-like structures on *Lotus pedunculatus* plants (Pankhurst *et al.*, 1979). These structures contain a central zone of small actively dividing cells surrounded by a cortex. However, the rhizobia do not infect the cells (Pankhurst *et al.*, 1979).

1.7 GENETICS OF NITROGEN FIXATION

The recent progress in genetic research on the *Rhizobium*-legume symbiosis can be attributed to advances in the methods available for the genetic analysis of *Rhizobium* (Kondorosi and Johnston, 1981; Beringer *et al.*, 1982). One important result was the demonstration that indigenous plasmids in *Rhizobium* carry the determinants necessary for nodule formation and nitrogen fixation (reviewed by Denarie *et al.*, 1981). One of the earliest reports suggesting that symbiotic genes were located on a plasmid was that by Higashi (1967). He demonstrated that the ability to nodulate clover could be transferred by conjugation from *R. trifolii* to *R. phaseoli* and also showed a loss of nodulation ability in these strains following acridine orange treatment. Further evidence showing a loss of nodulation and nitrogen fixation abilities following plasmid curing have also been reported (Dunican and Cannon, 1971; Zurkowski *et al.*, 1973; Dunican and Tierney, 1974). Johnston *et al.* (1978) reported that by using a *R. leguminosarum* strain carrying a conjugative plasmid pRL1J1 tagged with Tn5, the ability to nodulate peas could be transferred at a high frequency to a non-isogenic Nod$^-$ derivative of *R. leguminosarum* and to wild-type strains of *R. trifolii* and *R. phaseoli*. These results strongly implied that some of the genetic determinants for nodulation were plasmid-linked.

The development of techniques for the detection and isolation of large plasmids in *Rhizobium* (Sutton, 1974; Nuti *et al.*, 1977; Eckhardt, 1978; Casse *et al.*, 1979; Hirsch *et al.*, 1980; Schwinghamer, 1980) allowed direct comparisons to be made of the plasmid profiles in wild-type strains and derivatives that were altered in their symbiotic properties. Using these methods the loss of nodulation ability in *Rhizobium* was found to be associated with either the loss of a plasmid (Zurkowski and Lorkiewicz, 1978; Casse *et al.*, 1979; Hooykaas *et al.*, 1981) or the generation of a deletion within a plasmid (Beynon *et al.*, 1980; Hirsch *et al.*, 1980; Banfalvi *et al.*, 1981). In addition, the nitrogenase structural genes *nif* H, D and K have been identified on these symbiotic (Sym) plasmids by hybridisation with previously cloned and characterised *nif* HDK sequences from

Klebsiella pneumoniae (Nuti *et al.*, 1979; Banfalvi *et al.*, 1981; Hombrecher *et al.*, 1981; Prakash *et al.*, 1981). A number of interstrain and interspecific Sym plasmid transfer experiments have been carried out confirming the presence of nodulation (*nod*) genes on these plasmids. The presence of self-transmissible Sym plasmids in some strains has facilitated such gene transfer studies. Brewin *et al.* (1980a) found that transfer of the self-transmissible *R. leguminosarum* Sym plasmid, pJB5J1 (pRL1J1::Tn5) into a number of Nod$^-$ and Fix$^-$ derivatives of *R. leguminosarum* resulted in suppression of the mutant phenotype. In a series of plasmid transfer experiments, Hooykaas *et al.* (1981) showed that a self-transmissible *R. trifolii* Sym plasmid restored the nodulation ability of Nod$^-$ derivatives of *R. trifolii* and *R. leguminosarum*, and also conferred clover-specific nodulation ability to *R. leguminosarum* and *Agrobacterium*. Mobilization of a *R. trifolii* Sym plasmid has also been achieved by using R68.45 as a helper plasmid (Scott and Ronson, 1982). In these experiments the Sym plasmid was found to be mobilized as a stable cointegrate plasmid (Sym::R68.45) when crosses were carried out between the wild-type and the Sym plasmid-cured derivative. It is also well established for *R. meliloti* (Rosenberg *et al.*, 1981; Banfalvi *et al.*, 1981) and *R. phaseoli* (Lamb *et al.*, 1982) that some of the genes involved in nodulation and nitrogen fixation are located on large plasmids. The presence of large indigenous plasmids in *Bradyrhizobium* species has been reported (Cole and Elkan, 1973; Gross, *et al.*, 1979; Scott and Tait, 1980; Haugland and Verma, 1981; Masterson *et al.*, 1982). However, to date no evidence has been obtained demonstrating the presence of *nod* or *nif* genes on plasmids in these strains (Haugland and Verma, 1981; Masterson *et al.*, 1982).

Much of the recent *Rhizobium* research has focused on the identification and isolation of symbiotic genes. The *nod* and *nif* genes have been isolated by a number of different experimental strategies. Genes specific to the symbiosis have been identified by isolating mutants that are blocked at some stage in the symbiosis. A range of *Rhizobium* symbiotic mutants have been isolated by chemical or UV mutagenesis (Maier and Brill, 1976; Beringer *et al.*, 1977), but the most desirable method for isolating mutants is by transposon mutagenesis. Transposon-induced mutants are particularly useful for genetic studies because the site of mutation is defined by the inserted transposon, thus enabling the cloning of the flanking DNA sequences. A commonly used transposon is the well characterised Tn5 (Kleckner *et al.*, 1977; Jorgensen *et al.*, 1979). The introduction of Tn5 into the *Rhizobium* genome using a suicide plasmid pJB4J1 (pPH1::Mu::Tn5) was first described by Beringer *et al.* (1978). This system has been used to isolate Tn5-induced symbiotic mutants in *R. leguminosarum* (Buchanan-Wollaston *et al.*, 1980), *R. meliloti* (Banfalvi *et al.*, 1981; Meade *et al.*, 1982; Forrai *et al.*, 1983), *R. trifolii* (Rolfe *et al.*, 1981; Scott *et al.*, 1982), *R. phaseoli* (Noel *et al.*, 1984) and *Bradyrhizobium spp.* (*Parasponia*) (Cen *et al.*, 1982). Recently, Simon *et al.* (1983, 1984) constructed a group of broad host range mobilizable vectors based on *E. coli* specific narrow host range plasmids. These vetors provide an alternative system of Tn5 mutagenesis in *Rhizobium* (Simon *et al.*, 1984).

Symbiotic mutants of *Rhizobium* can be divided into two broad phenotypic classes: those blocked in nodule formation (Nod$^-$) and those incapable of nitrogen fixation (Nod$^+$ Fix$^-$). Mutant phenotypes are described in the context of nodule development, by using light and electron microscopic examinations to determine the developmental stage at which the symbiotic process is blocked. On this basis, Vincent (1980) devised a phenotypic code for a more detailed classification of *Rhizobium* symbiotic mutants (Table 1.1).

Nodulation genes have been isolated from *R. trifolii* (Scott *et al.*, 1982) and *R. leguminosarum* (Downie *et al.*, 1983a) by first isolating Tn5-induced Nod$^-$ mutants and then cloning sequences flanking the transposon for use as probes to isolate wild type sequences. The Tn5-induced Nod$^-$ derivatives were isolated by random Tn5 mutagenesis using the suicide plasmid pJB4J1 as a Tn5 donor. As Tn5 sequences contain no *Eco*RI restriction sites (Jorgensen *et al.*, 1979), the *Eco*RI containing Tn5 and flanking sequences were cloned from an *Eco*RI digest of mutant total DNA. The cloned *nod*::Tn5 sequences were labelled with [^{32}P] and used to identify wild type sequences from a gene library of the wild type strain.

In *R. meliloti*, Long *et al.* (1982) reported the isolation of *nod* genes by 'in planta' complementation of Nod$^-$ derivatives. The procedure involved the construction of a *R. meliloti* genomic library using the broad host range cosmid pLAFR1 (Friedman *et al.*, 1982). The genomic library was mass conjugated into Nod$^-$ derivatives in a triparental cross using HB101/pRK2013 as a source of helper plasmid (Ditta *et al.*, 1980). Transconjugants were inoculated onto host plants and the plants screened for the presence or absence of root nodules. Where complementation occurred, nodule isolates were recovered and cosmids responsible for the complementation were isolated.

Although transposon mutagenesis, coupled with molecular cloning, provides the potential for the isolation of other symbiotic genes of

Table 1.1 Proposed steps in Rhizobium-legume symbiosis

Sequence of events	Phenotypic code
(I) Preinfection	
(1) Root colonisation	Roc
(2) Root adhesion	Roa
(3) Hair branching	Hab
(4) Hair curling	Hac
(II) Infection and nodule formation	
(5) Infection	Inf
(6) Nodule initiation	Noi
(7) Bacterial release	Bar
(8) Bacteroid development	Bad
(III) Nodule function	
(9) Nitrogen fixation	Nif
(10) Complementary function	Cof
(11) Nodule persistance	Nop

Rhizobium, a more direct approach has been used to isolate *Rhizobium* nitrogenase structural genes (*nif*). The DNA sequences of *nif* structural genes are highly conserved between divergent species of nitrogen fixing bacteria (Ruvkun and Ausubel, 1980; Mazur *et al.*, 1980). Therefore, the cloned *nif* genes of *Klebsiella pneumoniae* can be used as a hybridisation probe to identify homologous sequences in *Rhizobium*. The plasmid pSA30, a pACYC184 derivative containing *nif* HDK from *Klebsiella pneumoniae* (Cannon *et al.*, 1977, 1979) was used as a probe to localise *nif* genes in *R. leguminosarum* (Ma *et al.*, 1982), *R. trifolii* (Scott *et al.*, 1982), *R. meliloti* (Corbin *et al.*, 1982) and in *B. japonicum* (Hennecke, 1981; Fuhrmann and Hennecke, 1982). The *Rhizobium nif* gene regions cloned on the basis of homology to *Klebsiella pneumoniae nif* genes were further characterised in *R. meliloti* (Ruvkun *et al.*, 1982) and in *B. japonicum* (Hahn and Hennecke, 1984) by generating transposon induced mutants by the site directed mutagenesis procedure described by Ruvkun and Ausubel (1981).

Genetic analysis of mutants and physical mapping have shown that the *nod* and *nif* genes are closely linked in *R. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981), *R. leguminosarum* (Buchanan-Wollaston *et al.*, 1980; Hirsch *et al.*, 1980; Downie *et al.*, 1983b), and *R. trifolii* (Schofield *et al.*, 1983; Scott *et al.*, 1984). In all these species, the *nod* genes identified were located on a DNA fragment less than 10kb in size and were mapped approximately 20kb away from the *nif* structural genes. Recently, the *nod* gene region involved in the early steps of nodule formation, including root hair curling (Hac), was found to share physical and functional homology between *R. trifolii*, *R. leguminosarum* and *R. meliloti* (Rolfe *et al.*, 1984; Kondorosi *et al.*, 1984).

In summary, the studies of symbiotic genes in *R. leguminosarum*, *R. trifolii*, *R. meliloti* and *R. phaseoli* indicate that genes involved in nodule formation and nitrogen fixation are located on large indigenous plasmids. A close linkage of *nod* and *nif* genes is a common feature of these *Rhizobium* species and at least one *nod* gene region, that involved in early nodule formation, is conserved in *Rhizobium* species studies to date.

1.8 AIMS OF THIS STUDY

(1) To determine the genetic relationship between the fast- and slow-growing *Lotus* rhizobia and between other selected groups of rhizobia.

(2) To determine if the nodulation genes of *Rhizobium loti* are located on plasmids.

(3) To isolate the nodulation genes from *R. loti* strain NZP2037.

(4) To examine the homology between the cloned nodulation genes of *R. loti* NZP2037 and those of *Bradyrhizobium spp.* (*Lotus*).

CHAPTER TWO

DNA HOMOLOGY STUDIES OF LOTUS RHIZOBIA

2.1 INTRODUCTION

Progress toward an improved classification for rhizobia was reviewed in chapter 1 and it was pointed out that rhizobia associated with many plant species are not included in the present classification. Fast-growing *Lotus* rhizobia have recently been included as a new species, *Rhizobium loti*, but the relationship between fast and slow-growing *Lotus* rhizobia and the relationships between slow-growing *Lotus* rhizobia and other members of the genus *Bradyrhizobium* requires further classification.

This chapter describes the use of DNA:DNA hybridisation techniques to study the DNA homology between the fast and slow-growing *Lotus* rhizobia and the relationship between *Lotus* rhizobia and two other groups of bacteria: DNA homology group 4 as described by Crow *et al*. (1981) and the DNA homology groups of *B. japonicum* as described by Hollis *et al*. (1981).

2.2 METHODS AND MATERIALS

2.2.1 Bacterial strains

These are described in Table 2.1.

2.2.2 Preparation of culture media

2.2.2.1 Liquid media

Tryptone yeast extract medium (TY; Beringer, 1974). Composition (g l^{-1}): tryptone (Difco), 5; yeast extract (Difco), 3; $CaCl_2.6H_2O$, 1.3.

Table 2.1 Strains of fast- and slow-growing rhizobia included in this study.

Strain	Host Plant	Growth rate[+]	Source[‡]
NZP2014*	*Lotus corniculatus*	fast	ABD Collection
NZP2037	*Lotus divaricatus*	fast	ABD
NZP2048	*Lotus corniculatus*	fast	ABD
NZP2077	*Lotus corniculatus*	fast	ABD
NZP2195	*Lotus tenuis*	fast	ABD
NZP2213	*Lotus pedunculatus* x *Lotus corniculatus*	fast	ABD
NZP2227=461	*Lotus corniculatus*	fast	Univ. Guelph
NZP2230=CC809a	*Lotus maroccanus*	fast	DPI
NZP2237=CC1005	*Hosackia spp.*	fast	DPI
NZP2238=LC265Da	*Lotus corniculatus*	fast	SCR
NZP2260	*Lotus tenuis*	fast	ABD
NZP2298=L72m103c	*Lotus corniculatus*	fast	Univ. Manitob.
NZP2300=L72m115b	*Lotus corniculatus*	fast	Univ. Manitob.
NZP5057	*Sophora tetraptera*	fast	ABD
NZP5198=L.densiflor85	*Lupinus densiflorus*	fast	SCR
NZP5201=Ca Wisc	*Caragana arborescens*	fast	ABD
NZP5223=3G2c2a	*Onobrychis viciifolia*	fast	USDA
NZP5266=3HOa1	*Cicer arietinum*	fast	USDA
NZP5268=CB712	*Psoralea pinnata*	fast	DTCP
NZP5275	*Sophora microphylla*	fast	ABD
NZP5302	*Robinia pseudoacacia*	fast	ABD
NZP5448=96DI	*Lupinus densiflorus*	fast	Nitragin
NZP5498=27A5	*Cicer arietinum*	fast	ABD
NZP5499=Revadim	*Cicer arietinum*	fast	ABD

Table 2.1 continued

Strain	Host Plant	Growth rate	Source
NZP2021=CC829=2012f	*Lotus pedunculatus*	slow	R. Callaghan
NZP2076	*Lotus hispidis*	slow	ABD
NZP2087	*Lotus pedunculatus*	slow	ABD
NZP2089	*Lotus pedunculatus*	slow	ABD
NZP2178	*Lotus pedunculatus*	slow	ABD
NZP2192	*Lotus corniculatus*	slow	ABD
NZP2201	*Lotus pedunculatus*	slow	ABD
NZP2228=CC803	*Lotus angustissimus*	slow	DPI
NZP2243	*Lotus angustissimus*	slow	ABD
NZP2257	*Lotus corniculatus*	slow	ABD
NZP2309=CC814s	*Lotus pedunculatus*	slow	DPI
NZP2308=CC814b	*Lotus pedunculatus*	slow	DPI
ATCC10325	*Lotus pedunculatus*	slow	ATCC
NZP2141	*Lupinus polyphyllus*	slow	ABD
NZP5044	*Lupinus arboreus*	slow	ABD
NZP5416=96KI	*Lupinus nanus*	slow	Nitragin
NZP5024=WU425	*Ornithopus compressus*	slow	Univ. W. Aus.
NZP5090	*Ornithopus pinnatus*	slow	ABD
NZP5112	*Ornithopus perpusillus*	slow	ABD
NZP5531=3Ilb76=US76	*Glycine max.*	slow	ABD
NZP5532=3Ilb31=US31	*Glycine max.*	slow	ABD
NZP5533=3Ilb123=US123	*Glycine max.*	slow	ABD
NZP5536=3Ilb505=US505	*Glycine max.*	slow	ABD
NZP5537=3Ilb110=US110	*Glycine max.*	slow	ABD
NZP5539=61A76	*Glycine max.*	slow	Nitragin
ATCC10324	*Glycine max.*	slow	ATCC

Table 2.1 continued

Footnotes

* NZP is the prefix for strains held in the Department of Scientific and Industrial Research Culture Collection, Applied Biochemistry Division, Palmerston North, New Zealand (Dr D.B. Scott, curator).

\+ Fast growing strains produce colonies $\geqslant 2$ mm diameter slow growing strains $\leqslant 2$ mm diameter after 5 days at 28°C.

‡ ABD, Applied Biochemistry Division, DSIR, Palmerston North, New Zealand; Univ. Guelph, G.G. Taylor, University of Guelph, Canada; DPI, J. Brockwell, Division of Plant Industry, Commonwealth Scientific and Industrial Research Organisation, Canberra, Australian Commonwealth Territory, Australia; SCR, The late H.L. Jensen, Government Laboratory for Soil and Crop Research, Department of Bacteriology, Lyngby, Denmark; Univ. Manitoba, K.W. Clarke, University of Manitoba, Canada; C.A. Parker, Department of Soil Science and Plant Nutrition, University of Western Australia, Perth, West Australia; USDA, D.F. Weber, United States Department of Agriculture, Beltsville, Maryland. DTCP, R.A. Date, Division of Tropical Crops and Pastures, Brisbane, Australia; Nitragin, J.C. Burton, Nitragin Company, Milwaukee, Wisconsin, U.S.A; ATCC, American Type Culture Collection.

Yeast extract mannitol medium (YM; Vincent, 1970). Composition (g l^{-1}): K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; sodium chloride, 0.1; mannitol, 10; yeast extract, 0.5.

2.2.2.2 Solid media

These were prepared by adding 15 g l^{-1} agar (Difco) to liquid media.

2.2.3 Maintenance of cultures

All cultures were maintained on YM slopes at 4°C and subcultured every two months. Freeze dried suspensions were used for long-term maintenance.

2.2.4 Growth of Bacteria

Rhizobium and *Bradyrhizobium* strains were incubated at 28°C in TY or YM medium for 2 or 6 days respectively.

2.2.5 Preparation of unlabelled DNA

Unlabelled DNA was prepared by a modification of the method of Brenner *et al*. (1969b).

Materials

(1) TE 50/20 buffer pH 8.0:50 mM-Tris/HCl, 20 mM-Na_2EDTA. (2) Lysozyme (Sigma; 10 mg ml^{-1}, stored at -20°C). (3) Pronase (Sigma; 10 mg ml^{-1}, self-digested at 37°C for 1h). (4) SDS (25% w/v). (5) Sodium perchlorate (5 M). (6) Chloroform: isobutyl alcohol (24:1, v/v). *(7) 1.4 M-Phosphate buffer (PB) pH 6.8. Composition (g l^{-1}): Na_2HPO_4, 99.4; $NaH_2PO_4 \cdot 2H_2O$, 109.2. (8) Hydroxyapatite (HA; Bio-Rad Laboratories, Richmond, California) was prepared in 0.0014 M-PB pH 6.8 according to the manufacturer's instructions. (9) 8 M-urea in 0.14 M-

PB pH 6.8. (10) Sarkosyl (Sigma; 0.1% w/v in TE 50/20 buffer pH 8.0). (11) Urea (Sigma). (12) Dialysis tubing (boiled in deionised water for 10 min, stored in 1 mM-Na_2EDTA solution at 4°C).

* All other phosphate buffers used in this work were prepared from the 1.4 M-PB pH 6.8 stock solution.

Method

Four litres of each bacterial strain were grown in YM broth at 28°C on a shaker. Fast and slow-growers were incubated for 2 to 6 days respectively. TY broth was used instead of YM broth to grow Bradyrhizobium japonicum strains to reduce extracellular polysaccharide (EPS) production.

Cells were harvested by centrifugation (10,000 g, 10 min, 4°C). Slow-growing strains that produced excessive EPS were pretreated before sedimentation by agitation in a Waring Blender for 5 min at full speed or by shaking at pH 12.0 for 30 min followed by neutralisation to pH 7.0.

Cell pellets of fast-growing strains were resuspended in 80 ml of TE (50/20) buffer, lysozyme was added to a final concentration of 1 mg ml^{-1}, and the suspension was incubated at 37°C for 1 h. After incubation the suspension was adjusted to 0.1 M with respect to sodium chloride, pronase was added to a final concentration of 0.5 mg ml^{-1} and incubation continued at 37°C for 1 h (Zaenen et al., 1974). Then SDS was added to a final concentration of 1% (w/v) and the mixture incubated for 30 min.

Cell pellets of slow-growing strains were washed with 0.1% sarkosyl and then resuspended in 80 ml of TE (50/20) buffer (Schwinghamer, 1980). Pronase and SDS were added to final concentrations of 0.5 mg ml^{-1} and 1% respectively and the mixture incubated at 50°C overnight (Fishcher and Lerman, 1979).

After incubation, sodium perchlorate solution was added to the lysate to a final concentration of 1M, and an equal volume of chloroform: isobutyl alcohol (24:1) was added, mixed until homogeneous and then centrifuged (6000 *g*, 10 min, 4°C). The upper aqueous phase was carefully removed, treated with RNase (200 g ml^{-1}, 37°C, 1h) and purified on hydroxyapatite (Britten *et al.*, 1970). The DNA solution was made 8 M with respect to urea and 0.14 M with respect to PB. Hydroxyapatite (HA) in 40 ml of 0.0014 M-PB was washed once with 100 ml of 8 M-urea in 0.14 M-PB solution and sedimented (6000 *g*, 2 min, 4°C). The DNA solution was added to the HA pellet which was then resuspended with the blender, incubated at room temperature for 10 min and sedimented (6000 *g*, 2 min, 20°C). The supernatant containing protein and RNA was discarded. The HA-DNA pellet was resuspended in 100 ml of 0.14 M-PB containing 8 M-urea urea and sedimented (6000 *g*, 2 min, 20°C). The HA was washed 10 times in 100 ml of 8 M-urea in 0.14 M-PB, followed by 4 washes with 100 ml of 0.014 M-PB to remove urea. The DNA was eluted from HA with 0.66 M-PB, and dialysed against one litre of 0.28 M-PB for 48 h with four changes of PB during this period.

DNA purity was determined from the UV absorption at 230 nm, 258 nm and 280 nm. The following spectral ratios were regarded as satisfactory: A_{258}/A_{230nm} 1.8-2.3, and A_{258}/A_{280nm} 1.8-2.0. DNA concentrations were determined at 258 nm using extinction coefficients of 20 for unsheared DNA and 24 for sheared DNA, for a

1 mg ml^{-1} DNA solution (Brenner and Falkow, 1971).

DNA solutions (5 ml) were sonicated for 75 s at 4°C with a 100 Watt ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., Buckingham Gate, London SW1, England) fitted with a 19 mm probe and tuned for maximum output.

2.2.6 Preparation of [^{32}P]-labelled DNA

DNA labelled with [^{32}P] was prepared by a modification of the method described in section 2.2.5. Cells were grown in 500 ml of YM broth without K_2HPO_4 and 10 mCi of [^{32}P] (carrier free orthophosphate in dilute hydrochloric acid pH 2-3, Amersham) was added to the culture after 4 h of incubation at 28°C. Cells were grown up to mid log phase and the DNA purified as described in section 2.2.5. The labelled DNA was sonicated for 56 s instead of 75 s and stored at -20°C.

2.2.7 DNA reassociation

Screw-capped Kimax-tubes (10 x 100 mm) containing 150 μg of unlabelled DNA and 0.1 μg of labelled DNA (specific activity 3-5 x 10^{4} cpm μg^{-1}) in 1 ml of 0.28 M-PB were placed in a boiling waterbath for 10 min, chilled rapidly on ice and held at 4°C. Samples were reassociated at 65°C for 40 h, diluted to 0.14 M-PB and held at 4°C until tested.

2.2.8 Separation of single and double stranded DNA on hydroxyapatite (HA)

The batchwise elution of DNA from HA with PB of appropriate molarity was used (Brenner *et al.*, 1969a; Jarvis *et al.*, 1977). The concentration of PB used to elute single-stranded DNA was determined for each new batch of HA and it ranged from 0.11 M - 0.13 M. The low molarity PB used to elute unbound single-stranded DNA contained 0.4% SDS to prevent non-specific binding of single-stranded DNA to HA. The HA suspension (5 ml) was sedimented in a Sorvall Type A centrifuge (1100 g, 2 min, 65^{o}C) and the HA washed once with low molarity PB + 0.4% SDS (10 ml). Reassociated DNA solution (0.5 ml) was added to the HA, followed by PB + 0.4% SDS (9.5 ml). The mixture was stirred with an overhead stirrer (Multispeed, Anderman and Co. Ltd), raised to 65^{o}C in a circulating water bath (Haake Type F, Haake circulators, Berlin, Germany) and immediately placed in a Sorvall bench centrifuge fitted with a type A rotor held at 65^{o}C in an incubator, centrifuged for 1 min at 1100 g, decelerated for 30 s, and stopped by hand. The supernatant was immediately poured into a scintillation vial. Further buffer was added and the process repeated four times. Double stranded DNA was eluted by increasing the buffer strength to 0.4 M-PB and washing four times as described above. The eluates were collected in scintillation vials and assayed by Cerenkov counting (Clausen, 1968). Counts eluted by 0.4 M-PB expressed as a percentage of the total counts recovered indicated the percentage of double-stranded DNA. Relative hybridisation values were obtained by adjusting the percent hybridisation of the reference DNA and homologous unlabelled DNA to 100% and applying a corresponding correction factor to hybridisations with heterologous DNA.

2.2.9 Thermal stability study of reassociated duplexes

A modification of the batch separation procedure (section 2.2.7) was used. After reassociation at 65^{o}C for 40 h, unbound single-stranded DNA was eluted by washing four times with PB (0.11-0.13 M) containing 0.4% SDS at 65^{o}C. The HA-DNA was resuspended in 10 ml of PB (0.11-0.13 M) without SDS, raised to 70^{o}C, sedimented (Sorvall bench centrifuge, type A rotor, 1100 g, 1 min, 65^{o}C) and the eluate collected in a scintillation vial. The process was repeated six times as the elution temperature was raised in 5°C steps to 100°C. Residual double-stranded DNA bound to HA at 100°C was eluted with 0.4 M-PB. Eluates were assayed by Cerenkov counting. The temperature at which 50% of the DNA which reassociated at 65°C eluted from HA is designated as the Tm(e). ΔTm(e) is the difference between the Tm(e) value for reassociated homologous DNA and Tm(e) for reassociated heterologous DNA (Brenner *et al.*, 1972). This value provides an index of sequence divergence in reassociated DNA fragments. Studies with synthetic polynucleotides have demonstrated that the presence of approximately 1% unpaired bases in a reassociated duplex lowers its thermal stability by 1°C (Bonner *et al.*, 1973). If the duplexes formed between homologous DNA strands are assumed to contain no unpaired bases, the value of ΔTm(e) is directly proportional to the percentage of unpaired bases in the heterologous duplexes.

2.3 RESULTS

2.3.1 DNA-DNA reassociation at 65°C among rhizobia from Lotus spp. and strains from other plants

The DNA homology among 53 strains of rhizobia (Table 2.1) was determined by DNA:DNA hybridisation as described in the methods using [^{32}P]-labelled reference DNA from two fast-growing *Lotus* rhizobia, strains NZP2230 and NZP2213, two slow-growing *Lotus* rhizobia strains NZP2309 (CC814s) and NZP2257, and *Bradyrhizobium japonicum* strain ATCC10324. The DNA homologies between these strains were determined in 0.28 M-PB at 65°C and are shown in Table 2.2. The strains were divided into two main DNA homology groups that shared less than 10% DNA homology with each other (Table 2.2). Group I contained fast-growing strains and group II slow-growing strains. Group I contained all the fast-growing

Table 2.2 Relative hybridisation[a] (%) of *Rhizobium* and *Bradyrhizobium* DNA at 65°C[b]

SOURCE OF UNLABELLED DNA Strain and plant origin[c]		SOURCE OF LABELLED DNA				
		NZP2230 (CC809a) *L. maroccanus*	NZP2213 *L. pedunculatus* x *L. corniculatus*	NZP2309 (CC814s) *L. pedunculatus*	ATCC10324 *Glycine max.*	NZP2257 *L. corniculatus*
FAST GROWERS						
NZP2230	*L. maroccanus*	100	56	4	4	2
NZP2213	*L. pedunculatus* x *L. corniculatus*	52	100	4	3	0
NZP5499	*C. arietinum*	82	58	4	4	0
NZP5498	*C. arietinum*	84	52	7	4	2
NZP5266	*C. arietinum*	78	56	4	11	2
NZP5448	*L. densiflorus*	62	52	4	15	5
NZP2260	*L. tenuis*	56	56	7	5	0
NZP5198	*L. densiflorus*	62	50	2	0	7
NZP5201	*C. arborescens*	52	58	7	3	2
NZP5223	*O. viciifolia*	52	52	0	5	0
NZP5302	*R. pseudoacacia*	46	50	4	8	0
NZP2300	*L. corniculatus*	40	45	-	2	0
NZP2014	*L. corniculatus*	42	48	5	5	2
NZP5275	*S. microphylla*	42	47	4	7	0
NZP2238	*L. corniculatus*	40	47	4	5	2
NZP2227	*L. corniculatus*	42	45	5	5	0
NZP2298	*L. corniculatus*	36	43	2	0	0
NZP2195	*L. tenuis*	30	48	11	9	0
NZP2048	*L. corniculatus*	36	40	4	-	0
NZP2037	*L. divaricatus*	30	40	2	3	2
NZP2237	*Hosackia spp.*	32	38	4	4	5
NZP5057	*S. tetraptera*	32	38	5	4	0
NZP2236	*A. vulneraria*	26	38	2	7	0
NZP5268	*P. pinnata*	24	30	5	5	0
NZP2077	*L. corniculatus*	20	32	5	0	3
Group Mean		46	47	4	5	1

Table 2.2 continued

SOURCE OF UNLABELLED DNA		SOURCE OF LABELLED DNA				
Strain and plant origin[c]		NZP2230 (CC809a) *L. maroccanus*	NZP2213 *L. pedunculatus* x *L. corniculatus*	NZP2309 (CC814s) *L. pedunculatus*	ATCC10324 *Glycine max.*	NZP2257 *L. corniculatus*
SLOW GROWERS GROUP SG1						
NZP2309	*L. pedunculatus*	2	5	100	-	-
NZP2308	*L. pedunculatus*	2	5	103	65	55
NZP2089	*L. pedunculatus*	4		102	53	50
ATCC10325	*L. pedunculatus*	-	-	91	62	52
NZP2141	*L. pedunculatus*	2	8	84	54	52
NZP2087	*L. pedunculatus*	2	5	82	61	52
NZP2021	*L. pedunculatus*	0	5	81	57	50
NZP5044	*L. arboreus*	4	7	75	63	50
Group mean		2	5	88	59	52
GROUP SG2						
ATCC10324	*Glycine max.*	-	-	65	100	55
ATCC10319	*L. angustifolius*	-	-	67	94	46
NZP5539	*Glycine max.*	0	5	70	94	53
Group mean		-	-	67	94	51
GROUP SG3						
NZP2257	*L. corniculatus*	0	3	51	56	100
NZP5537	*Glycine max.*	4	8	46	53	52
NZP5536	*Glycine max.*	2	7	42	55	41
NZP5533	*Glycine max.*	2	7	44	48	50
NZP5090	*O. pinnatus*	4	7	51	42	43
NZP2192	*L. corniculatus*	2	7	54	37	52
NZP5416	*L. nanus*	4	7	49	40	48
NZP5024	*O. compressus*	0	5	42	44	43
NZP2076	*L. hispidus*	2	3	49	35	50
NZP2201	*L. pedunculatus*	0	7	46	38	46

Table 2.2 continued

SOURCE OF UNLABELLED DNA Strain and plant origin[c]		SOURCE OF LABELLED DNA NZP2230 (CC809a) *L. maroccanus*	NZP2213 *L. pedunculatus* x *L. corniculatus*	NZP2309 (CC814s) *L. pedunculatus*	ATCC10324 *Glycine max.*	NZP2257 *L. corniculatus*
NZP5112	*O. perpusillus*	4	8	44	37	50
NZP2178	*L. pedunculatus*	0	3	42	39	45
NZP2228	*L. angustissimus*	2	5	53	34	50
NZP2243	*L. angustissimus*	2	5	46	31	52
Group Mean		2	6	47	42	48
GROUP SG4						
NZP5532	*Glycine max.*	2	8	21	32	26
NZP5531	*Glycine max.*	0	5	23	25	24
Group Mean		1	7	22	29	25

[a] Each relative hybridisation value is the mean of duplicate determinations for at least two hybridisations.

[b] Reassociation temperature

[c] Full generic names for the plants were listed in Table 2.1.

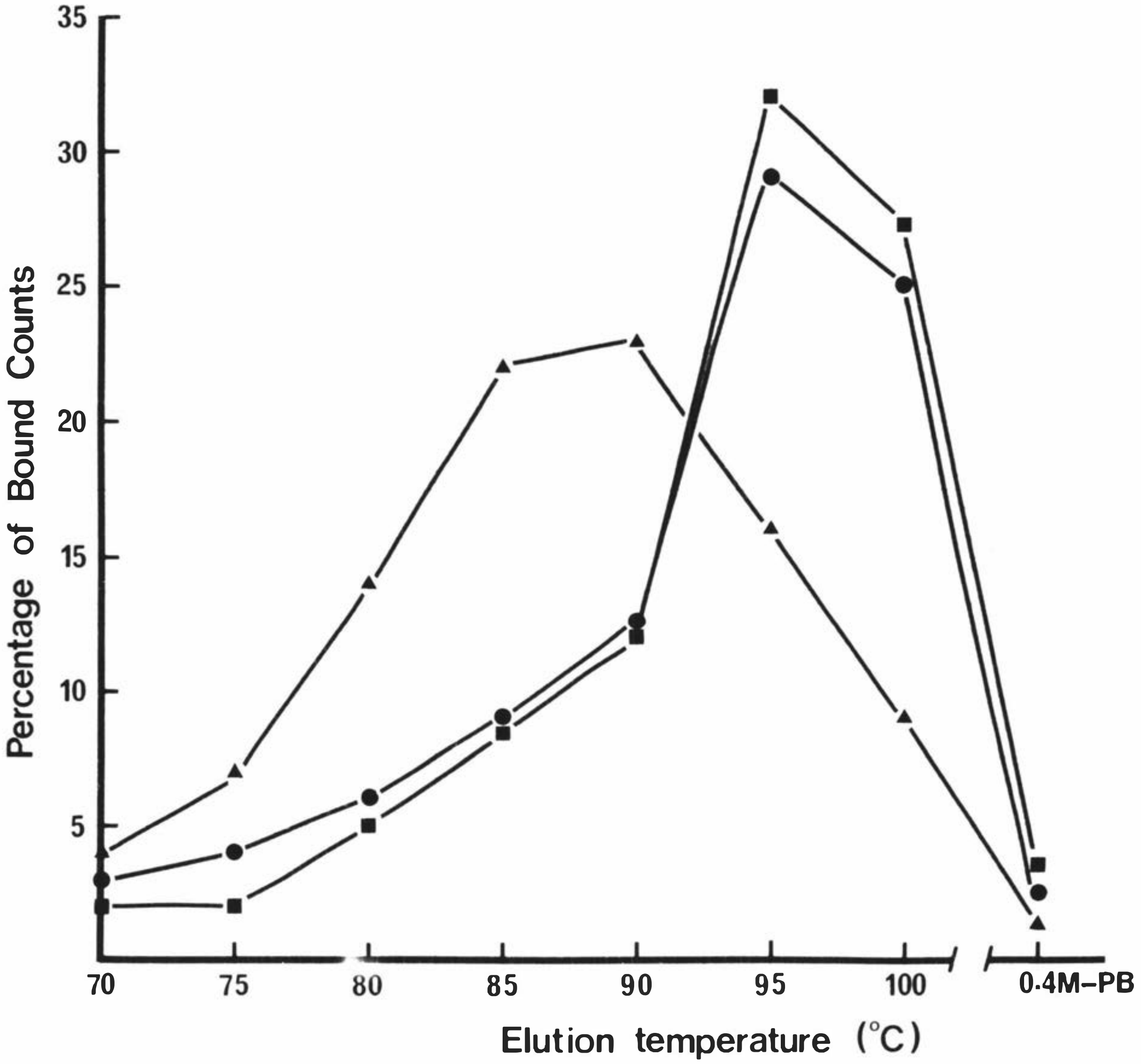
\- Not determined

Lotus strains and strains from DNA homology group 4 as defined by Crow *et al*. (1981). The relative DNA homologies between DNA from these strains and the reference DNA from strains NZP2230 and NZP2213 ranged from 20% to 84%. Hybridisations carried out using reference DNA from strains NZP2309, NZP2257 and ATCC10324 indicated that the 26 strains of slow-growers in group II could be divided further into 4 subgroups, designated as SG1-4. SG1 comprised 7 strains of rhizobia that nodulate *Lotus pedunculatus* and 1 strain that nodulates *Lupinus arboreus*. The mean relative homologies of DNA from SG1 strains with reference DNA from strains NZP2309 and ATCC10324 were 88% and 59% respectively. SG2 comprised three strains of *B. japonicum* including the type strain ATCC10324. Mean relative homologies for DNA from SG2 strains with reference DNA from strains NZP2309 and ATCC10324 were 67 and 94% respectively. SG3 comprised 14 strains of slow-growing rhizobia that were isolated from *Lotus*, *Ornithopus*, *Lupinus* and *Glycine max*. The mean relative DNA homologies between these strains and the three reference DNA's from strains NZP2309, ATCC10324 and NZP2257 were 47, 42 and 48% respectively. SG4 comprised only two strains, NZP5532 (*B. japonicum* US31) and NZP5531 (*B. japonicum* US76), and both showed relative DNA homologies of 22-29% with the three reference DNA's from the slow-growing strains used in this study.

2.3.2 Thermal stability of reassociated DNA duplexes

To complement the DNA homology data used to delineate DNA homology subgroups, the thermal stability of DNA duplexes formed between DNA of selected strains from SG1, SG2 and SG3 and reference DNA from slow-growing strains NZP2309, ATCC10324 and NZP2257 was determined (section 2.2.9). Representative thermal elution profiles are shown in Fig. 2.1. DNA duplexes formed between related strains such as NZP2309 and

Figure 2.1 Thermal elution profiles of reassociated DNA duplexes formed at 65^{o}C between B. japonicum ATCC10324 reference DNA and DNA from other selected strains. DNA from B. japonicum strain ATCC10324 (■). DNA from slow-growing Lotus strain NZP2309 (CC814s) (●). DNA from slow-growing Lotus strain NZP2192 (▲).


35
30
25
20
15
10
5
Percentage of Bound Counts
70
75
80
85
90
95
100
0·4M–PB
Elution temperature (°C)

ATCC10324 reference DNA, eluted at a higher temperature and had a sharper elution profile (Fig. 2.1) than did less closely related DNA such as the DNA duplexes formed between NZP2192 DNA and ATCC10324 reference DNA (Fig. 2.1). Thermal elution profiles could be characterised by the temperature at which half the reassociated DNA duplexes have dissociated, Tm(e) (section 2.2.9). Thermal stability data of the selected strains are summarised in Table 2.3.

2.4 DISCUSSION

The DNA hybridisation data shown in Table 2.2 indicate that *Lotus* rhizobia belong to two genetically distinct groups corresponding to fast-growing and slow-growing strains. The DNA homology data obtained with the fast-growing strains in this work support the results reported by Crow *et al*. (1981) that fast-growing *Lotus* rhizobia are genetically related to rhizobia obtained from a variety of plant hosts consisting of species from 12 genera. These fast-growing rhizobia appear to be genetically diverse since DNA homology values obtained with reference DNA from the 2 fast-growing *Lotus* strains NZP2230 and NZP2213 ranged from 20-84% at 65°C (Table 2.2). Similar results were obtained by Crow *et al*. (1981) with DNA from 2 fast-growing reference strains NZP2230 and CC811. These workers suggested that there may be two distinguishable subgroups corresponding to strains from the two related *Lotus* plant species, *L. corniculatus* and *L. tenuis*. However, when NZP2213, a strain associated with *L. corniculatus* and *L. tenuis* was used as a reference strain in this work, strains from *L. corniculatus* and *L. tenuis* shared a relative homology with NZP2213 ranging from 40-58%. There was no indication of subgroups within these *Lotus* strains. It is interesting to note that three *Cicer arietinum* strains included in the group showed a significantly higher relative DNA homology at 65°C with NZP2230 (81%)

Table 2.3 Thermal stability of rhizobial DNA duplexes formed at 65°C.[a]

Homology Group	Source of unlabelled DNA	ΔTm(e)[b] with reference DNA from		
		NZP2309	ATCC10324	NZP2257
SG[c]1	NZP2089	-	-	5.6
	NZP2141	-	1.4	-
	NZP2309	0	1.3	-
	ATCC10325	0.1	1.7	-
	NZP5044	0.9	-	5.2
SG2	NZP5539	-	0.4	5.8
	ATCC10324	1.5	0	-
SG3	NZP2178	-	7.5	-
	NZP2257	-	-	0
	NZP2228	4.7	-	6.9
	NZP2243	-	-	6.5
	NZP5416	4.9	-	-

[a] Reassociation temperature

[b] ΔTm(e) is the difference between the Tm(e) value for reassociated homologous DNA and Tm(e) for reassociated heterologous DNA. Each Tm(e) value is the mean of 4 duplicate determinations for two hybridisations.

[c] DNA homology sub-group.

\- Not determined

than with DNA from NZP2213 (51%). This may suggest that *Cicer arietinum* is nodulated by a separate group of rhizobia.

The fast-growing rhizobia strains considered here have recently been classified as a distinct group of rhizobia based on their DNA homology (this work, Crow *et al.*, 1981), phage typing patterns (Patel, 1976), 2D-protein patterns (Roberts *et al.*, 1981), the nodulation properties (Jensen, 1967), flagellation (Vincent, 1974) and other biochemical and physiological data (Jarvis *et al.*, 1982). Consequently, a new species was established and named *Rhizobium loti*. The type strain is NZP2213 (Jarvis *et al.*, 1982).

From this study, the slow-growing *Lotus* rhizobia can be divided into 2 DNA homology subgroups called SG1 and SG3. Strains from SG1 and SG3 share up to 50% relative DNA homology at 65^{o}C with strains from *Lupinus*, *Ornithopus* and *Glycine max*. Strains from SG1 shared mean relative DNA homologies of 88% and 52% at 65^{o}C with reference DNA from strain NZP2309 (SG1) and NZP2257 (SG3) respectively. The ΔTm(e) values (Table 2.3) indicated that the mismatched bases in DNA duplexes formed between NZP2309 reference DNA and DNA from strains in SG1 were no more than 1% (ΔTm(e) 0.0-0.9^{o}C) of the total genome assayed (Bonner *et al.*, 1973). In contrast, the DNA duplexes formed between NZP2309 reference DNA and DNA from strains in SG3 displayed approximately 5% of mismatched bases in the total genome assayed (ΔTm(e) 4.7-4.9^{o}C). These results support the relative DNA homology data used to delineate the two DNA homology subgroups.

Bradyrhizobium japonicum strains included in this study were separated into 3 DNA homology subgroups: SG2, SG3 and SG4 (Table 2.2).

The strains in SG2, SG3 and SG4 have mean relative DNA homologies of 94%, 52% and 29% repectively at 65°C with reference DNA from the *B. japonicum* type strain ATCC10324. These results are consistent with the report by Hollis *et al*. (1981) that strains classified as *B. japonicum* could be separated by DNA:DNA homology into at least 3 DNA homology groups: group I, group Ia and group II. Only group I was closely related to the type strain ATCC10324. Homology subgroups SG2, SG3 and SG4 include *B. japonicum* strains which Hollis *et al*. (1981) placed in group I, group Ia and group II respectively.

In general, there was no discrepancy between this work and that of Hollis *et al*. (1981) except in relation to the DNA homology of strain NZP5539 (61A76). Hollis *et al*. (1981) reported that this strain shared 24% relative DNA homology at 65°C with reference DNA from strain ATCC10324. In this study, NZP5539 was found to share 94% relative DNA homology at 65°C with strain ATCC10324 reference DNA. Elkan and Scholla (personal communication) have observed more than one colony type in their culture of 61A76 and this may be the cause of the discrepancy.

When DNA homologies of strains from *B. japonicum* in SG2 and *Lotus* strains in SG1 were compared it was found that although these two groups shared a mean relative DNA homology of 67% at 65°C. The $\Delta Tm(e)$ values (1.3-1.7°C) obtained from DNA duplexes formed between DNA from strains in SG1 and SG2 indicated that about 1.5% of their bases were mismatched, suggesting that SG1 and SG2 represent two genetically distinct groups. However, there is still insufficient data to support the designation of a new species for *Bradyrhizobium spp*. (*Lotus*) strains within the genus *Bradyrhizobium*.

Subgroup SG3 comprised 14 strains of slow-growing rhizobia from *Lotus*, *Ornithopus*, *Lupinus* and *Glycine max*. Both the relative DNA homology data (Table 2.2) and ΔTm(e) values (Table 2.3) indicated that these strains are genetically related. Although the taxonomic implications of this DNA homology requires further clarification, one possible implication is that some of the slow-growing strains from *Lotus*, *Lupinus* and *Ornithopus* are related to *B. japonicum* strains in DNA homology group Ia at the subspecies level.

The two *B. japonicum* strains in SG4 showed mean relative DNA homologies of 22%, 29% and 25% with reference DNA from strains NZP2309, ATCC10324 and NZP2257 respectively. These strains have diverged significantly from all other slow-growing strains analysed in this study. These results support the suggestion put forward by Hollis *et al*. (1981) that such strains should be classified under a separate species within the genus *Bradyrhizobium*.

Most of the slow-growing strains studied in this work had at least 40% relative DNA homology at 65^{o}C with the three reference DNA from slow-growing strains. This may represent a basic similarity among these slow-growing rhizobia. This is in contrast to the situation in the genus *Rhizobium* which comprises several distinct DNA homology groups with mean intergroup relative homologies of less than 20% (Jarvis *et al*., 1980; Crow *et al*., 1981).

This work demonstrates that the root nodule bacteria capable of nodulating *Lotus* species belong to two distinct genetic groups. The fact that both groups nodulate similar host plants would suggest that the genes responsible for symbiotic properties such as nodulation, nitrogen fixation and host specificity are conserved despite their lack of overall DNA homology.

2.5 SUMMARY

DNA homologies were determined among 24 strains of fast-growing rhizobia and 26 strains of slow-growing rhizobia with particular reference to those which nodulate *Lotus* species. Two major DNA homology groups were identified which showed less than 10% relative DNA homology. Fast-growing *Lotus* rhizobia were grouped with related fast-growing strains isolated from a range of plant species in DNA homology group I. These strains are designated *Rhizobium loti*. Slow-growing *Lotus* rhizobia and other slow-growing strains from *Glycine max*, *Ornithopus* and *Lupinus* formed DNA homology group II which was further divided into 4 subgroups (SG1-SG4). Thermal stability data for DNA duplexes formed between DNA from selected strains of slow-growers were also determined. Slow-growing *Lotus* rhizobia showed at least 40% relative DNA homology with strains from *Ornithopus*, *Lupinus* and *Glycine max* and they comprised DNA homology subgroups SG1 and SG3. Strains in SG1 are more closely related to the *B. japonicum* type strain ATCC10324 than those in SG3, but SG1 represents a genetically distinct group from *B. japonicum*.

CHAPTER THREE

CHARACTERISATION OF PLASMIDS IN LOTUS RHIZOBIA

3.1 INTRODUCTION

The presence of large plasmids of molecular weights greater than 100 MDal in size is a common feature of *Rhizobium* species (Prakash *et al.*, 1981; Denarie *et al.*, 1981). The genetic functions of most of these large indigenous plasmids are still largely unknown. However, it has been demonstrated by transposon mutagenesis, heat curing and plasmid transfer experiments that some of the symbiotic genes such as nodulation (*nod*), nitrogen fixation (*nif*) and host specificity (*hsp*) are located on large indigenous plasmids, designated as symbiotic (Sym) plasmids, in a number of *Rhizobium* species (reviewed by Denarie *et al.*, 1981). Among the closely related *Rhizobium* species, *R. leguminosarum*, *R. trifolii* and *R. phaseoli*, their Sym plasmids vary with respect to size (MW ranging from 120-550 MDal), transmissibility and incompatability group (Brewin *et al.*, 1983). In contrast, *R. meliloti* strains studied to date consistently contain Sym plasmids larger than 450 MDal, designated as megaplasmids (reviewed by Huguet *et al.*, 1983).

Large plasmids have also been identified in the slow-growing *Bradyrhizobium* species (Gross *et al.*, 1979; Scott and Tait, 1980; Haugland and Verma, 1981; Cantrell *et al.*, 1982; Masterson *et al.*, 1982). However, there is no evidence to date demonstrating that symbiotic genes are plasmid-borne in these strains (Haugland and Verma, 1981; Masterson *et al.*, 1982).

In *R. loti* and *Bradyrhizobium spp.* (*Lotus*), large plasmids have also been detected (Ronson and Scott, 1983; Pankhurst *et al.*, 1983), but the role of these indigenous plasmids is unknown. As demonstrated in Chapter 2, the two distinct groups of Lotus rhizobia shared less than 10% relative DNA homology, and so the possibility existed that the ability of these rhizobia to nodulate Lotus species was a plasmid-borne character.

This chapter describes the further characterisation of plasmids found in the Lotus rhizobia and attempts to isolate Nod^- mutants of R. loti NZP2213 by heat curing, or UV treatment, with the aim of determining whether the symbiotic genes in this strain are located on the single indigenous plasmid.

3.2 METHODS AND MATERIALS

3.2.1 Bacterial strains and plasmids

These are described in Table 3.1.

3.2.2 Preparation of media

3.2.2.1 Liquid media

Tryptone yeast extract medium (TY; section 2.2.2.1).

Yeast extract mannitol medium (YM; section 2.2.2.1).

Luria broth (LB; Miller, 1972). Composition (g l^{-1}): tryptone, 10; yeast extract, 5; NaCl, 10. pH 7.2.

S10 medium (Scott and Ronson, 1982). The following stock solutions were prepared: (1) Salts solution (g l^{-1}): $MgSO_4.7H_2O$, 25; $CaCl_2.7H_2O$, 2; Ferric EDTA, 1.5; NaCl, 20; distilled water, 1 litre. (2) Trace elements (per litre): $ZnSO_4.7H_2O$, 15 mg; $Na_2MoO_4.2H_2O$, 200 mg; H_3BO_3, 250 mg; $MnSO_4.4H_2O$, 200 mg; $CuSO_4.5H_2O$, 20 mg; $CoCl_2.6H_2O$ (0.2 g l^{-1}), 5 ml; distilled water, 995 ml. (3) Vitamins (per 50 ml): Thiamine HCl, 50 mg; calcium pantothenate, 100 mg, biotin (1 $mgml^{-1}$), 1 ml;

Table 3.1 Bacterial Strains and Plasmids

Strain	Relevant characteristics	Source or reference
Rhizobium loti		
NZP2037	Nod$^+$ Fix$^+$ (*Lotus tenuis*; *Lotus pedunculatus*)	DSIR culture collection, N.Z.
NZP2213	Nod$^+$ Fix$^+$ (*Lotus tenuis*) Nod$^+$ Fix$^-$ (*Lotus pedunculatus*)	DSIR culture collection, N.Z.
PN112	Nod$^+$ Fix$^+$ (*Lotus tenuis*) Nod$^+$ Fix$^-$ (*Lotus pedunculatus*) *str*-1 *rif*-1	C.E. Pankthurst - spontaneous mutant of NZP2213
NZP2227	Nod$^+$ Fix$^+$ (*Lotus tenuis*) Nod$^+$ Fix$^-$ (*Lotus pedunculatus*)	DSIR culture collection, N.Z.
NZP2048	Nod$^+$ Fix$^+$ (*Lotus tenuis*) Nod$^+$ Fix$^-$ (*Lotus pedunculatus*)	DSIR culture collection, N.Z.
NZP2238	Nod$^+$ Fix$^+$ (*Lotus tenuis*) Nod$^+$ Fix$^-$ (*Lotus pedunculatus*)	DSIR culture collection, N.Z.
Bradyrhizobium *spp*. (*Lotus*)		
NZP2309 (CC814S)	Nod$^+$ Fix$^+$ (*Lotus pedunculatus*) Nod$^+$ Fix$^-$ (*Lotus tenuis*)	DSIR culture collection, N.Z.
NZP2229	Nod$^+$ Fix$^+$ (*Lotus pedunculatus*)	collection, N.Z.
NZP2075	Nod$^+$ Fix$^+$ (*Lotus pedunculatus*)	collection, N.Z.
NZP2071	Nod$^+$ Fix$^+$ (*Lotus pedunculatus*)	collection, N.Z.
NZP2202	Nod$^+$ Fix $^+$ (*Lotus pedunculatus*)	DSIR culture collection

Table 3.1 continued

Strain	Relevant characteristics	Source or reference
Agrobacterium tumefacieus		
C58		C. Kado
Escherichia coli		
HB101	F^- *pro* *leu* *thi* *lac*Y Str^R r_k^- m_k^- Endo I^- *rec* A^-	Boyer and Roulland-Dussoix (1969)
Plasmids		
pRlo2213a	Indigenous plasmid of *R. loti* NZP2213	Pankhurst *et al.* (1983)
pRlo2037a	Indigenous plasmid of *R. loti* NZP2037	Pankhurst *et al.* (1983)

distilled water, 49 ml. (4) NH_4Cl (18 g l^{-1}). (5) *Potassium phosphate (g l^{-1}): K_2HPO_4, 100; KH_2PO_4, 100; distilled water, 1 litre. (6) *Sodium succinate (0.5 M). One litre of S10 medium consisted of 10 ml of (1); 1 ml of (2); 1 ml of (3); 6 ml of (4); 5 ml of (5); 20 ml of (6); 10 g of MES (Sigma); 0.1 g of histidine (Sigma); 953 ml of distilled water. The pH was adjusted to 6.2 with potassium hydroxide pellets.

* Added separately after autoclaving

Defined medium 2 (Schwinghamer, 1960). Composition per litre: NH_4NO_3, 1.0 g, K_2HPO_4, 0.3 g; K_2HPO_4, 0.3 g: $MgSO_4.7H_2O$, 0.1 g; $Ca(NO_3)_2$, 0.05 g; yeast extract, 1.0 g; glucose, 1.0 g; H_3BO_3, 0.1 μg; $ZnSO_4.7H_2O$, 1.0 μg; $CuSO_4.5H_2O$, 0.5 μg; $MnCl_2.4H_2O$, 0.5 μg; $NaMoO_4.2H_2O$, 0.1 μg; Fe-EDTA, 1.0 μg; distilled water, 1 litre. The pH was adjusted to 7.0.

3.2.2.2 Solid media (See section 2.2.2).

3.2.3 Maintenance of cultures

Rhizobium cultures were maintained on YM agar slopes at 4°C. E. coli cultures were maintained on LB agar plates at room temperature. For long term maintenance, all cultures were stored in 50% glycerol at -20°C.

3.2.4 Growth of bacteria

Solid and liquid TY media were used to grow Rhizobium loti and Agrobacterium tumefaciens at 28°C. Solid and liquid YM media were used to grow Bradyrhizobium spp. (Lotus) at 28°C.

Solid and liquid LB media supplemented as required with appropriate antibiotics: kanamycin (Km; 20 μg ml^{-1}), ampicillin (Ap; 100 μg ml^{-1}), tetracycline (Tc; 15 µg ml^{-1}), chloramphenicol (Cm; 25 µg ml^{-1}), were used to grow E. coli at 37°C. All antibiotics were supplied by Sigma Chemical Co.

3.2.5 Ultraviolet light mutagenesis of Rhizobium loti

The method described by Miller (1972) was used. A stationary phase culture of R. loti NZP2213 was diluted in S10 broth (section 3.2.2.1) and grown at 28°C to a density of 2-3 x 10^8 cells ml^{-1} (OD_{600} ≈0.2). Cells were pelleted by centrifugation (300 g, 5 min, 4°C), resuspended in 10 ml of 0.1 M-$MgSO_4.7H_2O$ and held on ice. A sample of the culture was plated on S10 plates to determine the number of cells present before irradiation. The cell suspension (5 ml) was spread thinly on an open glass petri dish and exposed for 15 s to UV radiation at 2000 μWcm^{-2}. The irradiated suspension was plated on S10 plates, covered with foil, and incubated at 28°C for 3 days. Surviving colonies were subcultured onto TY media and then used for plant tests and plasmid analysis.

3.2.6 Plant test

Seeds of Lotus tenuis, Waldst et Kit, were surface sterilized in a 1:1 (v/v) solution of 95% ethanol and hydrogen peroxide for 5 min, washed four times with 95% ethanol, dried in vacuo and germinated on water agar (1%). Seedlings were transferred aseptically to 150 x 24 mm test-tubes containing 10 ml of Thornton's seedling agar (Thornton, 1930) which had the following composition (g l^{-1}): $Ca_3(PO_4)_2$, 2; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FePO_4$, 1.0; $FeCl_3$, 0.01; agar, 1.5. A loopful of cells was used to inoculate each seedling, and plants were grown in a growth cabinet with a 12 h photoperiod (light intensity 180 W/m^2), and temperatures of 24°C by day and 20°C by night. Plants were examined for nodulation and growth at 2 and 6 weeks after inoculation respectively.

3.2.7 Isolation of bacteria from nodules

Nodules were surface sterilized in a 1:1 (v/v) solution of 95% ethanol and hydrogen peroxide for 2 min, rinsed in 95% ethanol for 1 min, washed three times with sterilized water, crushed and plated on YM medium. Single colonies were repurified and tested on TY medium supplemented with antibiotics for the appropriate genetic markers and retested on plants.

3.2.8 Heat curing experiment

An overnight culture of strain NZP2213 grown in YM liquid medium was diluted to about 2 x 10^8 cells ml^{-1}, incubated without shaking at 38°C for 7 days and aerated once each day by a brief shaking (Zurkowski and Lorkiewicz, 1978). The culture was then plated on YM agar and incubated at 28°C for 5 days. Two hundred colonies were single colony purified and tested for their ability to nodulate *Lotus tenuis* plants.

3.2.9 Bacteriophage susceptibility tests

Bacteriophage typing of *R. loti* cultures was carried out by the method of Patel (1976). Defined medium 2 (section 3.2.2.1) was used to grow cultures of the standard strain and test strain. The same medium was used for the preparation of double-layered agar plates for the assaying of the phage-susceptibility of rhizobia. Overnight cultures of *Rhizobium* (0.1 ml) were used to seed double-layered agar plates, and these plates were spotted with aliquots of hundred-fold dilutions (in defined medium 2) of the phage stock culture (a gift from Dr J.J. Patel, ABD, DSIR). The spots were air-dried in a laminar-flow cabinet, and the plates were incubated at 28°C for 24 h. Susceptibility of *Rhizobium* to a given phage was determined by plaque formation in the spotted area. Rhizobium strains were considered highly susceptible to phages when lysis occurred at phage stock dilutions of 10^{-6} or greater ($\leqslant 10^{6}$ PFU ml^{-1}), less susceptibile when lysis only occurred at dilutions of 10^{-4} or less ($> 10^{8}$ PFU ml^{-1}), and not susceptible when lysis was not produced by the undiluted phage stock (10^{12} PFU ml^{-1})

3.2.10 Tandem-crossed immunoelectrophoresis

For antigen preparation R. loti strains NZP2213 and NZP2037 were grown to mid log phase (OD_{600} = 0.3) in YM broth (100 ml) and harvested by centrifugation (300 g, 5 mins, 4°C). The cells were resuspended in 5 ml of sterile water, sonicated for 60 s to release internal proteins and then stored at -20°C until use. Antibodies to NZP2213 cells were a gift from Dr W.T. Jones, ABD, DSIR, and were prepared by a method of Jones et al. (1982). Immunoelectrophoresis was performed by Dr W.T. Jones by a method first described by Kroll (1973).

3.2.11 Plasmid isolation by Eckhardt method

The analysis of large plasmids in Rhizobium strains was carried out by the Eckhardt procedure (Eckhardt, 1978) with minor modifications described below.

Materials

(1) Tris-borate electrophoresis buffer pH 8.2: 89 mM-Tris base; 12 mM-Na_2EDTA; 8.9 mM-boric acid. (2) Lysozyme mixture: Ficoll (400,000, Sigma), 2 g; preboiled ribonuclease (Sigma, 1 mg ml^{-1}), 50 µl; bromophenol blue 0.05% (w/v); Tris-borate buffer pH 8.2, 10 ml; lysozyme (Sigma), 10 mg. (3) SDS mixture: Ficoll (400,000), 1 g; SDS, 0.2 g; Tris-borate buffer pH 8.2, 10 ml. (4) Overlay SDS

mixture: Ficoll (400,000), 0.5 g; SDS, 0.2 g; Tris-borate buffer pH 8.2, 10 ml. (5) TE (50/5) buffer pH 8.0: 50 mM-Tris-HCl; 5 mM-Na_2EDTA. (6) 0.1% (w/v) sarkosyl: N-lauroylsarkosine (Sigma), 0.1 g; TE (50/5) buffer pH 8.0, 100 ml. (7) Ethidium bromide (1 µg ml^{-1}). (8) Agarose (Sigma).

Method

Cells were grown to the log phase (OD_{600} = 0.4) in TY broth and harvested by centrifugation (microcentrifuge, top speed, 5 min). The cell pellet was washed once with sarkosyl solution (Schwinghamer, 1980), and once in 1 ml of TE (50/5) buffer, pH 8.0 and drained well. The cell pellet was resuspended in 40 µl of the lysozyme mixture, loaded into the well of a vertical 0.8% agarose gel (140 x 130 x 3 mm) and incubated for 10 min at room temperature. SDS mixture (10% Ficoll; 40 µl) was layered on top of the bacteria-lysozyme mixture and the two layers were gently mixed with a toothpick. The overlay SDS mixture (100 µl) was layered on top and the well was sealed with agarose. Plasmid DNA was separated by electrophoresis at 45V for 45 min and at 180V for 2.5 h. After electrophoresis gels were stained for 15 min in ethidium bromide (1 µg ml^{-1}), washed with distilled water, and photographed on a UV transilluminator with Kodak Tri-X film, using a No. 23A Wratten gelatine filter (Kodak). The molecular weight of the Rhizobium plasmids was determined by comparing their relative mobilities with those of the two plasmids of Agrobacterium tumefaciens strain C58, which contains plasmids of 130 Mdal (Holsters et al., 1978) and 275 Mdal (Denarie et al., 1981).

3.2.12 Isolation of plasmids by the Tait procedure

This procedure (Andersen et al., 1981) was used for screening plasmids found in Bradyrhizobium spp. (Lotus).

Materials

(1) 0.9% (w/v) sodium chloride. (2) TE (50/5) buffer pH 8.0: 50 mM-Tris-HCl, 5 mM-Na_2EDTA. (3) TE sucrose, pH 8.0: 50 mM-Tris-HCl, 5 mM-Na_2EDTA, 20% (w/v) sucrose. (4) Lysozyme (Sigma). (5) Pronase (Calbiochem, nuclease free, stored at -20°C). (6) 20% (w/v) SDS in distilled water. (7) Ribonuclease (1 mg ml^{-1}, heat treated for 2 min at 100°C). (8) Redistilled phenol saturated with TE buffer pH 8.0. (9) Urea-dye mixture: bromophenol blue, 0.05%; xylene cyanol FF (BDH) 0.05% in 10M-urea. (10) Ethidium bromide (1 µg ml^{-1}). (11) Tris-borate electrophoresis buffer, pH 8.2 (section 3.2.11). (12) Sarkosyl 0.1% (w/v) in TE buffer (section 3.2.11). All buffers were autoclaved before use.

Method

Cells (5 ml) grown in YM medium to mid-log phase ($OD_{600} \approx 0.5$) were harvested by centrifugation (300 g, 5 min, 4°C). The cell pellet was washed once in 5 ml of 0.1% sarkosyl solution (Schwinghamer, 1980), once in 0.9% sodium chloride (5 ml), once in TE buffer pH 8.0 and then frozen on dry ice. The cells were thawed, resuspended in 150 µl TE sucrose solution pH 8.0 and 1 mg of lysozyme in 150 µl of distilled water was added. The suspension was incubated with occasional mixing at 4°C for 30 min. Pronase (0.5 mg) was added, mixed and incubation continued at 4°C for 15 min. Fifteen microlitres of 20% SDS was added and mixed by rolling the tube until lysis occurred. The suspension was incubated on ice for a further 15 min with occasional mixing. After the incubation, distilled water (300 µl) and phenol (3 ml) were added to the lysate, which was mixed gently for 2 min and separated by centrifugation

(12,000 g, 10 min, 4^{o}C). The aqueous phase (100 μl) was carefully removed and mixed with 10 μl of urea-dye mixture and 70 μl was loaded onto a vertical 0.8% agarose gel (section 3.2.11). The DNA was separated by electrophoresis for 3 h at 90V. Staining and photography of gels were performed as described in section 3.2.11.

3.2.13 Preparation of R.loti plasmid DNA

In order to isolate pRlo2213a on a preparative scale the procedure of Kado and Liu (1981) was used with minor modifications.

Materials

(1) Tris-acetate buffer: 40 mM-Tris, 2 mM-Na_2EDTA, pH adjusted to 7.9 with glacial acetic acid. (2) Lysis mixture: 50 mM-Tris, 3% (w/v) sarkosyl, pH adjusted to 12.6 with 2 M-NaOH immediately before use. (3) 2 M-NaOH (freshly prepared). (4) 0.1% (w/v) sarkosyl in Tris-acetate buffer pH 7.9. (5) TE (50/10) buffer pH 8.0: 50 mM-Tris, 10 mM-Na_2EDTA. (6) Phenol-chloroform (1:1, v/v), saturated with TE (50/10) buffer pH 8.0. (7) 2 M-Tris pH 7.0 (8) 50% (w/v) PEG 6000. (9) Ethidium bromide (10 mg ml^{-1}). (10) Caesium chloride. (11) Isopropanol saturated with 5 M-sodium chloride. (12) Chloroform. (13) 95% ethanol. (14) 3 M-sodium acetate. (15) TE (10/1) buffer pH 8.0: 10 mM-Tris, 1 mM-Na_2EDTA.

Method

A 48 h culture of NZP2213 grown in 50 ml of TY broth was subcultured into 1.5 l of TY broth and incubated at 28^{o}C with shaking for 48 h (OD_{600} ≈ 0.3). Cells were harvested (10,000 g, 10 min, 4^{o}C), washed once with 50 ml of 0.1% sarkosyl solution, once with 50 ml of Tris-acetate buffer pH 7.9 and resuspended in 24 ml of Tris-acetate buffer pH 7.9. Solid lysozyme was added to the cell suspension to a final concentration of 10 mg ml^{-1} and incubated for 30 min at 4°C. After incubation, 48 ml of lysis mixture was added, mixed and incubated at 65°C for 1 h.

The lysate was cooled to room temperature, adjusted to pH 8.0 with 2 M-Tris pH 7.0, and 5 M-NaCl was added to a final concentration of 3% (w/v). An equal volume of phenol-chloroform mixture was added, mixed gently until emulsified and centrifuged (12,000 *g*, 15 min, 4°C). The aqueous phase was collected and an equal volume of chloroform added. The sample was mixed and re-centrifuged (12,000 *g*, 10 min, 4°C). The volume of the aqueous phase collected was determined and 0.2 volume of 50% (w/v) PEG 6000 added, mixed by inversion and held at 4°C overnight. The DNA was precipitated by low speed centrifugation (3000 *g*, 1.5 min, 4°C) and resuspended in TE (10/1) buffer pH 8.0.

Solid caesium chloride (CsCl) was added to the DNA solution to 1 g ml^{-1}, ethidium bromide added to 300 µg ml^{-1}, and the solution centrifuged in a Beckman Type 65 rotor at 250,000 *g* for 40 h at 16°C. After ultracentrifugation, the plasmid DNA was collected and extracted with sodium chloride-saturated isopropanol to remove ethidium bromide Amemura *et al.*, (1982). The extraction was repeated 3-4 times until the solution was clear. The CsCl in the plasmid DNA preparation was removed by dialysis against TE (10/1) buffer pH 8.0 (section 2.2.7)

After dialysis, the plasmid DNA was precipitated by adding 0.1 volume of 3 M-sodium acetate solution and 2.5 volumes of 95% cold ethanol to the DNA solution and leaving it overnight at -20°C. The DNA was harvested by centrifugation (27,000 *g*, 10 min, 4°C), washed twice in

95% ethanol, dried in vacuo and resuspended in TE (10/1) buffer. The purity and concentration of DNA were determined as described in section 2.2.5.

3.2.14 Isolation of genomic DNA

Total Rhizobium DNA was isolated by a modification of the procedure of Fischer and Lerman (1979).

Materials

The following materials were used: (1) TE (50/20) buffer: 50 mM-Tris/HCl, 20 mM-Na_2ETDA, pH 8.0. (2) TE (10/1) buffer: 10 mM-Tris/HCl, 1 mM-Na_2EDTA, pH 8.0. (3) 0.1% (w/v) sarkosyl in TE buffer. (4) Lysozyme (Sigma). (5) Proteinase K (Sigma). (6) N-lauroyl-sarkosine (Sigma). (7) 95% ethanol. (8) Phenol (saturated with TE (50/20) buffer pH 8.0). (9) 3 M-sodium acetate.

Method

A 20 ml culture of late-log phase R. loti NZP2213 cells was harvested by centrifugation (3000 g, 5 min, 4°C), washed with 10 ml of sarkosyl solution and then with 10 ml of TE (50/20) buffer. The cell pellet was resuspended in 5 ml of TE (50/20) buffer and solid lysozyme was added to a final concentration of 300 µg ml^{-1}. The mixture was incubated at 37°C for 30 min with occasional mixing. Proteinase K and sarkosyl were added to a final concentration of 600 µg ml^{-1} and 1% (w/v), respectively. This mixture was then incubated at 50°C for 16 h. The lysate was cooled to room temperature and extracted twice with phenol, followed by two chloroform extractions as described in section 3.2.13. The aqueous phase was then centrifuged to remove cell debris (12,000 g, 10 min, 4°C), and the DNA was precipitated with ethanol (section

3.2.13). The purity and concentration of the DNA were determined as previously described (section 2.2.5).

3.2.15 Digestion of DNA with restriction enzymes

Materials

(1) *Hae* III buffer (x10) pH 7.6: 0.06 M-Tris pH 7.6; 0.1 M-$MgCl_2.6H_2O$; 0.1 M-mercaptoethanol. (2) 1 M-sodium chloride. (3) SDS dye mixture: 20% (w/v) sucrose, 1% (w/v) SDS, 0.2% (w/v) bromophenol blue. (4) Restriction enzymes (New England Biolabs).

Method

All DNA digestions were carried out in *Hae* III buffer (x1) with the salt concentration adjusted as recommended by the manufacturer using 1 M-sodium chloride. Digestions were performed at 37°C for 1-2 h. An aliquot of the digest was checked on a minigel (section 3.2.17) to ensure the digestion had gone to completion before the reaction was stopped by adding 0.2 volume of an SDS dye mixture. If the DNA digestion was incomplete, more enzyme was added and the mixture further incubated or the DNA was further purified (section 3.2.16) and the experiment repeated.

3.2.16 Purification of DNA preparations

The following protocols were used to purify small quantities of DNA.

3.2.16.1 Purification by phenol chloroform extraction

A measured volume of DNA was transferred to a microcentrifuge tube and an equal volume of phenol-chloroform (1:1; v/v) added, mixed thoroughly and centrifuged at top speed for 3 min in a microcentrifuge.

The aqueous phase was collected, an equal volume of sterilized water added to the phenol-chloroform mixture and the extraction was repeated. The DNA was ethanol precipitated (section 3.2.13) *E. coli* transfer RNA (20 mg ml^{-1}) was added to a final concentration of (20-50 µg ml^{-1}) when the concentration of DNA was less than 1-5 µg ml^{-1}.

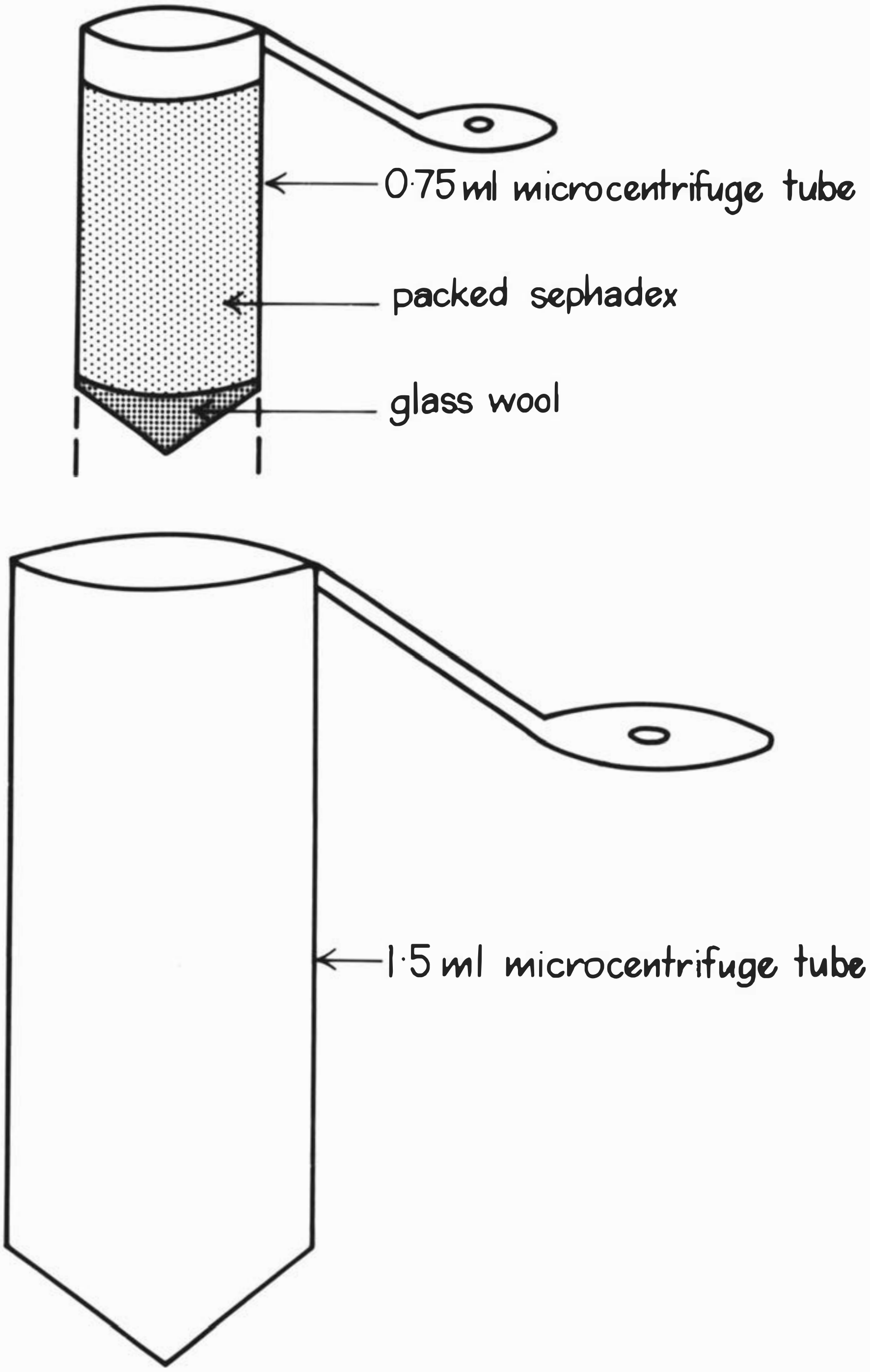
3.2.16.2 Desalting with Sephadex G-50

A method described by Helmerhorst and Stokes (1980) was used. Sephadex G-50 resin (2 g; Pharmacia, medium grade) was hydrated, deaerated and stored at 4°C. A Sephadex column was set up in a 0.75 ml microcentrifuge tube pierced by a heated #19 gauge needle, packed with a small quantity of glass wool and filled with the Sephadex suspension. The tube was placed in a 1.5 ml microcentrifuge tube, centrifuged in a table top centrifuge with swing out buckets (IEC) at 450 *g* for 10-15 s to pack the column (Fig. 3.1). More Sephadex was added to the column and centrifugation was repeated until the volume of the tightly packed column was at least 0.4 ml. The 1.5 ml microcentrifuge tube was replaced with a new one and the sample to be desalted (maximum volume = 100 µl) was loaded on the column and was centrifuged at 350 *g*, 1 min. If the recovered volume was significantly less than the original volume, an appropriatae volume of sterile water was added and the sample recentrifuged.

3.2.17 Horizontal agarose gel electrophoresis of DNA

The horizontal slab gels (200 x 155 x 4 mm) used contained 0.8% (w/v) agarose (Sigma, Type 1). The electrophoresis buffer pH 7.8, contained 40 mM-Tris, 1mM-Na_2EDTA and 5 mM-Na acetate. Electrophoresis was usually carried out at 1.5 V cm^{-1} for 16 h. Gels were stained and photographed as described in section 3.2.11. For rapid electrophoresis

Figure 3.1 A Sephadex G-50 column used for DNA purification. See section 3.2.16 for details.


0·75 ml microcentrifuge tube
packed sephadex
glass wool
1·5 ml microcentrifuge tube

of DNA, small samples were analysed on a minigel (93 x 68 x 1.5 mm) run at 13 Vcm^{-1} for 1 h.

3.2.18 Preparation of [^{32}P]-labelled DNA probes

The procedure described by Taylor *et al*. (1976) and Whitfeld *et al*. (1982) was used.

Materials

(1) Deoxycytidine 5' (α^{32}P) triphosphate (Amersham PB, 10205 3000 Ci mmol^{-1}). (2) Random primers (prepared by Dr D.B. Scott from herring sperm DNA). (3) DNA polymerase I (Klenow fragment from Boehringer Mannheim). (4) Deoxyribonucleoside triphosphates (Sigma): dATP (20 mM), dGTP (20 mM), dTTP (20 mM). (5) *Hae* III restriction enzyme (New England Biolabs). (6) Sephadex G-50 (Pharmacia, fine grade). (7) 0.25 M-Na$_2$EDTA, pH 8.0. (8) Phenol (equilibrated with TE (50/20) buffer, pH 8.0). (9) Chloroform. (10) TES (10/1/100) buffer pH 8.0: 10 mM-Tris, 1 mM-Na$_2$EDTA, 100 mM-sodium chloride.

Method

DNA (0.25-1.0 μg) to be labelled, was digested with *Hae* III restriction enzyme in a 25 μl reaction mixture for 30 mins at 37^{o}C (section 3.2.15). Random primers (100 g) were added and the mixture was boiled for 2 min then chilled rapidly on ice. Reagents were added in the order listed below: Sterilized distilled water, 2.5 μl; *Hae* III (x10) buffer, 1.5 μl; dTTP, 1 μl; dATP, 1 μl; dGTP, 1 μl; [^{32}P] CTP, 3 μl; DNA polymerase I, 1 μl. The mixture was incubated at 37^{o}C for 30 min, and the reaction was stopped by adding 2 μl of 0.25 M-Na$_2$EDTA. The reaction mixture was extracted with phenol:chloroform (section 3.2.16) and the aqueous phase was loaded onto a Sephadex G-50 column equilibrated with TES buffer pH 8.0. Fractions (approximately 150 μl) were collected and the DNA peak was pooled and stored at -20°C. DNA probes were usually labelled to a specific activity of 1-5 x 10^8 cpm per μg DNA.

3.2.19 DNA transfer and hybridisation

The procedure described by Southern (1975) was used.

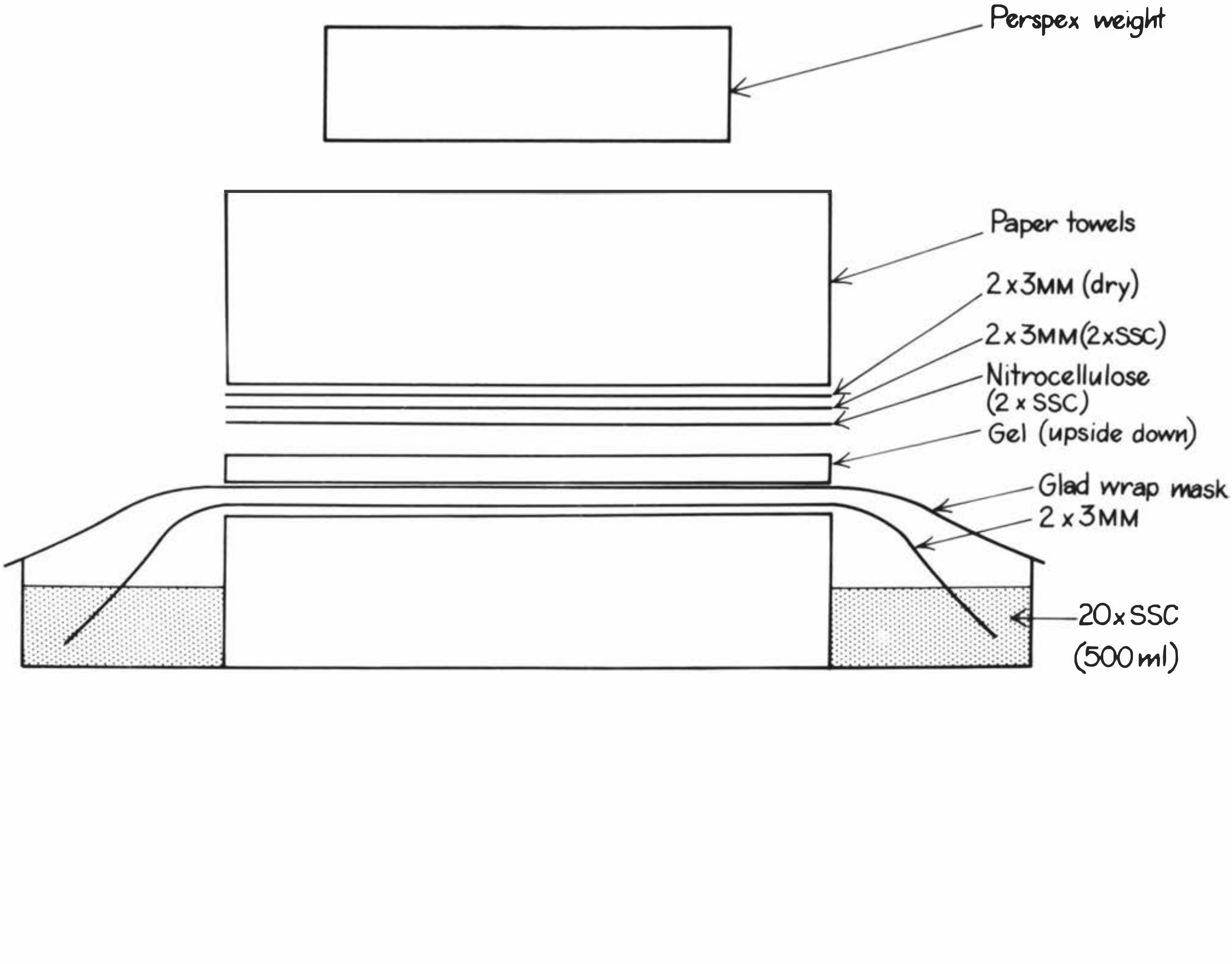
Materials

(1) 0.25 M-HCl. (2) 0.5 M-NaOH, 0.5 M-sodium chloride. (3) 0.5 M-Tris pH 7.4, 2.0 M-sodium chloride. (4) 20 x SSC (3 M-sodium chloride, 0.3 M-sodium citrate. (5) 2 x SSC. (6) Denhardts solution (10x) (Denhardt, 1966) contained: 1 M Hepes buffer pH 7.0, 25 ml; 20 x SSC, 75 ml; herring DNA (28 mg ml^{-1}), 0.32 ml; E. coli tRNA (10 mg ml^{-1}), 1 ml; 20% (w/v) SDS, 2.5 ml; Ficoll (Sigma 70), 1 g; bovine serum albumin (BSA), 1 g; polyvinylpyrrolidone (PVP); distilled water, 397 ml. (7) Nitrocellulose filter (Schleicher and Schull BA 85).

Method

Gels were stained and photographed as described in section 3.2.11. DNA was subjected to partial depurination by shaking the gel in 0.25 M-HCl for 15 min (Wahl et al., 1979), then denatured by shaking for 15 min in 0.5 M-NaOH, 0.5 M-sodium chloride, followed by shaking for 15 min in 0.5 M-Tris pH 7.4, 2.0 M-sodium chloride to neutralize the gel. The gel was washed in 2 x SSC for 2 min and placed on a blotting stand for DNA transfer to the nitrocellulose filter (Fig. 3.2). The nitrocellulose filter was removed after 16 h, washed in 2 x SSC for 5 min, blotted dry and baked in vacuo at 80°C for 2 h. The filter was sealed in a bag containing 20 ml (x10) Denhardts solution and prehybridised at 65°C for

Figure 3.2 Apparatus for DNA transfer by Southern blotting. See section 3.2.19 for details.


Perspex weight
Paper towels
2 x 3MM (dry)
2 x 3MM (2 x SSC)
Nitrocellulose (2 x SSC)
Gel (upside down)
Glad wrap mask
2 x 3MM
20 x SSC (500 ml)

at least 2 h. Most of the liquid in the bag was drained off, boiled probe (10^6 cpm) was added (section 3.2.18), the bag resealed and incubated in a shaking waterbath at 65°C overnight. After incubation, the filter was removed from the bag, washed in 3 changes of 2 x SSC, blotted dry, covered with "Gladwrap" and exposed to Ilford Curix X-ray film in the presence of Cronex intensifying screens for 1-5 d at -70°C. When filters were reused the probe DNA was removed by washing for 20 min in 20 mM-NaOH, 15 min in 0.5 M-Tris, pH 7.4, 2.0 M-NaCl and 15 min in 2 x SSC.

3.3 RESULTS

3.3.1 Physical characterisation of plasmids in Lotus rhizobia

The plasmid content of five *R. loti* strains and five *Bradyrhizobium spp.* (*Lotus*) strains were determined by the Eckhardt procedure and the Tait procedure respectively. The results obtained are summarised in Table 3.2.

3.3.2 DNA homology between pRlo2213a and pRlo2037a

Both NZP2213 and NZP2037 contain a single large indigenous plasmid (Table 3.2). In order to determine if there was any homology between these plasmids, pRlo2213a was purified as described in the methods (section 3.2.13) and used as a probe against NZP2037 plasmid DNA (Fig. 3.3a, lane 1) and an *Eco*RI digest of NZP2037 total DNA (Fig. 3.3a, lane 3). No hydridisation was observed in either cases (Fig. 3.3b, lanes 1 and 3) although strong hybridisation was observed to the control samples of NZP2213 plasmid DNA (Fig. 3.3b, lane 2) and the *Eco*RI digest of NZP2213 total DNA (Fig. 3.3b, lane 4).

Table 3.2 Plasmids in *Lotus rhizobia*

Bacterial strains	Number of plasmids present	Plasmid designation[a]	Estimated size[b] (MDal)
Rhizobium loti[c]			
NZP2213	one	pRlo2213a	120
NZP2037	one	pRlo2037a	240
NZP2048	one	pRlo2048a	240
NZP2227	one	pRlo2227a	240
NZP2238	one	pRlo2238a	240
Bradyrhizobium spp. (*Lotus*)[d]			
NZP2309 (CC814s)	one	-	220
NZP2071	one	-	230
NZP2075	two	-	240,280
NZP2229	two	-	130,280
NZP2202	three	-	180,220,240

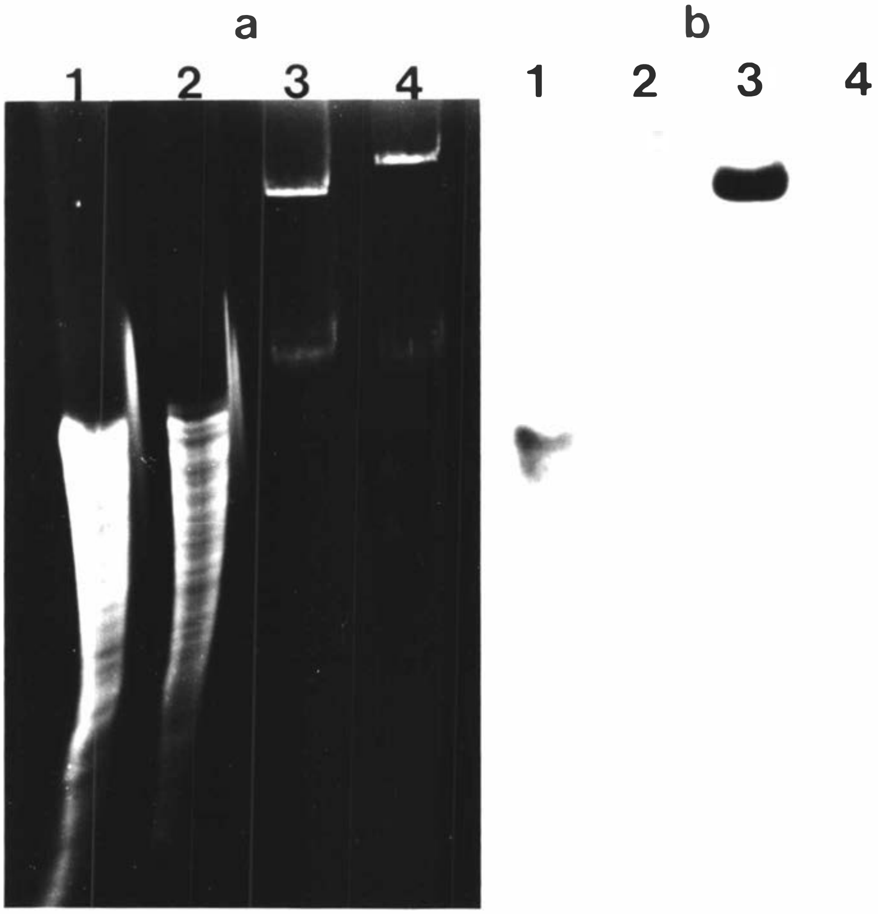
[a] The plasmid nomenclature proposed by Casse *et al*. (1979) is used. The indigenous plasmid of *R. loti* was designated by a prefix p, followed by an abbreviated species name Rlo and strain number in which the plasmid was found, the serial letters were used here to indicate the possible occurrence of multiple plasmids.

[b] *Agrobacterium tumefaciens* strain C58 contains two plasmids (MW 130 MDal and 275 MDal) and these were used for molecular size comparison.

[c] Plasmids of *R. loti* strains were analysed by the Eckhardt procedure.

[d] Plasmids of *Bradyrhizobium spp.* (*Lotus*) strains were analysed by the Tait procedure.

Figure 3.3 DNA homology between pRlo2213a and pRlo2037a. (a) Agarose gel of pRlo2037a DNA (lane 4) and pRlo2213a DNA (lane 3) isolated by the Eckhardt procedure and EcoRI digests of total DNA from NZP2037 (lane 2) and NZP2213 (lane 1). (b) Autoradiograph of the same gel hybridised with [^{32}P]-labelled pRlo2213a DNA.


a
b
1
2
3
4
1
2
3
4

3.3.3 Attempt to isolate Nod$^-$ derivatives of NZP2213 by heat curing

In an attempt to isolate non-nodulating (Nod$^-$) derivatives of NZP2213, a TY broth culture of NZP2213 was incubated at 38°C for 1 week. During this period the cell number dropped from 1×10^9 cells ml^{-1} to approximately 1×10^3 cells ml^{-1}. The surviving bacteria were plated on YM agar medium and incubated at 28°C for 6 days. Two hundred single colonies were picked and tested on *Lotus tenuis* plants. All the colonies tested had a Nod$^+$Fix$^+$ phenotype.

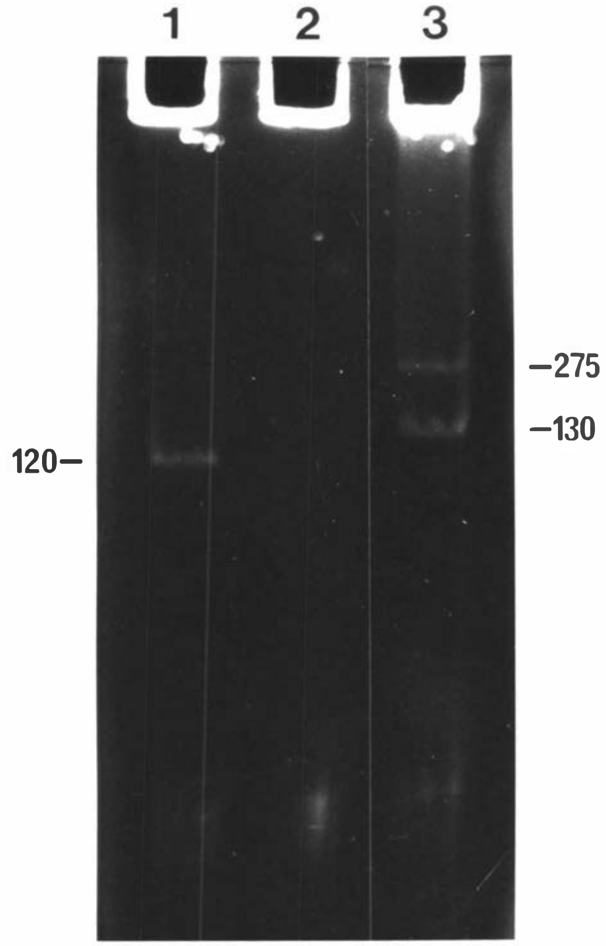
3.3.4 Attempt to isolate a Nod$^-$ derivative of NZP2213 by UV mutagenesis

Since the isolation of a Nod$^-$ derivative of NZP2213 by heat treatment was unsuccessful, UV mutagenesis was used in a second attempt to isolate Nod$^-$ mutants from a Str^R Rif^R derivative, PN 112, of NZP2213. The UV treatment of a PN112 broth culture (section 3.2.5) resulted in a 99.9% kill. Two hundred colonies were single colony purified and tested on *Lotus tenuis* plants. All the inoculated plants were Nod$^+$.

To determine whether the stability of the indigenous plasmid, pRlo2213a, was affected by the UV treatment, twenty out of the 200 colonies were analysed by the Eckhardt procedure. One of the twenty colonies tested had lost pRlo2213a (Fig. 3.4, lane 2). However this plasmid-cured derivative, strain PN225, retained the ability to nodulate *Lotus tenuis* plants effectively. Bacteria isolated from these nodules were found to be Str^R Rif^R, induced Nod$^+$Fix$^+$ nodules when retested on *Lotus tenuis* plants and were found to lack pRlo2213a.

Although the nodulation properties of the plasmid-cured strain were

Figure 3.4 Agarose gel of plasmids in *R. loti* strains. Lane 1, *R. loti* NZP2213 showing the single indigenous plasmid, pRlo2213a; lane 2, *R. loti* PN225, a plasmid-cured derivative of NZP2213; lane 3, *A. tumefaciens* C58 reference strain showing two plasmids. Numbers indicate molecular size of plasmids in megadaltons (MDal).


1
2
3
—275
—130
120—

identical to that of NZP2213, their growth rates appeared to be different. Colonies of NZP2213 reached 1 mm in diameter after 3 days on YM agar medium, whereas colonies of PN225 required 4 days incubation to reach the same size.

3.3.5 Hybridisation of pRlo2213a to total DNA of R. loti strains NZP2213 and PN225

To determine whether the absence of pRlo2213a in PN225, was due to curing of the plasmid or to integration of the plasmid DNA into the chromosome, pRlo2213a DNA was purified (section 3.2.13) and used as a hybridisation probe against a Southern blot of EcoRI digests of total DNA from NZP2213 (Fig. 3.5a, lane 1) and PN225 (Fig. 3.5a, lane 2). The results showed that [^{32}P]-labelled pRlo2213a hybridised to the total DNA digest of NZP2213 (Fig. 3.5b, lane 1) but not to the total DNA digest of PN225 (Fig. 3.5b, lane 2).

3.3.6 Identification of strain PN225 as a derivative of R. loti NZP2213

3.3.6.1 Strain identification by phage typing

When R. loti strains NZP2213 and PN225 were typed against Ø2037/1, a phage specific for R. loti strains (Patel, 1976), both strains showed identical patterns of susceptibility (Fig. 3.6).

3.3.6.2 Strain identification by tandem crossed-immunoelectrophoresis

Strain PN225 was also compared with strain NZP2213 by the technique of tandem crossed-immunoelectrophoresis. The crossed-immunoelectrophoresis pattern of strain NZP2213 against itself is shown in Fig. 3.7a. Fig. 3.7b shows the crossed-immuno-electrophoresis pattern of strain NZP2213 reference antigen (well 3) against strain PN225 antigen (well 4). Fig. 3.7c shows the crossed-immunoelectrophoresis pattern of strain NZP2213

Figure 3.5 Hybridisation of pRlo2213a to *EcoRI* enzyme digests of *R. loti* total DNA. (a) An ethidium bromide stained agarose gel showing the total DNA digests of NZP2213 (lane 1) and PN225 (lane 2). (b) Autoradiograph of the same gel hybridised with [^{32}P]-labelled pRlo2213a DNA.

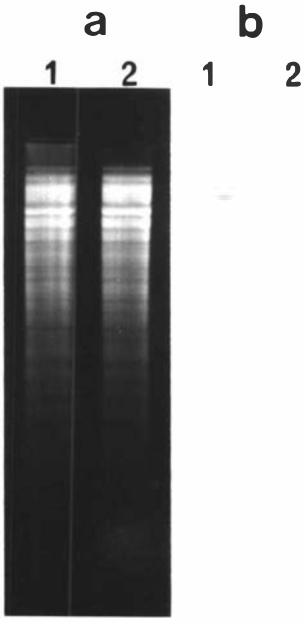

a
b
1
2
1
2

Figure 3.6 Identification of R. loti strains by phage typing. Susceptibility pattern of R. loti strain NZP2213 to phage Ø2037/1. Numbers indicate the serial dilution factors.

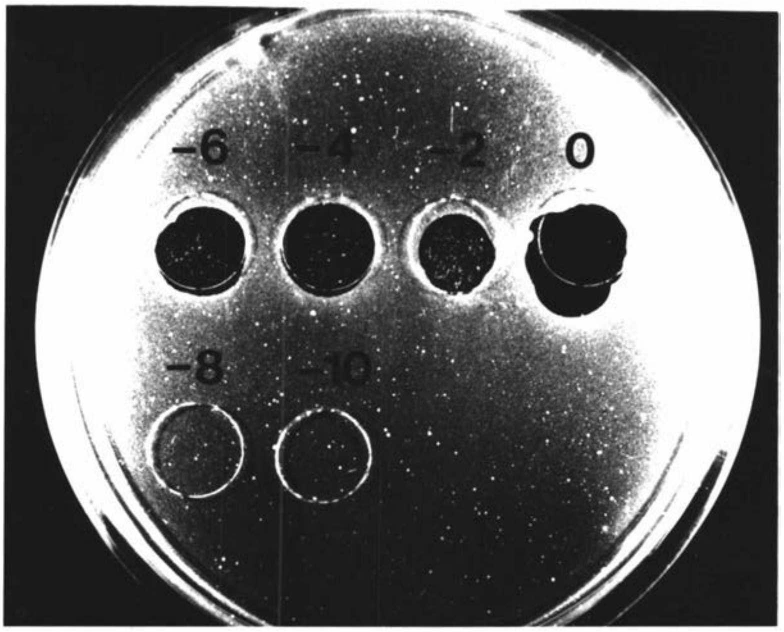
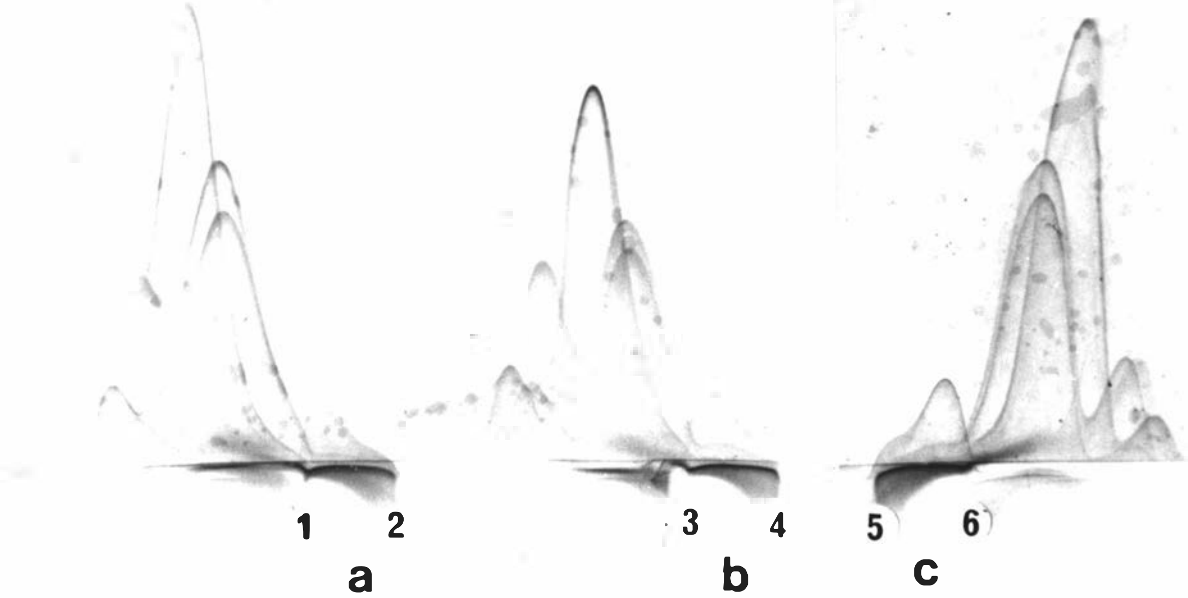

-6
-4
-2
0
-8
-10

Figure 3.7 Tandem crossed-immunoelectrophoresis patterns of R. loti strains. (a) The reference crossed-immunoelectrophoresis pattern of NZP2213 using NZP2213 antigens in wells 1 and 2. (b) The crossed-immunoelectrophoresis pattern of NZP2213 antigen, well 3, against PN225 antigen, well 4. (c) The crossed-immunoelectrophoresis pattern of NZP2213 antigen, well 5, against NZP2037 antigen, well 6. Sonicated bacterial cells were used as the source of antigen and antibodies to NZP2213 cells were used as the reference antiserum in these experiments.


1
2
3
4
5
6
a
b
c

reference antigen (well 5) against strain NZP2037 antigen (well 6).

3.3.6.3 Strain identification by restriction enzyme digest patterns

Comparison of restriction enzyme digest patterns of total DNA is also a useful method for strain identification. Using this method *Eco*RI digest patterns of total DNA from *R. loti* strains NZP2213, PN225 and NZP2037 were compared (Fig. 3.8).

3.3.7 DNA homology between the nod gene region of R. loti NZP2037 and the plasmid-cured derivative, PN225

The plasmid-cured derivative of *R. loti* NZP2213, strain PN225, was found to still form effective nodules on *Lotus tenuis* (section 3.3.4). Confirmation that the plasmid-cured derivative, PN225, retained its *nod* gene region was obtained by Southern hybridisation. Total DNA from strains NZP2213 and PN225 were digested with *Eco*RI enzyme, blotted and hydridised to the [^{32}P]-labelled *nod* gene region of *R. loti* NZP2037 (Chapter 5). The *nod* gene probe used in this hybridisation experiment was a 12.8 kb *Eco*RI fragment carrying *nod*::Tn5 sequences from pPN301 (section 5.3.1). An *Eco*RI digest of NZP2037 total DNA was included as a control. Hybridisation was found to 9.6 and 3.8 kb *Eco*RI fragments in both NZP2213 (Fig. 3.9, lane 1) and PN225 (Fig. 3.9, lane 2) and to a 7.1 kb *Eco*RI fragment in NZP2037 (Fig. 3.9, lane 3).

3.4 DISCUSSION

Using the Eckhardt (Eckhardt, 1978) and the Tait (Andersen *et al.* 1981) procedures, both fast- and slow-growing *Lotus* rhizobia were found to harbour large indigenous plasmids. The five strains of *R. loti* analysed by the Eckhardt procedure were found to contain a single

Figure 3.8 *EcoRI* restriction enzyme digest patterns of total DNA from *R. loti* strains. Lane 1, NZP2213; lane 2, PN225; lane 3, NZP2037.

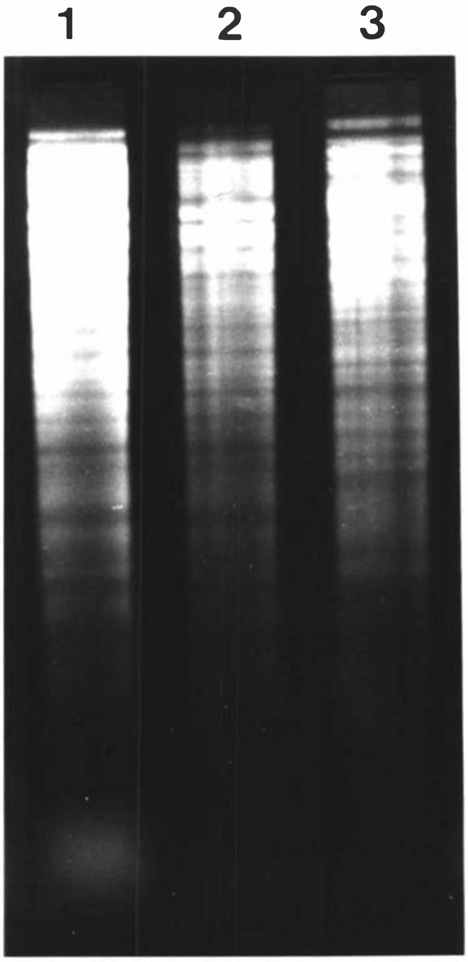
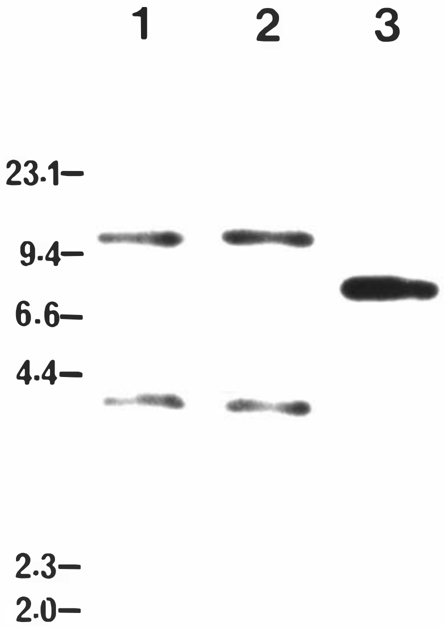

1
2
3

Figure 3.9 Hybridisation of the *nod* gene region of NZP2037 to *Eco*RI digests of DNA from strains NZP2213, PN225, and NZP2037. Autoradiograph of a Southern blot of *Eco*RI digests of *R. loti* total DNA hybridised with a [^{32}P]-labelled 12.8kb *Eco*RI (*nod*::Tn5) fragment from pPN301. Lane 1, NZP2213; Lane 2, PN225; Lane 3, NZP2037.


1
2
3
23.1
9.4
6.6
4.4
2.3
2.0

indigenous plasmid (Table 3.2). Four out of the 5 strains tested carried a plasmid of molecular weight approximately 240 MDal, whereas strain NZP2213 carried a smaller plasmid of about 120 MDal. Identical results were obtained when these strains were analysed by the Tait procedure, a method that involves a more efficient lysis step. No DNA homology was observed between pRlo2213a and pRlo2037a, suggesting that these 2 plasmids do not carry common gene sequences.

Although the presence of an additional large plasmid in R. loti cannot be totally excluded, the technique used here for screening plasmids in R. loti was satisfactory for the isolation of the large cryptic plasmid (>300 MDal present in Agrobacterium tumefaciens (Holsters et al., 1978) and the megaplasmid (>300 MDal) present in Rhizobium meliloti (Ronson and Scott, 1983). In addition Kondorosi et al. (1982) detected the 1000 MDal R. meliloti megaplasmid (Burkardt and Burkardt, 1984) after it was transferred to R. loti NZP2037, but did not detect any further plasmids in NZP2037.

Presence of a single large plasmid in R. loti strains contrasts with the observation that all the other fast-growing Rhizobium species examined to date, carry multiple plasmids of molecular weights ranging in size from 100-200 MDal (reviewed by Prakash et al., 1980).

Large plasmids were also a common feature of Bradyrhizobium spp. (Lotus). In contrast to R. loti, Bradyrhizobium spp. (Lotus) contained multiple plasmids of molecular weights ranging in size from 130-280 MDal. Although these plasmids could not be isolated by the Eckhardt procedure, the more efficient lysis obtained by the Tait procedure proved to be satisfactory for the isolation of these plasmids. Multiple plasmids are a common feature of other *Bradyrhizobium spp*. (*B. japonicum*) (Gross *et al*., 1979; Haugland and Verma, 1981; Cantrell *et al*., 1982). It appears that the presence of indigenous plasmids of molecular weight greater than 100 MDal is a general feature of both fast and slow-growing root nodule bacteria.

In order to determine whether genes for nodulation of *Lotus spp*. were carried on the large indigenous plasmids identified in *R. loti*, an attempt was made to isolate Nod⁻ derivatives of *R. loti* strain NZP2213 by heat treatment (section 3.2.8). However, no Nod⁻ derivatives were obtained. As an alternative approach, UV mutagenesis was used in a second attempt to isolate Nod⁻ derivatives of NZP2213. Although this method was used successfully by Russell and Jones (1973) in the isolation of symbiotic mutants of *R. trifolii*, the attempts described here to isolate Nod⁻ derivatives of *R. loti* were unsuccessful. One possible explanation for this result was that the number of colonies screened (200) was not high enough to detect any symbiotic mutants. Alternatively, the combination of the high frequency of reversion due to the enzymatic repair mechanisms that operate in bacteria (Witkin, 1976) and the strong plant selection for revertants during the plant assay may have contributed to the difficulty of screening for symbiotic mutants. However, the UV treatment did affect the stability of the indigenous plasmid, pRlo2213a, of NZP2213. One out of the 20 colonies tested by the Eckhardt procedure (section 3.3.4) had lost pRlo2213a. This was confirmed by the fact that [^{32}P]-labelled pRlo2213a hybridised to a total DNA digest of NZP2213 but not with that of the plasmid-cured derivative of NZP2213, PN225. Confirmation that PN225 was indeed derived from NZP2213 was shown by the species specific phage typing (section 3.3.6.1), the strain specific serological test (section

3.3.6.2) and the EcoRI enzyme restriction pattern of total DNA (section 3.3.6.3).

When the pRlo2213a-cured strain PN225 was tested on Lotus tenuis, it induced effective nodules on the tested plants. In addition, a pRlo2037a-cured derivative of R. loti strain NZP2037 obtained by heat treatment was found to nodulate Lotus pedunculatus effectively (C.E. Pankhurst, personal communication). These results suggest that both pRlo2213a and pRlo2037 do not carry genes necessary for nodulation. This was confirmed by the observation that a cloned nod region isolated from strain NZP2037 hybridised strongly with total DNA digests of both NZP2213 and PN225 (section 3.3.7). In addition, Pankhurst et al. (1983) reported that purified pRlo2037 DNA did not hybridise with the nif region of R. meliloti and Dr D.B. Scott (personal communication) found that [^{32}P]-labelled pSA30, a pACYC184 clone containing the nif HDK genes of Klebsiella pneumoniae (Cannon et al., 1979) did not hybridise with either pRlo2213a or pRlo2037a. All these observations suggest that the genes for nodulation and the nif structural genes are not located on the detectable large indigenous plasmid in R. loti. This is in contrast to the finding that some of the genes involved in nitrogen fixation, nodulation and host specificity are located on large indigenous plasmids in R. trifolii (Zurkowski and Lorkiewicz, 1979; Prakash et al., 1981; Hooykaas et al., 1981; Schofield et al., 1983; Scott et al., 1984), R. leguminosarum (Johnston et al., 1978; Nuti et al., 1979; Downie et al., 1983b), R. phaseoli (Beynon et al., 1980; Lamb et al., 1982), and R. meliloti (Banfalvi et al., 1981; Rosenberg et al., 1981; Long et al., 1982; Buikema et al., 1983; Kondorosi et al., 1984). However, studies to date on the slow-growing Bradyrhizobium japonicum strains showed that nif HDK genes were not located on the large plasmids present in the strains studied (Haugland and Verma, 1981; Masterson *et al.*, 1982). It is unknown whether the plasmids of *Bradyrhizobium spp.* (*Lotus*) carry any symbiotic genes.

The work reported here shows that the large plasmids of *R. loti* strains do not carry *nod* and *nif* genes. The function of these plasmids is unknown. However, it was observed that the growth rate of the plasmid-cured derivative of strain NZP2213, PN225, was slower than that of the wildtype (section 3.3.4) suggesting that pRlo2213a may carry genes involved in some catabolic pathways.

3.5 SUMMARY

Large plasmids were identified in both the fast-growing *R. loti* and slow-growing *Bradyrhizobium spp.* (*Lotus*). A derivative lacking the large plasmid found in *R. loti* strain NZP2213 still retained the capacity to nodulate *Lotus tenuis* effectively. This plasmid-cured strain was shown to be a derivative of strain NZP2213 by phage typing, serological tests and by comparison of the restriction enzyme patterns of total DNA. No DNA homology was observed between pRlo2213a and pRlo2037a. These results suggest that genes for nodulation and nitrogen fixation are carried on the chromosome of NZP2213. The function of the plasmids in both *R. loti* and *Bradyrhizobium spp.* (*Lotus*) is still unknown.

CHAPTER FOUR

ISOLATION AND CHARACTERISATION OF TN5-INDUCED SYMBIOTIC MUTANTS OF RHIZOBIUM LOTI

4.1 INTRODUCTION

In chapter 3 an attempt was made to isolate a Nod$^-$ derivative of *R. loti* strain NZP2213 by UV mutagenesis as at the time a suitable vector system for introducing a transposon into the *R. loti* genome was not available. During the course of this work, a vector system (Simon *et al.*, 1983) became available for introducing Tn5 into the genome of *R. loti* and as outlined below this is a more desirable method for generating mutations.

Transposons have been used to induce mutations in a range of bacterial species (Kleckner *et al.*, 1977; Van Vliet *et al.*, 1978). A commonly used transposon is the well characterised Tn5 (Kleckner *et al.*, 1977; Jorgensen *et al.*, 1979). Transposon Tn5 (5.7 kb) encodes resistance to neomycin and kanamycin and thus is suitable for direct selection in the isolation of mutants. Transposition of Tn5 appears to be a singular and nonspecific event, hence multiple mutations are avoided. If Tn5 insertion occurs within an operon it has a polar effect on genes downstream of the insertion (Kleckner *et al.*, 1977). The absence of an *Eco*RI restriction site in Tn5 (Jorgensen *et al.*, 1979) facilitates identification and molecular cloning of the Tn5 mutated genes from an *Eco*RI digest of total mutant DNA.

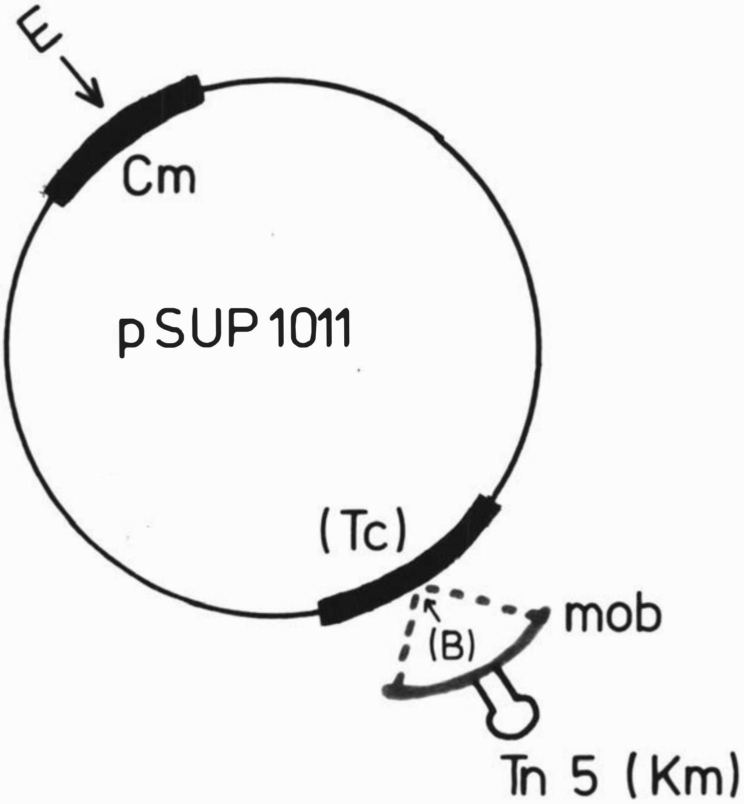
A method for introducing Tn5 into the *Rhizobium* genome by using the suicide plasmid pJB4JI (pPH1J1::Mu::Tn5) was first described by Beringer *et al.* (1978). This system has been used successfully in the isolation of Tn5-induced symbiotic mutants in *R. leguminosarum* (Buchanan-Wollaston *et al.*, 1980), *R. meliloti* (Banfalvi *et al.*, 1981; Meade *et al.*, 1982; Forrai *et al.*, 1983), *R. trifolii* (Rolfe *et al.*, 1981; Scott *et al.*, 1982), *R. phaseoli* (Noel *et al.*, 1984) and a *Bradyrhizobium spp.* (*Parasponia*) (Cen *et al.*, 1982). However, attempts to use pJB4JI for Tn5 mutagenesis were unsuccessful in certain strains of *R. meliloti* (Meade *et al.*, 1982), *R. trifolii* (Rolfe *et al.*,1981; Scott and Ronson, 1982) as well as *R. loti* strains NZP2037 and NZP2213 (Pankhurst *et al.*, 1983). An additional problem was the coinsertion of Mu phage as well as Tn5 sequences into the genome (Meade *et al.*, 1982; Noel *et al.*, 1984). The recent construction of a group of mobilizable vector plasmids by Simon *et al.* (1983, 1984) has provided an alternative system of Tn5 mutagenesis for *Rhizobium*. These vector plasmids are derived from *E. coli* specific narrow host range replicons that contain the broad host range RP4 specific Mob-site and Tn5 sequences cloned into unique *Bam* HI sites of the vector plasmids. One such plasmid is pSUP1011 which is a pACYC184 (Chang and Cohen, 1978) derivative carrying the transposon Tn5 in mob (refer Fig. 4.1). Plasmid pSUP1011 encodes kanamycin and chloramphenicol resistance and can be mobilized to *Rhizobium* by using *E. coli* strain SM10, which contains plasmid RP4 on its chromosome (Simon *et al.*, 1983). This vector system has been used successfully in random Tn5 mutagenesis of *R. meliloti* (Simon *et al.*, 1984) and *R. loti* (Pankhurst *et al.*, 1983).

This chapter describes the use of the SM10/pSUP1011 system for isolating symbiotic mutants of *R. loti* strain NZP2037. Strain NZP2037 was used instead of strain NZP2213 because the seedlings of *Lotus tenuis*, the effective host of NZP2213, did not always nodulate with wild type strains when grown in test-tubes or petri-dishes containing plant

Figure 4.1 The vector plasmid pSUP1011 used for Tn5 mutagenesis

Mob system.

pACYC 184 - mob :: Tn 5



E = EcoR 1 site
B = BamHI site

nutrient agar. In contrast Lotus pedunculatus, an effective host for NZP2037, gave reliable plant test results with both the test-tube and plate assay methods and was a more suitable plant for isolating Tn5-induced symbiotic mutants. The scheme devised by Vincent (1980) (refer Table 1.1) was used to describe the mutant phenotypes resulting from the various blockages in the nodule development of Lotus pedunculatus.

4.2 METHODS and MATERIALS

4.2.1 Bacterial strains and plasmids

These are described in Table 4.1.

4.2.2 Preparation of culture media

4.2.2.1 Liquid media

Tryptone yeast extract medium (TY, section 2.2.2.1)

Yeast extract mannitol medium (YM, section 2.2.2.1)

Luria broth (LB; section 3.2.2.1)

S20 medium (Modified M9 medium). Composition (g l^{-1}): Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1. The following were added as sterile solutions after autoclaving (ml l^{-1}): 10% (w/v) $MgSO_4 \cdot 7H_2O$, 2.5; 1% (w/v) $CaCl_2 \cdot 2H_2O$, 1.5, 20% (w/v) sucrose, 12.0; (500 μg ml^{-1}) biotin, 1.0.

4.2.2.2 Solid media (see section 2.2.2.2)

4.2.3 Maintenance of cultures

Rhizobium and E. coli cultures were maintained as described in section 3.2.3. Rhizobium loti Tn5-induced mutants were maintained at 4°C on S20 (see section 4.2.2.1) slopes supplemented with neomycin (500 μg ml^{-1}).

Table 4.1 Bacterial strains and plasmids

Bacteria	Relevant Characteristics	Source/reference
Rhizobium loti		
NZP2037	Nod$^+$Fix$^+$ (Lotus pedunculatus; Lotus tenuis)	DSIR culture collection, N.Z.
PN184	NZP2037 str-1, Nod$^+$Fix$^+$	This study; spontaneous mutant of NZP2037
PN233	PN184 str-1, Nod$^-$	This study; Tn5-induced mutant of PN184
PN234-PN246	PN184 str-1, Nod$^+$Fix$^-$	This study; Tn5-induced mutants of PN184
Escherichia coli		
SM10	C600 thi thr leu ton A lac Y sup E rec A$^-$ [RP4.2 Tc::Mu] Ap^S Tc^S Muc$^+$ Km^R Tra$^+$	Simon et al. (1983)
HB101	F$^-$ pro leu thi lacY str^R r$_k$$^-$ m$_k$$^-$ Endo I$^-$ rec A$^-$	Boyer and Roulland-Dussoix (1969)
Plasmids		
pACYC184	C$_m$^{R} T$_c$^{R}	Chang and Cohen (1978)
pSUP1011	Cm^R Km^R Derivative of pACYC184 carrying a 2.6 kb mob DNA fragment from RP4 and Tn5	Simon et al. (1984)

Table 4.1 continued

Bacteria	Relevant Characteristics	Source/reference
pBR322	Ap^R Tc^R	Bolivar *et al*. (1977)
pKan2	Ap^R Tc^S Km^R, pBR322 containing the internal *Hind* III fragment of Tn5	Scott *et al*. (1982)
pSA30	Cm^S Tc^R, *Klebsiella pneumoniae nif* HDKY genes cloned in pACYC184	Cannon *et al*. (1979)

4.2.4 Growth of bacteria

Rhizobium loti (see section 3.2.4)

Rhizobium loti Tn5-induced mutants were incubated at 28°C on S20 medium supplemented with streptomycin (250 μgm l^{-1}) and neomycin (500 μgm l^{-1}).

E. coli (see section 3.2.4)

4.2.5 Conjugation

R. loti strain PN184, the Tn5 recipient strain, was grown in 5 ml of TY broth at 28°C for 2 days. The Tn5 donor strain, E. coli SM10/pSUP1011, was cultured overnight at 37°C in LB, diluted in 9 volumes of fresh warm LB, and grown for 4h at 37°C before it was crossed with PN184. A modification of the method described by Jacob et al. (1976) was used to perform the cross. Aliquots (1 ml) containing approximately 10^9 donor and recipient cells were mixed on a TY plate and left to dry in a laminar flow cabinet for 1 h. The bacteria were incubated for 20h at 28°C and eluted from the plate with 5 ml of sterile distilled water, washed once (5 ml sterile water), and resuspended in 5 ml of distilled water. Serial dilutions of the cell suspension were plated on S20 containing 250 μgm l^{-1} streptomycin and 500 μgm l^{-1} neomycin. The cells were incubated for 5 days at 28°C. Transconjugants were single colony purified and tested on plants for their symbiotic response.

4.2.6 Screening method for symbiotic mutants

The plate assay described by Rolfe et al. (1980) was used. Seeds of Lotus pedunculatus Cav. 'Grassland Maku' were sterilized and germinated as described in section 3.2.6. Inocula were prepared from single colony purified transconjugants (section 4.2.5) and streaked over half the surface of an agar plate. Lotus pedunculatus seedlings were placed on the agar surface just above the line of the streaked rhizobia. The agar plates were prepared using Fahraeus medium (Fahraeus, 1957) which has the following composition (g l^{-1}): KH_2PO_4, 0.1, $Na_2HPO_4 \cdot 12H_2O$, 0.15; $MgSO_4 \cdot 7H_2O$, 0.12; $CaCl_2 \cdot 2H_2O$, 0.1; Ferric citrate, 0.05 and 1 ml of trace elements stock solution which contained the following microelements (g l^{-1}): H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$, 0.08; $CoSO_4 \cdot 7H_2O$, 0.095; $Na_2MoO_4 \cdot 2H_2O$, 0.054. The medium was solidified with 1.5% agar.

Inoculated plates were sealed with Nescofilm strips (2.5 cm wide and 10 cm long; NIPPON SHOJI KAISHI Ltd., Osaka, Japan). Three small slits were made in the Nescofilm at the top of the plate to allow gas exchange. The plates were then stacked in rows and incubated in a greenhouse (Day temperature 20-26^{o}C, night temperature, 16^{o}C). Root nodules first appeared after 10 days. Plants were examined at 3 and 6 weeks after inoculation for nodulation and growth.

4.2.7 Plasmid analysis by the Eckhardt procedure (see section 3.2.11)

4.2.8 Bacteriophage susceptibility tests (see section 3.2.9)

4.2.9 Preparative isolation of plasmid DNA from E.coli by the cleared lysate method

A modification of the cleared lysate procedure of Clewell and Helinski (1969) was used.

Materials

(1) 25% (w/v) sucrose solution in 0.05 M-Tris pH 8.0. (2) Lysozyme (20 mg ml^{-1}) freshly prepared in 25% (w/v) sucrose solution.
(3) TE (10/1) buffer pH 8.0: 10 mM-Tris-HCl, 1 mM-Na_2EDTA.
(4) Triton lysis mixture consisted of 0.25 M-Na_2EDTA (25 ml), 1 M-Tris

pH 8.0 (50 ml), 20% (v/v) Triton X-100 in 0.01 M-Tris-HCl pH 8.0 (10 ml) and sterilised water (60 ml). (5) Caesium chloride. (6) Ethidium bromide (10 mg ml^{-1}).

Method

An overnight culture of E. coli in LB (25 ml) was inoculated into 1 litre of LB and incubated at 37°C with shaking (200 r.p.m.). For isolation of amplifiable plasmid DNA, chloramphenicol (Cm) was added (170 µg ml^{-1}) when OD_{600} was approximately 0.4. Spectinomycin (399 µg ml^{-1}) was used if the plasmid contained a Cm^R gene . Cells were further incubated for 12 to 16 h and harvested by centrifugation (10,000 g, 10 min, 0°C). The remaining operations were performed on ice with chilled solutions. The cell pellet was resuspended in 8 ml of sucrose solution, lysozyme (2 ml) and Na_2EDTA (4 ml) were added and the mixture was incubated for 10 min. Triton lysis mixture (12 ml) was added to the mixture and gentle agitation was maintained until lysis was completed. The lysate was centrifuged (27,000 g, 45 min, 0°C) and the clear supernatant was collected. Care was taken to avoid the gelatinous chromosomal DNA pellet. Caesium chloride was added to the supernatant to a final concentration of 1 µg ml^{-1}, the mixture was incubated at room temperature for 1 h and centrifuged (27,000 g, 15 min, 4°C) to remove cell debris. Ethidium bromide was added to a final concentration of 300 µg ml^{-1} and the mixture was held at 4°C overnight. The next day, the mixture was centrifuged (27,000 g, 15 min, 4°C) to pellet the ribonucleoprotein. The refractive index was adjusted to n=1.393-1.397 and the mixture was centrifuged in a Beckman Type 65 rotor at 250,000 g for 40 h at 16°C. The plasmid DNA band was collected and the ethidium bromide and caesium chloride were removed as described in section 3.2.13. The DNA solution was concentrated by ethanol precipitation (section 3.2.13) and the purity and concentration of the DNA determined spectrophotometrically as described in section 2.2.5. The DNA solution was stored at 4°C.

4.2.10 Isolation of genomic DNA

Total *Rhizobium* DNA was isolated as described in section 3.2.14.

4.2.11 Digestion of DNA with restriction enzymes

DNA (0.2-5.0 μg) was digested with restriction enzymes as described in section 3.2.15.

4.2.12 Purification of DNA (see section 3.2.16)

4.2.13 Agarose gel electrophoresis of DNA (see section 3.2.17)

4.2.14 Preparation of [^{32}P] labelled DNA-probes (see section 3.2.18)

4.2.15 DNA transfer and hybridisation (see section 3.2.19)

4.2.16 Determination of molecular weights

A *Hind* III digest of λ DNA was used as a standard to determine the molecular weight of DNA fragments. The relative mobilities of DNA fragments were measured and molecular weights were determined graphically from a plot of relative mobility versus $\log_{10}$ molecular weight (Daniels *et al.*, 1980; Sanger *et al.*, 1982) or by using a computer programme (Cubic spline, M. Pritchard, Grasslands Division, DSIR, Palmerston North) designed to transform mobilities to molecular weights.

4.2.17 Light and electron microscopy of nodules

Root nodules and nodule-like structures studied by light and electron microscopy were removed from the roots of plants with a scalpel blade. The specimens were cut in half in cold (4°C) Karnovsky fixative

(2% (w/v) formaldehyde, 3% (w/v) glutaraldehyde, 0.1 M-phosphate buffer pH 7.2) and left for 2 h. The tissues were then washed in three changes of 0.1 M-phosphate buffer pH 7.2 for 30 min, and post-fixed in 1% (w/v) osmium tetroxide in the same buffer for 2 h. Following a further buffer wash (2 changes for 30 min) the tissues were dehydrated in 25%, 50%, 75%, and 95% ethanol and infiltrated with epoxy resin through propylene oxide. Embedding was done under vacuum and blocks containing the specimens were polymerised at 60°C for 72 h.

For light microscopy thick sections (2-3 μm) were cut with an LKB "Ultratome" using glass knives and stained with 0.5% toluidine blue in 0.1 M phosphate buffer pH 4.5. The sections were photographed using a Reichert "Zetopan" research microscope fitted with a Reichert "Photo-Automatic" camera.

For electron microscopy thin sections (pale gold interference colour) were cut using a diamond knife and stained with saturated uranyl acetate in 50% (v/v) ethanol followed by lead citrate (Venable and Coggeshall, 1965) and examined in a Phillips EM200 electron microscope at 80 KV.

4.2.18 Examination of root hairs by light microscopy

A root specimen obtained from a 5-week-old *Lotus pedunculatus* plant was placed on a microscopic slide and stained in 1% methylene blue for 5 min, washed, dried, examined and photographed.

4.3 RESULTS

4.3.1 Transposon mutagenesis of Rhizobium loti

To isolate symbiotically defective mutants of *Rhizobium loti*

NZP2037, Tn5 mutagenesis was carried out using the broad host range mobilizable vector pSUP1011 (Simon et al., 1984). E. coli SM10/pSUP1011 was crossed into a Str^R derivative of NZP2037, strain PN184, as described in section 4.2.5. Selection was carried out on S20 medium containing 250 μg ml^{-1} of streptomycin and 500 μg ml^{-1} of neomycin. S20 defined medium was used instead of a complete medium to avoid selection of symbiotically defective auxotrophs. The frequency of transfer of neomycin resistance was $1x10^{-4}$. From this cross 1060 Str^RNm^R transconjugants were single colony purified and then inoculated onto Lotus pedunculatus seedlings to determine their symbiotic phenotype. Of the 1060 transconjugants tested, one was nodulation defective (Nod^-) (Fig. 4.2a(1)) and 12 were defective in nitrogen fixation (Nod^+Fix^-) (e.g. Fig 4.2a(2) and (3)) when compared with the wild type strain R. loti PN184 (Nod^+Fix^+) (Fig. 4.2a(4)). Repeated plant tests showed that all were stable mutants except PN245 (Nod^+Fix^-). Plants inoculated with this mutant showed a variable response, two out of six plants showed some nitrogen fixation, four remained nitrogen deficient.

The plasmid profiles, the phage typing patterns and the EcoRI restriction enzyme digestion patterns of all the mutant strains were compared with those of the parental strain R. loti PN184, and the results confirmed that they were derivatives of R. loti PN184.

4.3.2 Physical analysis of presumptive Tn5-induced symbiotic mutants by Southern hybridisation

To confirm the presence of Tn5 sequences in the symbiotic mutants isolated, Southern blots of EcoRI digests of total DNA from the mutants were hybridised with [^{32}P]-labelled pKan2, a pBR322 derivative

Figure 4.2 Lotus pedunculatus plants inoculated with R. loti wild type and Tn5-induced symbiotic mutant strains. (a) Plants inoculated with (1) PN233 (Nod^-); (2) PN236 (Nod^+Fix^-); (3) PN238 (Nod^+Fix^-); (4) PN184 (Nod^+Fix^+). (b) A close-up view of the root systems of the same plants.


a
1
2
3
4
b

containing the HindIII fragment of Tn5 (Scott et al., 1982). Of the 13 mutants analysed, 10 showed hybridisation to a single EcoRI fragment (e.g. Fig. 4.3, lanes 1,2 and 3), the remaining 3 mutants showed hybridisation to several bands (e.g. Fig. 4.3, lane 4). A summary of the sizes of the EcoRI fragments hybridising to pKan2 is shown in Table 4.2. All the mutants were also analysed for pACYC184 vector sequences. As shown in Table 4.2 the 3 mutants that had mutiple bands hybridising to pKan2 were found to contain vector sequences.

4.3.3 Hybridisation of total DNA from R. loti Tn5-mutants with [^{32}P]-labelled nif HDK probe

To determine whether any of the symbiotic mutants isolated carried Tn5 in the nif structural gene region of the genome, pSA30, a derivative of pACYC184 containing the nif HDK genes of Klebsiella pneumoniae, and pKan2 were both hybridised to EcoRI digests of total DNA from the R. loti Tn5-induced mutants. Two separate bands (e.g. Fig. 4.4, lane 1, 2 and 3) were obtained for 10 of the 13 mutants. In each case the upper band (Fig. 4.4, lanes 1, 2 and 3) corresponded in size to the EcoRI fragment carrying Tn5 (Fig. 4.3) and the lower band was in all cases a 5 kb EcoRI fragment, corresponding in size to the nif fragment present in the wild type strain NZP2037 (Fig. 4.4, lane 5). The three remaining mutants PN240, PN241 and PN242 showed multiple hybridising bands (e.g. Fig. 4.4, lane 3).

4.3.4 Light microscopic examination of Lotus pedunculatus root hairs inoculated with a Nod$^-$ mutant of R.loti

To examine the response shown by the root hairs of Lotus pedunculatus plants inoculated with the Nod$^-$ mutant strain PN233, root samples were stained and examined by light microscopy (section 4.2.18).

Figure 4.3 Physical analysis of presumptive Tn5 mutants by Southern hybridisation. Autoradiograph showing hybridisation of [^{32}P]-labelled pKan2 to *EcoRI* digests of total DNA from *R. loti* Tn5-induced mutants. Lane 1, PN234 DNA; Lane 2, PN233 DNA; Lane 3, PN235 DNA; Lane 4, PN240 DNA; Lane 5, NZP2037 (wild type) DNA. Size markers arein kilobases (kb).

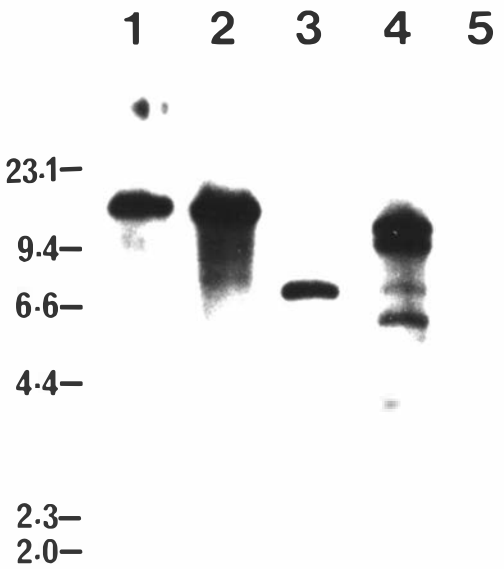

1
2
3
4
5
23·1
9·4
6·6
4·4
2·3
2·0

Table 4.2 Characterisation of Tn5-induced mutants of R. loti NZP2037

Strain number	Phenotype	Size of EcoRI fragment (kb) containing Tn5[a]	Size of EcoRI fragment (kb) in wild type strain[b]	Presence of vector DNA (pACYC184)[c]	Class of mutant[d]
PN233	Nod^-	12.8	7.1	-	Hac
PN234	Nod^+Fix^-	13.2	7.5	-	Noi
PN235	Nod^+Fix^-	7.2	1.5	-	Cof
PN236	Nod^+Fix^-	17.3	11.6	-	Noi
PN237	Nod^+Fix^-	17.3	11.6	-	Noi
PN238	Nod^+Fix^-	7.8	2.1	-	Noi
PN239	Nod^+Fix^-	13.7	8.0	-	Bar
PN240	Nod^+Fix^-	multiple bands		+	N.D.
PN241	Nod^+Fix^-	multiple bands		+	N.D.
PN242	Nod^+Fix^-	multiple bands		+	N.D.
PN244	Nod^+Fix^-	12.1	6.4	-	Noi
PN245	Nod^+Fix^-	13.1	7.4	-	Cof
PN246	Nod^+Fix^-	14.2	8.5	-	Cof

[a] Determined by Southern hybridisation of [^{32}P]-labelled pKan2 (Scott et al., 1982) to EcoRI digests of total DNA.

[b] Predicted size of EcoRI fragments carrying the wild-type gene(s) in R. loti NZP2037 as Tn5 is 5.7 kb in size (Jorgensen et al., 1979).

[c] Determined by Southern hybridisation of [^{32}P]-labelled pACYC184 (Chang and Cohen, 1978) with EcoRI digests of total DNA.

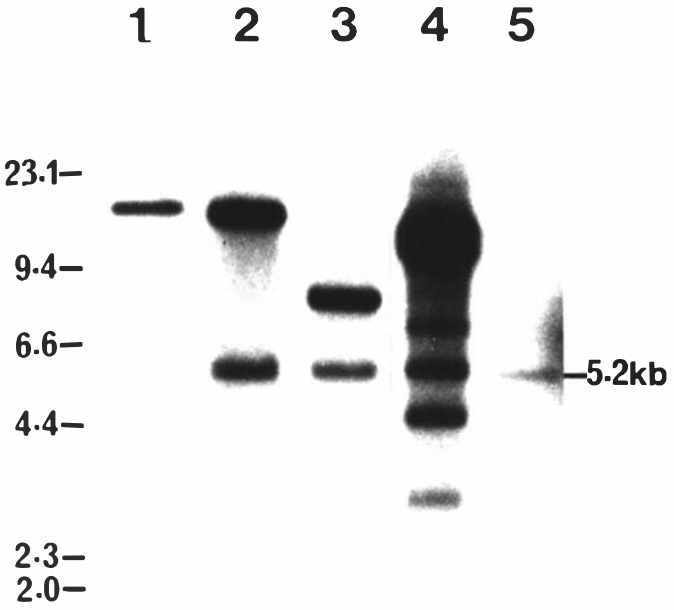
[d] Classified according to a phenotypic code devised by Vincent (1980).

Hac = hair curling; Noi = nodule initiation (tumour-like structures);

Bar = bacterial release; Cof = complementary function

N.D. = not determined

Figure 4.4 Hybridisation of Tn5 and *nif* HDK sequences to total DNA from *R. loti* Tn5-induced mutants. Autoradiograph showing hybridisation of [^{32}P]-labelled pKan2 and pSA30 probes with *Eco*RI digests of total DNA. Lane 1, PN234; Lane 2, PN233; Lane 3, PN235; Lane 4, PN240; Lane 5, NZP2037 (wild type). Size markers are in kilobases (kb).


1
2
3
4
5
23.1
9.4
6.6
4.4
2.3
2.0
5.2kb

The root hairs of the Lotus pedunculatus plants inoculated with PN233 were long, slender and straight (Fig. 4.5(a)) whereas plants inoculated with the wild-type strain NZP2037 had short root hairs that were curled or distorted (Fig. 4.5(b)).

4.3.5 Light and electron microscopic examination of nodules induced by the Fix$^-$ mutants

To further characterise the phenotypes of the various Tn5-induced Fix$^-$ mutants, root nodules or 'tumour-like' structures formed on Lotus pedunculatus were examined by light and electron microscopy.

4.3.5.1 Morphology of nodules induced by the wild-type R. loti strain NZP2037

Root nodules induced by NZP2037 on Lotus pedunculatus plants appeared 10 to 12 days after inoculation. In `transverse section, the newly formed nodules consisted of a spherical mass of meristematic cells, many of which contained rhizobia-filled infection threads (Fig. 4.6(c)). The rhizobia released from the infection threads into the host cell cytoplasm were enclosed within a peribacteroid membrane (Robertson et al., 1978; Fig 4.6b and d). The infected plant cells increased in size as they filled with membrane-enclosed bacteria and became irregularly shaped (Fig. 4.6b and d). In the fully developed nodule the infected plant cells were surrounded externally by a cortex containing vascular traces (Fig. 4.6a). The rhizobia within the peribacteriod membrane had divided one or two times before enlarging into bacteriods (Fig. 4.6 d and e). The bacteroids generally lacked inclusions although small deposits characteristic of poly-β-hydroxybutyrate (PHB) were often observed (Fig. 4.6e).

Figure 4.5 Light micrographs of root hairs of Lotus pedunculatus plants. (a) Root hairs of a Lotus pedunculatus plant inoculated with the Nod^- mutant PN233. (b) Root hairs of a Lotus pedunculatus plant inoculated with the wild-type strain NZP2037. x 140.

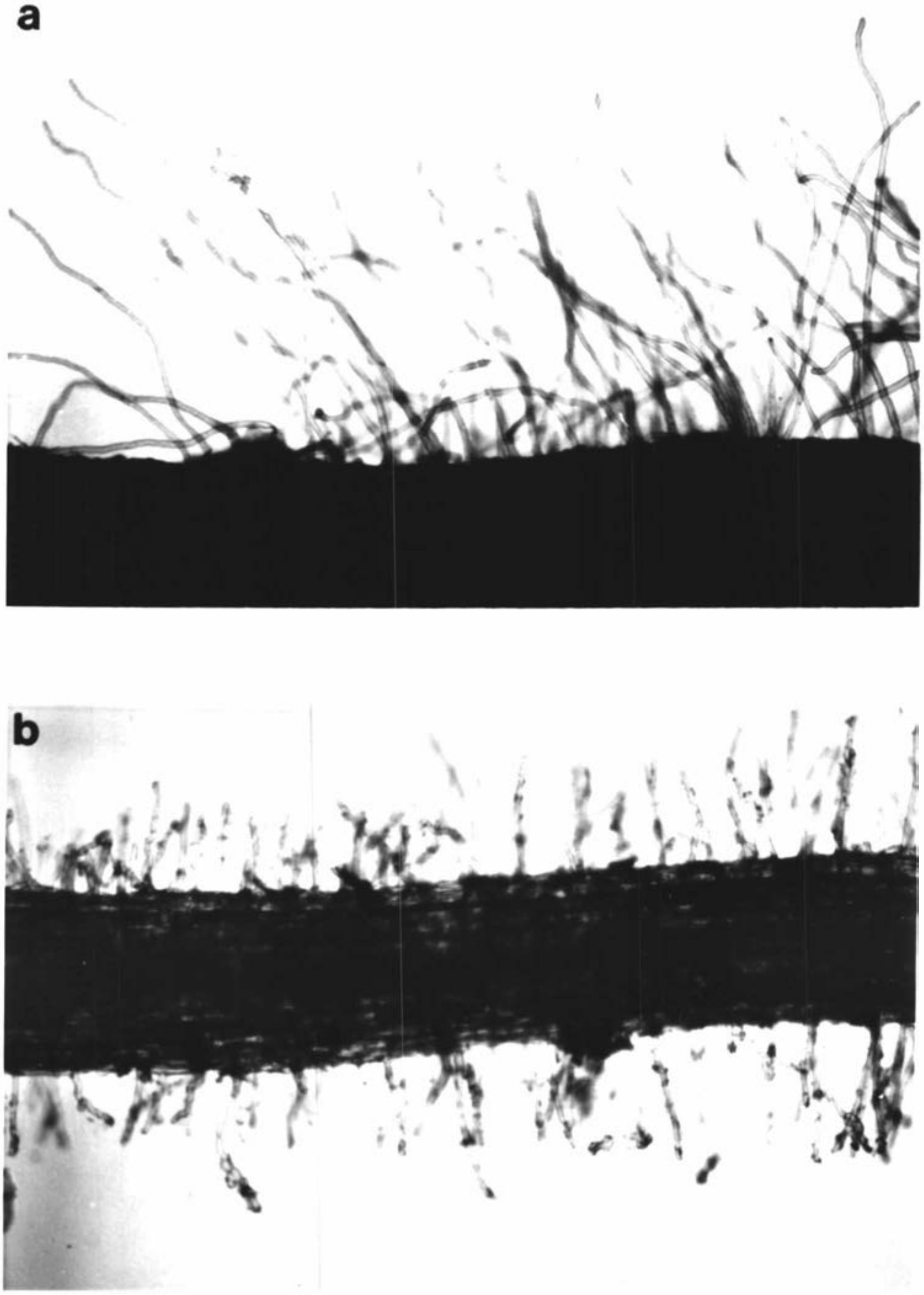

a
b

Figure 4.6 Light and electron micrographs showing the structure of root nodules formed by R. loti strain NZP2037 on L. pedunculatus. B = bacteroid, BZ = bacteroid zone, CT = cortex, CW = plant cell wall, F = flavolan, IT = infection thread, PBM = peribacteroid membrane, PBS = peribacteroid space, R = rhizobia, S = starch deposits, VT = vascular trace, PHB = poly-β-hydroxybutyrate.

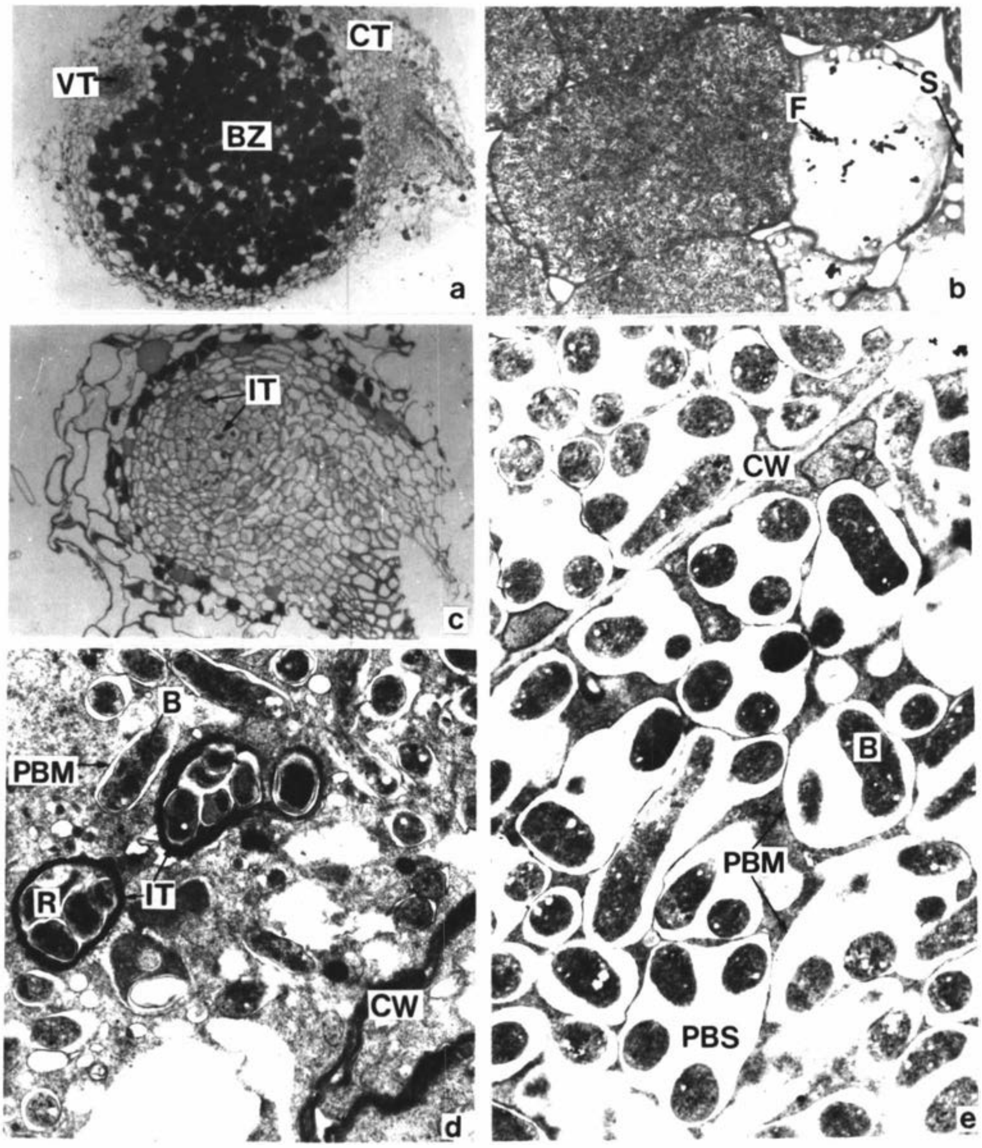
(a) Light micrograph of a median transverse section of a 28-day-old nodule showing a central bacteroid zone containing bacteroid-filled plant cells surrounded by a cortex which contains vascular traces. x 160.

(b) Light micrograph of a bacteroid-filled plant cell and an uninfected plant cell. Small deposits of condensed tannin (flavolan) and starch are visible in the uninfected plant cell. x 340.

(c) Light micrograph of a median transverse section of a young 14-day-old nodule showing a group of meristematic cells, some of which contain infection threads. x 160.

(d) Electron micrograph of part of a newly invaded plant cell in a 14-day-old nodule showing the enlarged and peribacteroid membrane enclosed bacteroids. x 13,850.

(e) Electron micrograph of a mature bacteroid-containing cell showing swollen bacteroids enclosed within the peribacteroid membrane. The space between the bacteroids and the peribacteroid membrane is the peribacteroid space. x 12,600.


CT
VT
BZ
a
F
S
b
IT
c
B
PBM
R
IT
CW
d
CW
B
PBM
PBS
e

4.3.5.2 Morphology of tumour-like nodules

Several Nod$^+$Fix$^-$ Tn5 mutants (PN234, PN236, PN237, PN238 and PN244) formed small structures resembling small nodules or large irregularly shaped tumours. In transverse section these structures contained a mass of meristematic cells surrounded by cortical cells, many of which contained flavolans (Pankhurst *et al.*, 1979). There was no evidence of vascular differentiation within the structure and no evidence of rhizobia or infection threads within the cells (Fig. 4.7a). Rhizobia were readily seen among the root hairs, on the surface of the epidermal cells, and within the intercellular spaces of the epidermal and outer cortical cells (Fig. 4.7b), but no rhizobia were present within the host plant cells. When located within intercellular spaces the rhizobia were surrounded by copious deposits of an electron-dense material that was probably polysaccharide (Fig. 4.7c). In mutants PN234 and PN238, evidence of infection thread initiation in root hair cells was occasionally observed (Fig. 4.7d). It appears that Tn5 insertion in each of these strains has resulted in a blockage in nodule initiation, and so these mutants were designated as Noi mutants (Vincent, 1980).

4.3.5.3 Morphology of ineffective nodules

Mutants PN235, PN239 and PN246 formed nodules which did not fix nitrogen. Mutant PN239 formed spherical nodules of normal external morphology. The light micrographs showed that the nodule contained many enlarged plant cells, some of which contained infection threads, but there were no rhizobia released into plant cells (Fig. 4.8a and b). Some of the enlarged threads were intercellular while others appeared to be intracellular and to have ramified between many cells (Fig. 4.8b and c). Extensive multiplication within the greatly enlarged infection threads was evident and the rhizobia were tightly packed within the enlarged

Figure 4.7 Light and electron micrographs showing the morphology of the tumour-like nodules formed by the Noi mutants PN234, PN236, PN237, PN238 and PN244 on L. pedunculatus. CT = cortex, F = flavolan, IT = infection thread, MC = meristematic cells, R = rhizobia, RH = root hair.

(a) Light micrograph of a median longitudinal section of a 28-day-old tumour-like nodule formed by PN234 showing a zone of meristematic cells surrounded by cortical cells, many of which contain flavolans. x 250.

(b) Light micrograph of a group of outer cortical cells and root hair cells from the tumour-like nodule shown in (a). Several of the cortical cells contain flavolans. Many rhizobia (see arrows) can be seen among the root hair cells. The development of an infection thread between two cells is visible. x 1,620.

(c) Electron micrograph showing a large mass of rhizobia grouped on the surface of several epidermal cells (28-day-old tumour-like nodule formed by PN235). The rhizobia are contained within a structure resembling a large infection thread. x 2,600.

(d) Electron micrograph of a median transverse section through the root hair cells showing evidence of infection thread formation (28-day-old tumour-like nodule formed by PN244). x 5,000.

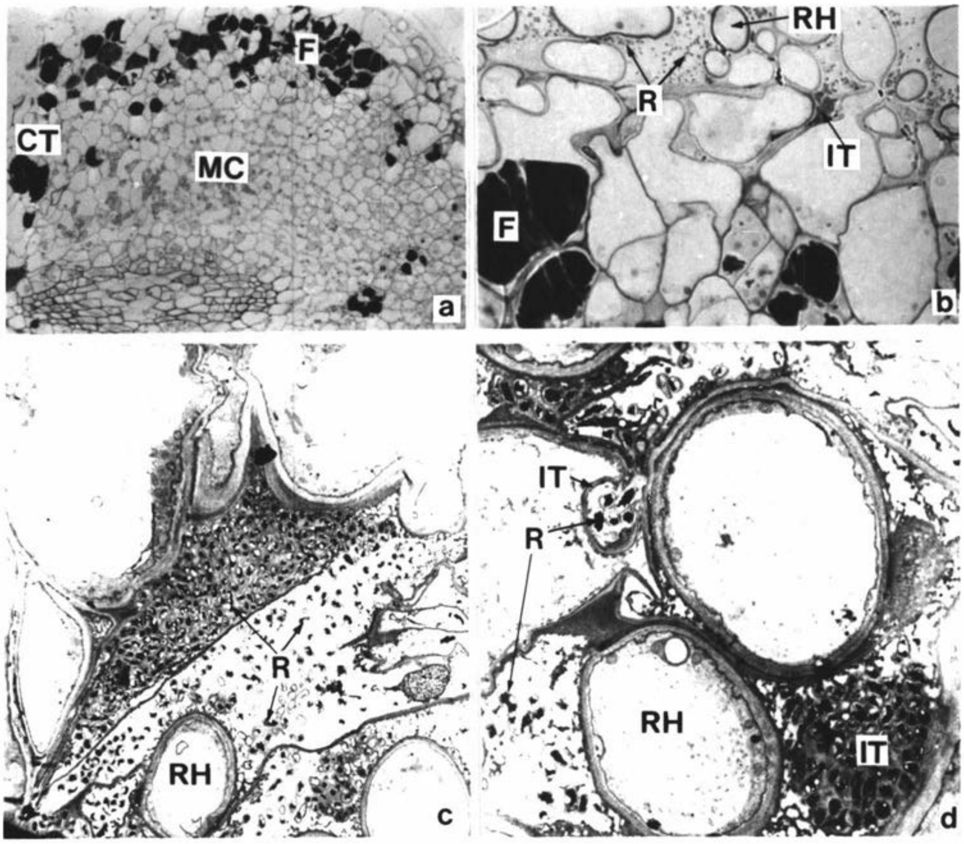

F
CT
MC
a
RH
R
IT
F
b
R
RH
c
IT
R
RH
IT
d

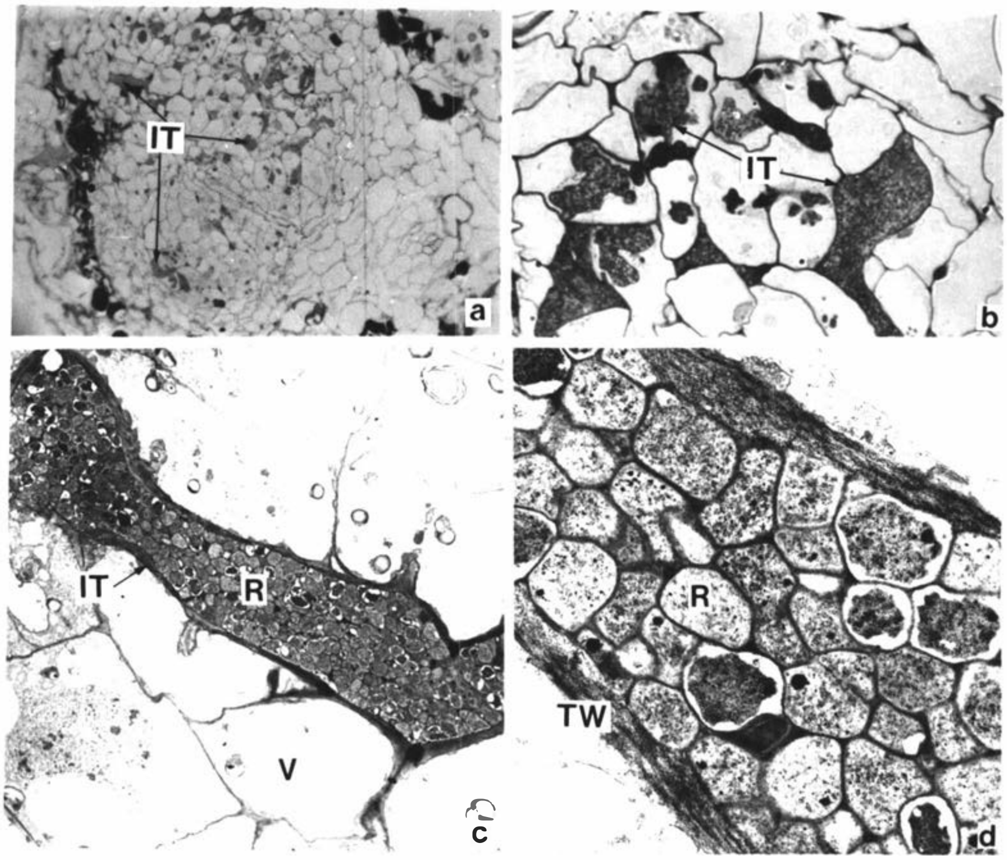
Figure 4.8 Light and electron micrographs showing the structure of the nodules formed by the Bar mutant PN239 on L. pedunculatus. IT = infection thread, R = rhizobia, TW = infection thread wall, V = vacuole.

(a) Light micrograph of a median transverse section through a 28-day-old nodule. The central zone of the nodule consists of many enlarged plant cells, some of which contain infection threads. x 250.

(b) Light micrograph of a group of plant cells from the nodule shown in (a). The cells contain one or several enlarged infection threads. Rhizobia have not been released from the infection threads into the plant cell cytoplasm. x 1,620.

(c) Electron micrograph showing an enlarged infection thread containing numerous rhizobia. The infection thread has traversed several plant cells (c.f. Fig 4.6d). x 3,700.

(d) Electron micrograph showing rhizobia embedded within the infection thread shown in (c). The rhizobia are tightly packed within the thread as shown by their compressed outline. x 17,500.


IT
a
IT
b
IT
R
V
c
R
TW
d

threads as seen by their compressed appearance and the lack of significant matrix materials between them (Fig. 4.8c and d). In contrast to the wild type nodule section there was little or no starch deposits in the infected plant cells. This mutant appeared to be blocked in bacterial release and hence was referred to as a Bar mutant (Vincent, 1980).

Two other mutants PN235 and PN236 formed ineffective nodules which contained plant cells filled with bacteriods (Fig. 4.9 and Fig. 4.10). Light and electron micrographs of a transverse section of nodules formed by PN235 showed that the infected plant cells appeared normal but there was extensive deposits of starch in plastids within the infected cells and particularly in the uninfected cells of the central zone and cells of the cortex (Fig. 4.9 a,b and c). The bacteroids within the infected cells contained many poly-β-hydroxybutyrate granules and the peribacteroid space was much smaller than in normal NZP2037 nodules (Fig. 4.9c and d; c.f. Fig. 4.6e) whereas a transverse section of a nodule formed by PN246 showed a different morphology. The plant cells of the nodule were larger than normal and contained many small vacuoles in addition to the more commonly observed central vacuole (Fig. 4.10a). The small vacuoles were usually located around the periphery of the infected cells (Fig. 4.10b). A common feature of these vacuoles was the presence of large quantities of plant cell membrane within them (Fig 4.10c and d). On numerous occasions this membrane material was seen to be part of the peribacteroid membrane enclosing a bacteroid bordering the vacuole (Fig. 4.10). The bacteroids contained significant amount of poly-β-hydroxybutyrate and the peribacteroid space was much reduced, but there was little or no accumulation of starch in plastids within either the infected or uninfected cells (Fig. 4.10b and c).

Figure 4.9 Light and electron micrographs showing the structure of the nodules formed by the Cof mutant PN235 on *L. pedunculatus*. B = bacteroid, BZ = bacteroid zone, CT = cortex, CW = plant cell wall, M = mitochondrion, PBM = peribacteroid membrane, PHB = poly-β-hydroxy-butyrate, S = starch deposit, VS = vesicle.

(a) Light micrograph of a median transverse section of a 28-day-old nodule. The central zone of the nodule contain bacteroid-filled plant cells. Starch deposits in plastids are observed within the infected and uninfected cells of the central zone and within the cells of the nodule cortex. x 250.

(b) Light micrograph of a group of infected bacteroid-filled plant cells and several cortical cells showing prominent starch deposits. x 1,620.

(c) Electron micrograph of part of an infected plant cell showing bacteroids, mitochondria and starch deposits. In addition to the bacteroids enclosed within the peribacteroid membranes, the plant cell cytoplasm contains many small vesicles. x 5,000.

(d) Electron micrograph showing a group of bacteroids. Note the presence of many granules of poly-β-hydroxybutyrate within the bacteroids (c.f. Fig. 4.6e). The peribacteroid membrane is also very closely aligned to the bacteroid surface. x 26,200.

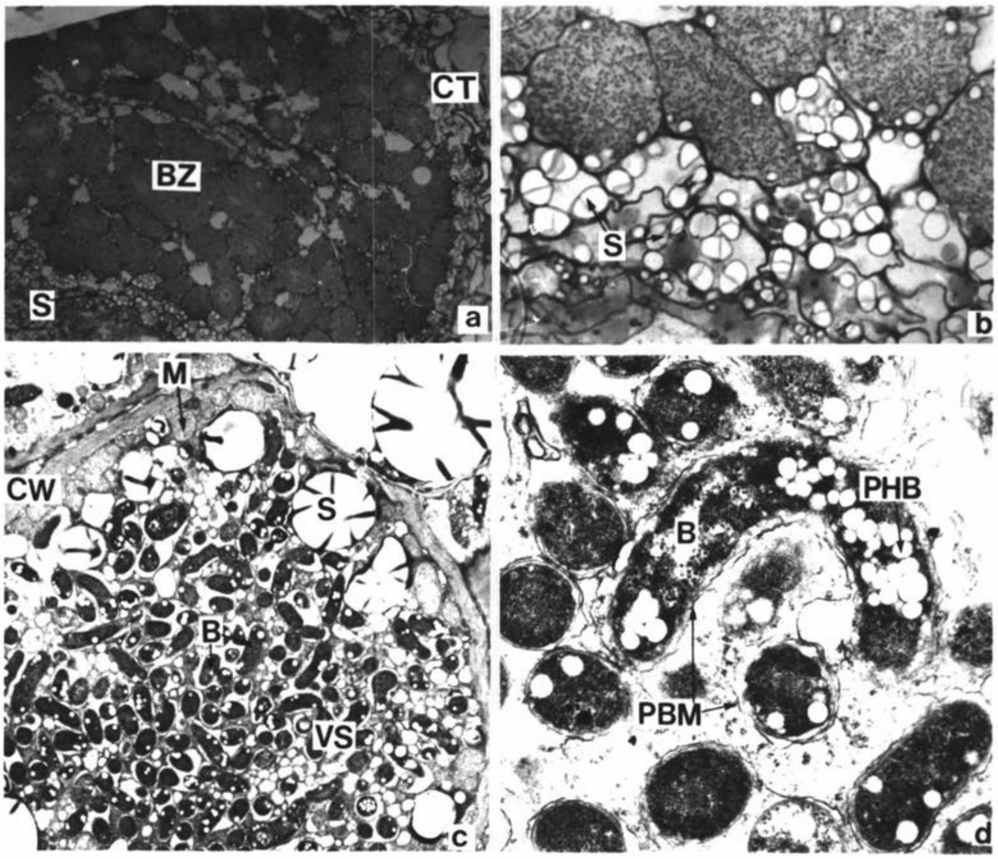

CT
BZ
S
a
S
b
M
CW
S
B
VS
c
B
PHB
PBM
d

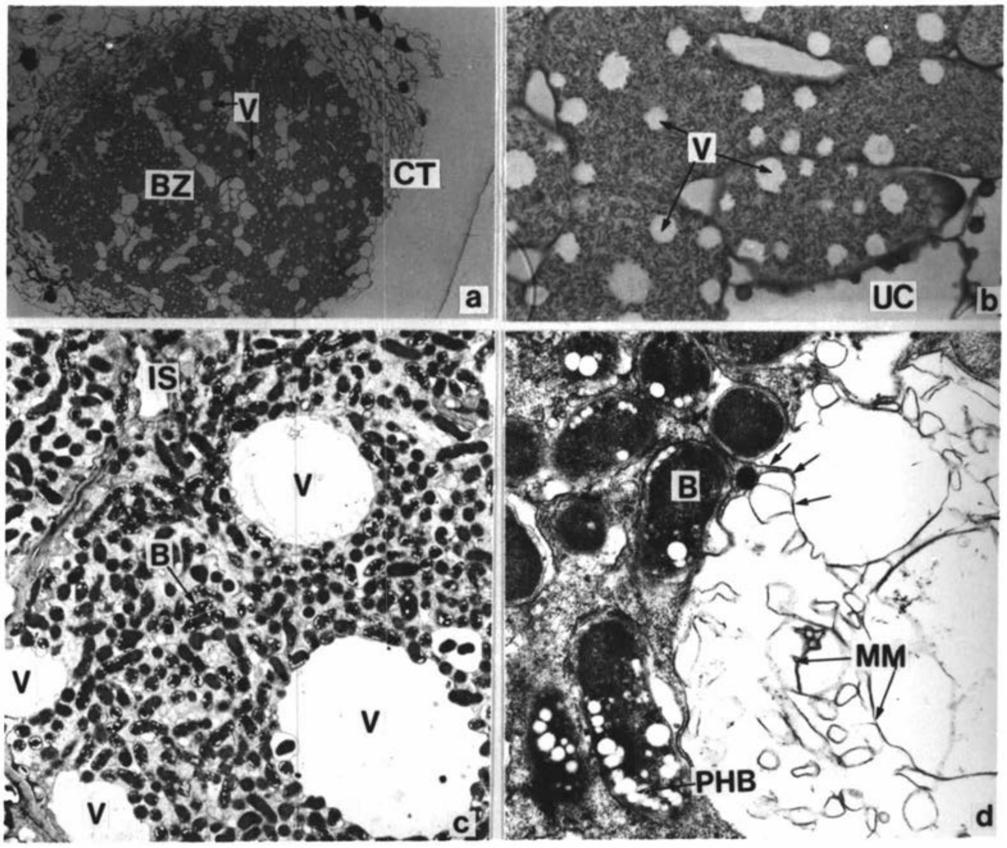
Figure 4.10 Light and electron micrographs showing the structure of nodules formed by the Cof mutant, PN246, on L. pedunculatus. B = bacteroid, BZ = bacteroid zone, CT = cortex, IS = intercellular space, MM = membrane material, PHB = poly-β-hydroxybutyrate, UC = uninfected cell, V = vacuole.

(a) Light micrograph of median transverse section of a 28-day-old nodule. The central zone of the nodule contains large bacteroid-filled plant cells with many vacuoles. x 200.

(b) Light micrograph of a group of bacteroid-filled plant cells showing the presence of numerous small vacuoles. The vacuoles tend to be located around the periphery of the bacteroid-filled cell. x 1,620.

(c) Electron micrograph of part of an infected plant cell showing several small vacuoles. x 3,700.

(d) Electron micrograph showing bacteroids bordering one of the vacuoles in an infected plant cell. This vacuole contains large quantities of membraneous material, presumably of plant origin. Part of this membrane seems to be contiguous with the peribacteroid membrane surrounding two of the bacteroids shown (see arrows). Note the presence of many large granules of poly-β-hydroxybutyrate in the bacteroids. x 26,200.


V
BZ
CT
a
V
UC
b
IS
V
Bl
V
V
V
c
B
MM
PHB
d

It is evident that the Tn5 insertions in these two mutants have blocked genes involved in late nodule functions. The EcoRI fragment carrying Tn5 in each of these mutants is different to that which hybridises with a probe for nif HDK (section 4.3.3) suggesting that the genes blocked in these 2 mutants are not the nif structural genes but perhaps genes involved in other processes essential for bacteroid function and maintenance. Such mutants are described as blocked in complementary functions (Cof) (Vincent, 1980).

4.4 DISCUSSION

By using the broad host range mobilizable vector plasmid pSUP1011 (Simon et al., 1984), the transposon Tn5 was successfully introduced into the genome of Rhizobium loti NZP2037 at a high frequency. Thirteen Tn5-induced symbiotic mutants of NZP2037 were isolated, one that was completely defective in nodulation (Nod^-) and 12 others that induced nodules or tumour-like structures that were ineffective (Nod^+Fix^-). Analysis of the plasmid profiles, phage susceptibility tests and EcoRI enzyme digest patterns of total DNA confirmed that these mutant strains were all derivatives of NZP2037.

All presumptive Tn5 mutants were shown to contain Tn5 sequences. As shown in Table 4.2, 10 of the 13 mutants showed a single hybridisation band with the Tn5 probe and each carried Tn5 on a different size EcoRI fragment, except PN236 and PN237 which had Tn5 inserted into EcoRI fragments of identical sizes. These results suggest that Tn5 inserts into the R. loti genome in a singular and random manner. Random insertion of Tn5 into the genome of R. leguminosarum (Buchanan-Wollaston et al., 1980), R. meliloti (Meade et al., 1982) and R. trifolii (Scott et al., 1982) has also been reported. The multiple bands that hybridised with pKan2 in mutants PN240, PN241 and PN242 were due to the presence of pACYC184 vector DNA sequences, suggesting that pSUP1011 has the capacity to cointegrate into the genome of *R. loti* NZP2037. This was confirmed by hybridisation experiments using [^{32}P]-labelled pACYC184 as a DNA probe to *Eco*RI digests of total DNA isolated from the mutants (Table 4.2). None of the 12 Nod$^+$Fix$^-$ mutants carried Tn5 in the *nif* structural gene region as the *Eco*RI fragment carrying the Tn5 in each of the mutants analysed was different to that which hybridised to the *nif* HDK probe (Fig. 4.4).

The mutants carrying just Tn5 sequences were characterised further, using the system developed by Vincent (1980), to define the phenotype of the various blockage in *Lotus pedunculatus* nodule development. Light microscopic examination of the root hairs of *Lotus pedunculatus* seedlings inoculated with the Nod$^-$ mutant, strain PN233, showed that this mutant was blocked at the root hair curling (Hac) stage, a very early step in nodule formation (section 4.3.4). Hac mutants generated by transposon mutagenesis have also been isolated from *R. meliloti* (Meade *et al.*, 1982; Hirsch *et al.*, 1982; Forrai *et al.*, 1983; Dusha *et al.*, 1983), *R. leguminosarum* (Buchanan-Wollaston *et al.*, 1980; Ma *et al.*, 1982) and *R. trifolii* (Rolfe *et al.*, 1981; Djordjevic *et al.*, 1983). In each of these cases, there was a low recovery of Nod$^-$ mutants, and this has also been observed in this work where one Nod$^-$ mutant was isolated from the 1060 transconjagants screened. There are two possible explanations to account for such a low frequency of recovery of Nod$^-$ mutants. First, there is always a certain frequency of reversion from Nod$^-$ to Nod$^+$ and such revertants are strongly selected for by plants. Secondly, there is perhaps a relatively low number of bacterial genes involved specifically in the nodulation process, resulting in a low frequency of mutation in the *nod* region (Downie *et al.*, 1983a). Repeated plant tests with the Hac mutant PN233 showed that this mutant was very stable. Although some reports (Raleigh and Signer, 1982; Stacey *et al.*, 1982) have shown an alteration in phage susceptibility associated with the loss of nodulation ability, no such differences were observed here in the phage susceptibility of the wild type and Nod$^-$ mutant.

Light and electron microscopic studies of the nodule structures induced by the 9 Nod$^+$Fix$^-$ mutants showed that these strains could be divided into 3 classes. The first class consisting of strains PN234, PN236, PN237, PN238 and PN244, all formed tumour-like structures on the roots of *Lotus pedunculatus* and were classified to be of the Noi (nodule initiation) phenotype (Vincent, 1980). It is interesting to note that the Tn5-containing *Eco*RI fragment in each of these mutants (except PN236 and PN237) is different (Table 4.2), but all resulted in a Noi phenotype. This implies that there are at least four different loci in the *R. loti* genome that can give rise to a Noi phenotype. Widely scattered chromosomal *fix* alleles have been identified in *R. meliloti* (Forrai *et al.*, 1983), although the resulting nodule phenotypes were not described. Recently, Noel *et al.* (1984) also reported symbiotic specific chromosomal mutations in *R. phaseoli*, resulting in at least two discernible phenotypic classes. The possibility that some of the *R. loti* Noi mutants described above are blocked in an important metabolic pathway cannot be totally excluded, despite the fact that these mutants were originally selected on a defined medium to exclude symbiotically defective auxotrophic mutants.

The tumour-like structures induced by these *R. loti* Noi mutants are similar to those formed on *Lotus pedunculatus* plants by *R. loti* strain NZP2213, a strain which nodulates *Lotus tenuis* and *Lotus corniculatus* effectively (Pankhurst *et al.*, 1979). The 'reactive' Nod^- mutants of *R. meliloti* described by Hirsch *et al.* (1982) also appeared to be blocked at this early stage of nodule formation.

The second class of Nod^+Fix^- mutant identified contained a single mutant strain PN239 which was designated as a Bar (bacterial release) mutant. Nodules formed by this mutant contained enlarged infection threads with more bacteroids than found in a normal nodule (Fig. 4.8c and d) and no evidence was found for the release of these bacteria into the plant cells. In contrast to the normal nodule section (Fig 4.6) there was little starch deposits in the infected plant cells of nodules induced by PN239, this could suggest that the plant may not mobilise carbon substrates to the nodule until after the rhizobia are released from the infection thread into the plant cell cytoplasm. Although auxotrophic or antibiotic resistant mutants with a Bar phenotype have been well documented (see review by Vicent, 1980), no transposon induced Bar mutants have been reported to date. Ma *et al.* (1982) isolated several Tn5-induced *R. leguminosarum* Nod^+Fix^- mutants which formed nodules with a partial block at the bacterial release stage, and found that the mutant alleles mapped at several different loci on the Sym plasmid, pRL1JI. However, it is unknown at this stage the number of alleles in the *R. loti* genome involved in this significant stage of nodule development.

Mutants PN235 and PN246 with a Cof phenotype (Fig. 4.9 and Fig. 4.10) formed the third class of Nod^+Fix^- mutants identified. Nodules induced by PN235 showed an accumulation of starch in the plant cells and poly-β-hydroxybutyrate in the bacteroids (Fig 4.9b and d) suggesting that carbon substrates were not being mobilised to the bacteroids. This is consistent with the lack of nitrogen fixation in the nodules induced by this mutant. The morphology of the nodules induced by PN246 suggested that the block in nodule function was different to that for PN235. There was little accumulation of starch within either the infected or uninfected plant cells but there were numerous poly-β-hydroxybutyrate granules in the bacteroids and the peribacteroid space was much reduced (Fig. 4.10b and c). One significant feature of the nodule was the presence of a large number of small vacuoles in the infected plant cells, indicating a defect in the normal transport process of substances into or out of the cells.

4.5 SUMMARY

Using pSUP1011 as a vector for introducing Tn5 into R. loti NZP2037, thirteen symbiotic mutants were isolated. One that was completely defective in nodulation (Nod^-) and 12 others that induced ineffective nodules or tumour-like structures on Lotus pedunculatus. All mutants were shown to contain Tn5 sequences and each carried Tn5 on a different sized EcoRI fragment, except 2 mutants which had Tn5 inserted into EcoRI fragments of identical sizes. It was found that the Nod^- mutant was blocked at the root hair curling (Hac) stage, an early step in nodule formation. Nine Fix^- mutants that were characterised further by light and electron microscopy studies could be divided into three phenotypic classes. The first class consisted of 5 Fix^- mutants that formed tumour-like structures on Lotus pedunculatus and were classified as blocked in nodule initiation (Noi). The second class consisted of only 1 mutant which was defective in bacterial release (Bar) from the infection thread. The 2 mutants in the third class formed nodules containing bacteroid filled plant cells but were unable to fix N_2 and therefore were classified to be of the Cof phenotype.

CHAPTER FIVE

MOLECULAR CLONING AND GENETICAL ANALYSIS OF NODULATION GENES IN RHIZOBIUM LOTI

5.1 INTRODUCTION

To date *nod* genes have been isolated from *R. trifolii* (Schofield *et al.*, 1983; Scott *et al.*, 1984), *R. meliloti* (Long *et al.*, 1982; Kondorosi *et al.*, 1984) and *R. leguminosarum* (Downie *et al.*, 1983a). The main strategy used to isolate *nod* genes in *Rhizobium* has involved the isolation of a Tn5-induced Nod$^-$ mutant of the strain followed by either subcloning of the mutagenised DNA fragment to obtain an appropriate DNA probe or to complement the mutant with wild type sequences cloned on a broad host range plasmid. In cases like *R. leguminosarum* (Downie *et al.*, 1983a) and *R. trifolii* (Schofield *et al.*, 1983), the Tn5::*nod* region from the Tn5-induced Nod$^-$ mutant was cloned and used as a hybridisation probe to identify wild type sequences from a wild type genomic library. Alternatively, *nod* genes from *R. meliloti* have been isolated by direct 'in planta' complementation of a Nod$^-$ mutant using a *R. meliloti* genomic library constructed in the broad host range cosmid pLAFR1 (Long *et al.*, 1982).

Plasmid pLAFR1 is one of the best vectors available for cloning *Rhizobium* DNA. This vector (21.6kb) carries λ cos DNA (1.6kb) cloned into the unique *Bgl* II site of the broad host range cloning vector pRK290 (Ditta *et al.*, 1980). It encodes tetracycline resistance and contains a unique *Eco*RI site (Friedman *et al.*, 1982). It is a useful vector for the construction of gene libraries because the RK2 replicon enables recombinant plasmids to be mobilized into and stably maintained within *Rhizobium* cells, and so direct complementation of mutants is possible. Mobilization of pLAFR1 (pRK290) requires the presence of the helper plasmid pRK2013 which contains the RK2 transfer genes and encodes neomycin resistance (Figurski and Helinski, 1979). The pLAFR1/pRK2013 binary plasmid system has been used successfully to shuttle rhizobial genes between *E. coli* and *Rhizobium* species in genetic studies (Long *et al.*, 1982; Downie *et al.*, 1983a; Ronson *et al.*, 1984).

Physical and genetical studies have demonstrated that *nod* and *nif* genes are closely linked in *R. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Long *et al.*, 1982; Kondorosi *et al.*, 1984), *R. leguminosarum* (Buchanan-Wollaston *et al.*, 1980; Hirsch *et al.*, 1980; Downie *et al.*, 1983b), and *R. trifolii* (Schofield *et al.*, 1983; Scott *et al.*, 1984). The *nod* gene regions in these *Rhizobium* species are confined to a region of DNA less than 10kb. Comparative physical and genetical studies of *nod* regions from different *Rhizobium* strains have shown that these species share some structural and functional conserved *nod* gene sequences that are involved in root hair curling (Hac) (Rolfe *et al.*, 1984). Kondorosi *et al.* (1984) reported the isolation of two different *nod* gene regions in *R. meliloti*. One of them was confined to a region of about 3kb of DNA and carried genes involved in root hair curling. The genes in this region have been called 'common' *nod* genes as they show strong conservation with other *Rhizobium* species. Recently these 'common' *nod* genes have also been identified in *R. trifolii, R. leguminosarum* and in some strains of *Bradyrhizobium* species, and have recently been named *nod*ABC (K.F. Scott, personal communication).

This chapter describes the isolation of a *nod* gene region from *R. loti* strain NZP2037 by an experimental strategy which first involved the isolation of a Nod$^-$ mutant by random Tn5 mutagenesis (Chapter 4). This was followed by the cloning of the mutated gene sequence which was then used to probe a pLAFR1-NZP2037 gene library for the wild type sequences. Comparative physical and genetical studies of the cloned nod gene region among the Lotus rhizobia and strains from other Rhizobium species will also be described.

5.2 METHODS AND MATERIALS

5.2.1 Bacterial strains and plasmids

These are described in Table 5.1

5.2.2 Preparation of culture media

5.2.2.1 Liquid media

Tryptone yeast extract medium (TY; see section 2.2.2.1)

Luria broth (LB; see section 3.2.2.1)

S20 medium (see section 4.2.2.1)

LM medium. Composition per litre: sodium chloride, 5 g, tryptone, 10 g; yeast extract, 5 g; maltose, 2 g; 1 M-$MgCl_2$, 10 ml. pH 7.2.

5.2.2.2 Solid media (see section 2.2.2.2)

5.2.3 Maintenance of cultures (see section 4.2.3)

5.2.4 Growth of bacteria (see section 4.2.4)

5.2.5 Isolation of genomic DNA (see section 3.2.14)

5.2.6 Isolation of plasmid DNA

5.2.6.1 Cleared lysate method (see section 4.2.9)

5.2.6.2 Alkaline lysis method

The method of Ish-Horowitz and Burke (1981) was scaled up for the preparation of the pLAFR1 recombinant plasmids from E. coli.

Table 5.1 *Bacterial strains and plasmids*

Strain	Relevant characteristics	Source or reference
Rhizobium loti		
NZP2037	Nod$^+$Fix$^+$ (*Lotus pedunculatus*, *Lotus tenuis*)	DSIR culture collection, N.Z.
NZP2213	Nod$^+$Fix$^+$ (*Lotus tenuis*) Nod$^+$Fix$^-$ (*Lotus pedunculatus*)	DSIR culture collection, N.Z.
PN184	NZP2037 *str*-1, Nod$^+$Fix$^+$	This study, spontaneous mutant
PN233	PN184 *str*-1, Nod$^-$	This study, Tn5-induced mutant
Bradyrhizobium spp. (*Lotus*)		
NZP2309 (CC814s)	Nod$^+$Fix$^+$ (*Lotus pedunculatus*) Nod$^+$Fix$^-$ (*Lotus tenuis*) A slow-growing *Lotus* strain	DSIR culture collection, N.Z.
Rhizobium trifolii		
NZP561	Nod$^+$Fix$^+$	DSIR culture collection, N.Z.
PN100	Nod$^+$Fix$^+$ *str*-1 *rif*-1	Scott and Ronson (1982)
PN104	Nod$^-$ *str*-1 *rif*-1	Scott and Ronson (1982)
Rhizobium meliloti		
NZP4009 (SU47)	Nod$^+$Fix$^+$	DSIR culture collection, N.Z.
Escherichia coli		
HB101	F$^-$ *pro* *leu* *thi* *lacY* Str^R r_K^- m_K^- Endo I$^-$ *recA*$^-$	Boyer and Roulland-Dussoix (1969)
PN226	HB101/pPN301	This study
PN231	HB101/pPN305	This study
PN232	HB101/pPN306	This study
PN200	HB101/pPN1	Ronson and Scott (1983)
PN250	HB101/pPN20	D.B. Scott, ABD, DSIR, N.Z.

Table 5.1 continued

Strain	Relevant characteristics	Source or reference
RRI/pRt572		Schofield *et al*. (1983)
PN382	HB101/pPN329	This study
Plasmids		
pBR328	Ap^R Tc^R Cm^R	Bolivar *et al*. (1977)
pRK290	IncP rep_{RK2} Tc^R	Ditta *et al*. (1980)
pRK2013	rep_{ColE1} Nm^R	Ditta *et al*. (1980)
pLAFR1	Derivative of pRK290, containing 1.6 kb λ cos DNA cloned into *Bgl* II site. Tc^R	Friedman *et al*. (1982)
pPN301	pBR328 clone containing 12.8 kb Tn5::*nod* region of NZP2037	This study
pPN305	pLAFR1 cosmid containing *nod* DNA of NZP2037	This study
pPN306	pLAFR1 cosmid containing *nod* DNA of NZP2037	This study
pRtr514a	symbiotic plasmid from *R. trifolii* NZP514	Scott and Ronson (1982)
R68.45	IncP $Km^R Tc^R Cb^R$	Haas and Holloway (1976)
pPN1	pRtr514a::R68.45	Ronson and Scott (1983)
pPN20	pLAFR1 clone containing 11.7 kb *EcoRI* *nod* DNA fragment from pRtr514a	D.B. Scott, ABD DSIR, N.Z.
pRt572	pBR328 clone containing 7.2 kb *EcoRI* *nod* DNA from *R. trifolii* strain ANU843	Schofield *et al*. (1983)
pPN329	pLAFR1 clone containing *nod* DNA of NZP2309	This study

Materials

(1) Solution I: 5 mM-glucose; 25 mM-Tris/HCl pH 8.0; 10 mM-Na_2 EDTA. (2) Solution II: 0.2 M-sodium hydroxide, 1% (w/v) SDS. (3) Solution III: 5 M-potassium acetate pH 4.8. (4) Lysozyme. (5) Isopropanol. (6) 70% ethanol. (7) TE 10/1 buffer pH 8.0: 10 mM-Tris/HCl; 1 mM-Na_2 EDTA. (8) Caesium chloride (CsCl). (9) Ethidium bromide (10 mg ml^{-1}).

Method

E. coli cells (1 l) grown to late exponential phase were harvested by centrifugation (10,000 g, 10 min, 4°C) and resuspended in 10 ml of solution I. 50 mg of lysozyme was added and the mixture was incubated at room temperature for 5 min. Solution II (20 ml) was added, mixed by inversion and incubated on ice for 5 min. Solution III (15 ml) was added and the mixture was agitated briefly, incubated for 5 min at 4°C and centrifuged (3000 g, 5 min, 4°C). DNA was precipitated by adding 0.54 volume of isopropanol, incubated for 5 min at room temperature and centrifuged (3000 g, 5 min, 4°C). The pellet (DNA + RNA) was washed twice with 70% ethanol at room temperature and resuspended in TE 10/1 buffer. CsCl and ethidium bromide were added as described in section 3.2.13. The DNA-CsCl mixture was centrifuged (vertical rotor, Vi Type 65, 900,000 g, 4 h, 15°C) and the plasmid DNA removed and purified as previously described (see section 3.2.13). The purity and concentration of the DNA was determined spectrophotometrically as described (see section 2.2.5).

5.2.6.3 Rapid boiling method

The method of Holmes and Quigley (1981) was used for small scale preparation of plasmid DNA from E. coli.

Materials

(1) STET buffer contained 8% (w/v) sucrose; 5% (w/v) Triton X-100; 50 mM-Na_2 EDTA pH 8.0, 50 mM-Tris/HCl, pH 8.0. (2) Lysozyme (10 mg ml^{-1}) in 10 mM-Tris, pH 7.6. (3) Isopropanol. (4) RNAase (1 mg ml^{-1}), preboiled for 10 min.

Method

E. coli cells (1.5 ml) grown to late exponential phase were harvested in a microfuge tube, resuspended in 300 µl of STET buffer and 25 µl of lysozyme was added, boiled for 40s and immediately centrifuged (microcentrifuge, top speed, 10 min). About 350 µl of supernatant was collected and an equal volume of isopropanol was added, incubated at -20°C for 10 min and centrifuged (microcentrifuge, top speed, 5 min). The pellet (DNA + RNA) was washed with 95% ethanol, dried *in vacuo* and resuspended in 50 µl of sterile water. The RNA was removed by incubation at 37°C for 2 min with 2 µl of RNAase.

5.2.7 Electroelution of DNA from agarose

Preparative 1% seaplaque agarose (FMC marine colloids) gels were stained with ethidium bromide and DNA was visualised under long wavelength (350 nm) UV light. The required DNA band was cut out and transferred to a small dialysis sac filled with sterile 0.5x Tris-borate-EDTA buffer (TBE; see section 3.2.11). The sac was then submerged in 0.5x TBE buffer and the DNA electroeluted (120 V for 3 h). After electrophoresis, the current was reversed for 30 s then the contents of the sac were recovered and centrifuged (microcentrifuge, top speed, 2 min) to remove agarose fragments. DNA was concentrated by ethanol precipitation (see section 3.2.13), resuspended in water and used directly for cloning or preparation of a [^{32}P]-labelled probe.

5.2.8 DNA ligation

Materials

(1) *Hae* III buffer (x10) pH 7.6 (see section 3.2.15).
(2) 10 mM-ATP. (3) T_4 DNA ligase (New England Biolabs).

Method

Ligation mixtures (20 µl) containing 0.2-1.0 µg of vector DNA, 0.2-0.5 µg of insert DNA, 2 µl of *Hae* III buffer (x10) pH 7.6, 2 µl of 10 mM-ATP, 1 µl of ligase (400 units) and an appropriate volume of sterile water to make up the final volume, were incubated at 4°C for 16 h. An aliquot (1 µl) was checked on a 1% minigel (see section 3.2.17) before and after ligation. The ligated DNA was then used directly for transformation.

5.2.9 Transformation of *E. coli*

A modified procedure of Cohen *et al.* (1972) was used.

Materials

(1) 10 mM-sodium chloride. (2) 60 mM-$CaCl_2.2H_2O$.
(3) TEC buffer pH 8.0: 10 mM-Tris/HCl pH 8.0; 0.25 mM-Na_2EDTA pH 8.0; 30 mM-$CaCl_2.2H_2O$.

Method

A stationary phase culture of *E. coli* strain HB101 was diluted 1/50 into x ml of LB and incubated at 37°C. The cells (OD_{600} = 0.4) were harvested by centrifugation (3000 *g*, 5 min, 4°C). All subsequent steps were carried out at 4°C. The cells were washed once in 10 ml of sodium chloride solution (10 mM) resuspended in 10 ml of calcium chloride solution (60 mM) and incubated on ice for 20 min. These cells were then harvested and resuspended in x/100 ml of calcium chloride solution (60 mM) and used in transformation experiments. The transformation mixture containing 100 µl competent cells, 10 µl ligated DNA and 90 µl of TEC buffer was incubated at 4°C for 1 h, heat shocked at 42°C for 2 min, then diluted 1/5 in LB and incubated at 37°C for 2 h. The culture (0.1 ml) was plated on LB medium supplemented with the appropriate antibiotic and incubated at 37°C overnight.

5.2.10 Colony hybridisation

The method of Grunstein and Hogness (1975) was used.

Materials

These were described in section 3.2.18.

Method

About 300 colonies from the pLAFR1 cosmid gene bank were grown on LB medium supplementd with tetracycline (15 µg ml^{-1}) in a petri dish (90 mm diameter) at 37°C overnight. The colonies were transferred to a nitrocellulose filter (Schleicher and Schüll BA85) by placing it over the colonies on the agar to obtain a colony print. The plate was reincubated for 4 h at 37°C and stored at room temperature to serve as a reference plate. The filter and the bottom of the plate were marked in such a way that the positively hybridised colonies could be readily identified. The filter was wetted with a solution of 0.5 M-NaOH, 2.0 M-NaCl for 5 min to lyse the cells and denature the DNA, and neutralised by a 5 min treatment in 0.5 M-Tris pH 7.4, 2.0 M-NaCl. Care was taken to avoid mixing DNA from different colonies on the filter. The filter was washed in 2 changes of 2x SSC, air dried, and baked *in vacuo* at 80°C for 2 h. The prehybridisation and hybridisation were performed as described in section 3.2.19.

5.2.11 Preparation of $[^{32}P]$-labelled DNA probe (See section 3.2.18)

5.2.12 DNA transfer and hybridisation (See section 3.2.19).

5.2.13 Conjugation

Triparental crosses (Ditta *et al.*, 1980) were performed by mixing 10^9 cells from each of the three cultures involved on a TY plate as described in section 4.2.5. Cells were diluted, plated on S20 medium supplemented with neomycin (500 μg ml^{-1}) and tetacycline (2 μg ml^{-1}) and incubated at 28°C for 5 days.

5.2.14 Plant test (See section 3.2.6)

5.2.15 Isolation of bacteria from nodules (See section 3.2.7).

5.2.16 Light and electron microscopy of nodules (See section 4.2.17).

5.2.17 Construction of a pLAFR1 gene bank to Bradyrhizobium spp. (Lotus) strain NZP2309 (CC814s)

Materials

(1) SM buffer pH 7.5. Composition per litre: sodium chloride, 5.8 g; $MgSO_4.7H_2O$, 2.0g; 1 M-Tris/HCl pH 7.5, 50 ml; 2% gelatin, 5 ml. The remaining of the materials used have been described previously as indicated in the text.

Method

Total DNA from strain NZP2309 (CC814s) was isolated (section 3.2.14) and partially digested with *EcoRI* to an average size of 25kb, then separated by electrophoresis (1.5 V cm^{-1} for 6 h) in a horizontal 1.0% seaplaque agarose (marine colloids) gel. DNA in the size range of 15-30 kb was electroeluted from the agarose (section 5.2.7), extracted with phenol-chloroform (section 3.2.16) and concentrated by ethanol precipitation (section 3.2.13). Vector DNA, pLAFR1, (Friedman *et al.*, 1982) was isolated by the cleared lysate method (section 4.2.9) and cut with *Eco*RI enzyme (section 3.2.15). The electroeluted rhizobial DNA (5 µg) was mixed with *Eco*RI digested pLAFR1 (0.5 µg) and ligated overnight at 4°C in a 10 µl reaction mixture containing 6 mM-Tris pH 7.6, 10 mM-$MgCl_2$, 10 mM-β-mercaptoethanol, 1 mM-ATP and 400 units of T_4-DNA ligase.

DNA packaging was carried out by the method of Hohn (1979) using the packaging extracts prepared from the lysogenic strains BHB2688 and BHB2690 (prepared by C.E. Pankhurst). A 3 µl aliquot of ligated mixture was added to packaging extract (40 µl) immediately following thawing and mixed by stirring with a pipette tip and incubated at 37°C for 1 h. After incubation, 500 µl of SM buffer (prewarmed to 37°C) was added and 3 drops of chloroform were added as a preservative. *E. coli* HB101 cells grown overnight in 20 ml of SM broth were harvested by centrifugation (3000 *g*, 5 min, 4°C), resuspended in 10 ml of SM buffer at room temperature, and 4.5 ml of the cell suspension mixed with the 500 µl of DNA-packaging mixture prepared above. The mixture was incubated at 37°C for 45 min without shaking, centrifuged (3000 *g*, 5 min, room temperature), resuspended in 5 ml of LB and incubated at 37°C for 60 min with slow-shaking. The cells were harvested by centrifugation (3000 *g*, 5 min, room temperature), resuspended in 1 ml of SM buffer, and 200 µl aliquots were plated on LB plates containing 15 µg ml^{-1} of tetracycline. After overnight incubation at 37°C the Tc^R colonies were washed off with 50% glycerol and stored at -20°C.

To check the quality of the gene bank, recombinant plasmid DNA from 20 randomly selected Tc^R colonies were isolated by the rapid boiling method (section 5.2.6.3), restricted with EcoRI enzyme and separated on an agarose gel. The sizes of rhizobial DNA inserts were then determined as described (see section 4.2.16).

5.2.18 'In planta' complementation of the Rhizobium loti Nod$^-$ mutant

A gene bank of Bradyrhizobium spp. (Lotus) strain NZP2309 (CC814s) (see section 5.2.17) was crossed en masse with the Tn5-induced Nod$^-$ mutant of R. loti strain PN233, in a triparental cross using E. coli HB101/pRK2013 as a source of helper plasmid (see section 5.2.13). Batches of about 300 Tc^R transconjugants from the cross were used to inoculate Lotus pedunculatus seedlings (see section 3.2.6) and plants were examined for nodulation and growth six weeks after inoculation.

5.3 RESULTS

5.3.1 Cloning of Tn5::nod region of NZP2037

To clone the 12.8 kb EcoRI fragment that carried the Tn5 sequence in the Nod$^-$ mutant PN233, total DNA (20 μg) from the mutant was digested with EcoRI enzyme, fragments of 10-20 kb in size were electroeluted from seaplaque agarose (section 5.2.6), ligated to EcoRI digested pBR328 DNA, and transformed into E. coli HB101. The frequency of kanamycin resistant (Km^R) transformants obtained was approximately 1×10^{-8} per μg DNA. Sixteen Km^R transformants were grown up overnight and plasmid DNA isolated and digested with EcoRI enzyme. Each clone was shown to contain a 4.9 kb EcoRI fragment corresponding to pBR328 DNA and a 12.8 kb EcoRI fragment of insert DNA (e.g. Fig. 5.1). One such transformant strain, PN226 was kept and the recombinant plasmid in this strain was designated as pPN301.

Figure 5.1 Agarose gel of an EcoRI digest of pPN301.

Lane 1, Hind III restriction enzyme digest of λ DNA;

Lane 2, EcoRI restriction enzyme digest of pPN301 DNA.

Numbers indicate size (kb) of DNA fragments.


1
2
23.1
9.4
6.6
4.4
2.3
2.0
12.8
4.9

5.3.2 Isolation of wild type nodulation genes by colony hybridisation

To isolate the wild type *nod* genes of *R. loti* strain NZP2037, pPN301 (section 5.3.1) DNA was digested with *Eco*RI enzyme, separated on a 1% seaplaque agarose gel and the 12.8 kb *Eco*RI fragment was purified by electroelution (section 5.2.7). This *Eco*RI DNA fragment was labelled with [^{32}P] (section 3.2.18) and used as a probe to identify clones carrying the corresponding sequences in a pLAFR1 cosmid gene bank of NZP2037 (constructed by C.E. Pankhurst) using the technique of colony hybridisation (section 5.2.9). Of 600 colonies screened, three positive colonies were identified and designated PN230, PN231 and PN232. Cosmid DNA was isolated from each of these strains by the rapid boiling method (section 5.2.5) and digested with *Eco*RI enzyme. Analysis of these DNA digests showed that PN230 and PN231 carried identical cosmids with *Eco*RI fragments of 8.0 kb, 7.1 kb, 4.2 kb, 2.6 kb and 0.8 kb (Fig. 5.2a, lane 1) and were designated pPN304 and pPN305 respectively. Strain PN232 carried a cosmid pPN306 with *Eco*RI fragments of 8.0 kb, 7.5 kb, 7.1 kb, 4.2 kb and 2.6 kb (Fig. 5.2a, lane 3). When pPN305 and pPN306 DNA were digested with *Hind* III, pPN305 contained fragments of 30.3 kb and 14.0 kb (Fig 5.2a, lane 2); pPN306 contained fragments of 31.8 kb, 14.0 kb and 5.2 kb (Fig 5.2a, lane 4). When the *Eco*RI and *Hind* III digests of pPN305 (Fig 5.2a, lanes 1 and 2) and pPN306 (Fig. 5.2a, lanes 3 and 4) were blotted and hybridised with [^{32}P]-labelled 12.8 kb *Eco*RI fragment from pPN301 (section 5.2.1), the 7.1 kb *Eco*RI (Fig 5.2b, lanes 1 and 3) and the 14.0 kb *Hind* III (Fig 5.2b, lanes 2 and 4) fragments hybridised.

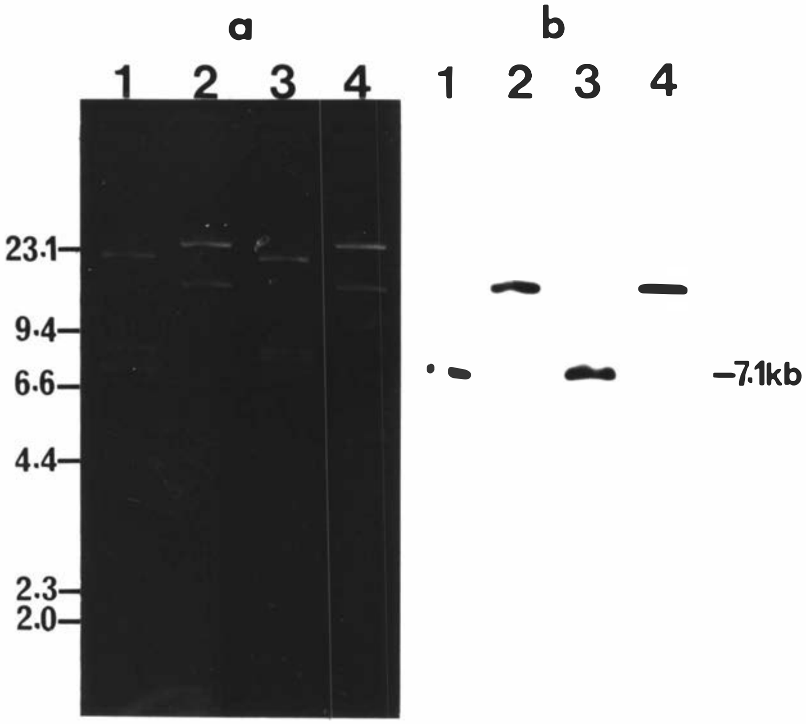
5.3.3 Construction of a physical map in the nodulation gene region of R. loti NZP2037

A *Hind* III and *Eco*RI restriction enzyme map of the *nod* gene region of *R. loti* NZP2037 was constructed using the two cosmids, pPN305 and

Figure 5.2 Hybridisation of Tn5::*nod* region from pPN301 to cosmids carrying *R.loti* wild type DNA sequences.

(a) Agarose gel of *Eco*RI (Lanes 1 and 3) and *Hind* III (Lanes 2 and 4) cosmid DNA digests. Lane 1, pPN305 DNA; Lane 2, pPN305 DNA; Lane 3, pPN306 DNA; Lane 4, pPN306 DNA.

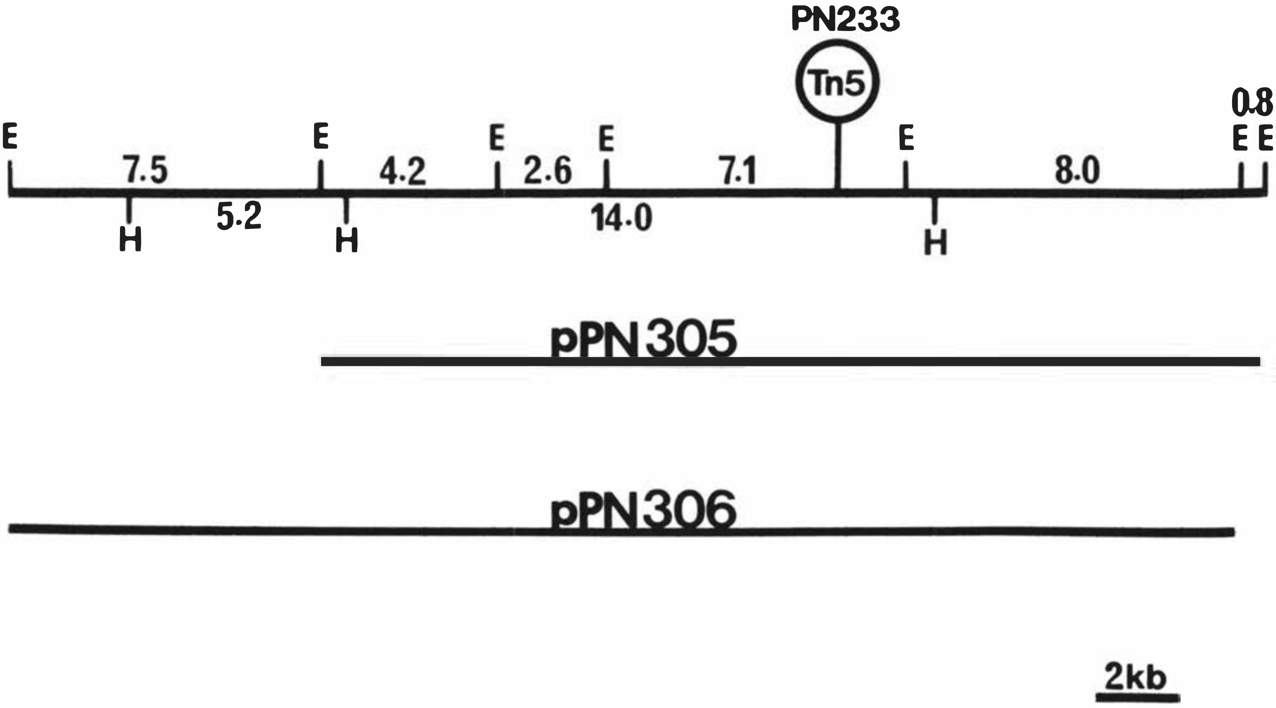
(b) Autoradiograph of the same gel hybridised with [^{32}P]-labelled 12.8 kb *Eco*RI fragment (Tn5::*nod* region) from pPN301. Size markers are in kilobases (kb).


a
b
1
2
3
4
1
2
3
4
23·1
9·4
6·6
4.4
2.3
2.0
7·1kb

pPN306, that were found to complement the Nod$^-$ mutant, PN233. Cosmid DNA was purified by the alkaline lysis method (section 5.2.6.2) and subjected to single and double digestions using *EcoRI* and *Hind* III enzymes. The DNA samples were separated by electrophoresis on a 0.7% agarose gel and the molecular weight (kb) of each fragment was estimated by reference to a *EcoRI* and *Hind* III digest of λ DNA.

The physical maps of the cosmids were constructed using both single and double restriction enzyme digest data and DNA hybridisation data by the following deductions: (1) The *EcoRI* digests of the cosmids pPN305 and pPN306 showed that the 8.0 kb, 7.1 kb, 4.2 kb and 2.6 kb fragments were common to both cosmids. A 0.8 kb fragment was unique to pPN305 (Fig. 5.2a, lane 1) and a 7.5 kb fragment was unique to pPN306 (Fig. 5.2a, lane 3). Therefore by definition, the unique fragments were end fragments on each cosmid. (2) The *Hind* III digests of pPN305 and pPN306 (Fig. 5.2a, lanes 2 and 4) showed that each yielded a big fragment of 30.3 kb and 31.8 kb respectively and a common 14 kb fragment. A 5.2 kb *Hind* III fragment was unique to pPN306 (Fig. 5.2a, lane 4). As there is no *Hind* III site in pLAFR1, the biggest fragment in each cosmid must contain pLAFR1 and the end fragments of the cloned DNA. When the 5.2 kb *Hind* III fragment from pPN306 was hybridised to a Southern blot of an *EcoRI* digest of pPN306, the 4.2 kb and 7.5 kb *EcoRI* fragments hybridised, thus demonstrating that these 2 fragments were contiguous. Using the 14 kb *Hind* III fragment as a hybridisation probe, hybridisation was observed to the 8.0 kb, 7.1 kb, 4.2 kb and 2.6 kb *EcoRI* fragments of pPN306. *EcoRI* and *Hind* III double digests of pPN305 and pPN306 showed that *Hind* III sites were present within the 8.0 kb and 4.2 kb *EcoRI* fragments demonstrating that the 4.2 kb and 8.0 kb *EcoRI* fragments were on either side of the 7.1 kb and the 2.6 kb *EcoRI*

Figure 5.3 Hind III (H) and EcoRI (E) restriction enzyme map of the nod gene region of *R. loti* NZP2037. Plasmid pPN305 and pPN306 were isolated from a NZP2037 pLAFR1 gene library by colony hybridisation with the Tn5::*nod* (12.8 kb) fragment from pPN301. They were found to complement the Tn5-induced NZP2037 Nod$^-$ mutant, strain PN233. The 7.1 kb *EcoRI* fragment in cosmids pPN305 and pPN306 was shown to contain the *nod* gene region of *R. loti* NZP2037. The site of Tn5 insertion in this fragment is also indicated.


PN233
Tn5
E
7.5
E
4.2
E
2.6
E
7.1
E
8.0
0.8
E E
H
5.2
H
14.0
H
pPN305
pPN306
2kb

fragments. (3) To locate the position of the 2.6 kb EcoRI fragment relative to the 7.1 kb EcoRI fragment, total DNA from the Tn5-induced Nod$^-$ mutant, strain PN233, was digested with Hind III enzyme, blotted and hybridised with [^{32}P]-labelled 4.2 kb and 2.6 kb EcoRI fragments separately. Hybridisation to a 13.0 kb Hind III fragment was observed in both cases. As Tn5 contains Hind III sites 1.2 kb away from the ends of the inverted repeats (Jorgensen *et al.*, 1979), this experiment enabled precise localisation of the Tn5 within the 7.1 kb EcoRI fragment and demonstrated that the 2.6 kb EcoRI fragment was located between the 4.2 kb and 7.1 kb fragments.

5.3.4 Complementation of Nod$^-$ mutant of R.loti strain NZP2037 by cosmids carrying presumptive wild type nod DNA of NZP2037

To test whether the cosmids isolated by colony hybridisation using the 12.8 kb EcoRI fragment from pPN301 would complement the *R. loti* NZP2037 Nod$^-$ mutant strain PN233, each cosmid was crossed into this strain in a triparental cross (section 5.2.9). Eight tetracycline resistant transconjugants from each cross were single colony purified and inoculated in duplicate onto *Lotus pedunculatus* plants (section 5.2.10). All the inoculated plants formed nodules 2 weeks after inoculation but were still Fix$^-$ after 6 weeks. However, 8 weeks after inoculation, three out of 16 plants tested in each cross showed a delayed nitrogen fixing response. The effective nodules formed on these plants were the same size and colour as the nodules formed by wild type strain NZP2037, but fewer in number (Fig. 5.4 a and b, plant 3). Bacteria isolated from these effective nodules were sensitive to tetracycline (2 μg ml^{-1}) and neomycin (500 μg ml^{-1}), and formed Nod$^+$Fix$^+$ nodules when retested on *Lotus pedunculatus* plants. The other 13 plants remained Nod$^+$Fix$^-$ and there were two types of nodules formed.

Figure 5.4 Complementation of the Tn5-induced Nod$^-$ mutant with R. loti NZP2037 wild type gene sequences.

(a) Gross morphology of Lotus pedunculatus plants 8 weeks after inoculation. Plant 1 was inoculated with the Tn5-induced Nod$^-$ mutant strain PN233 (Nod$^-$); Plant 2 was inoculated with a transconjugant of PN233 containing pPN305 (Nod$^+$Fix$^-$); Plant 3 was inoculated with a transconjugant of PN233 containing pPN305 (Nod$^+$Fix$^+$); Plant 4 was inoculated with R. loti strain NZP2037 (Nod$^+$Fix$^+$).

(b) A close-up view of the roots of the same Lotus pedunculatus plants.

a

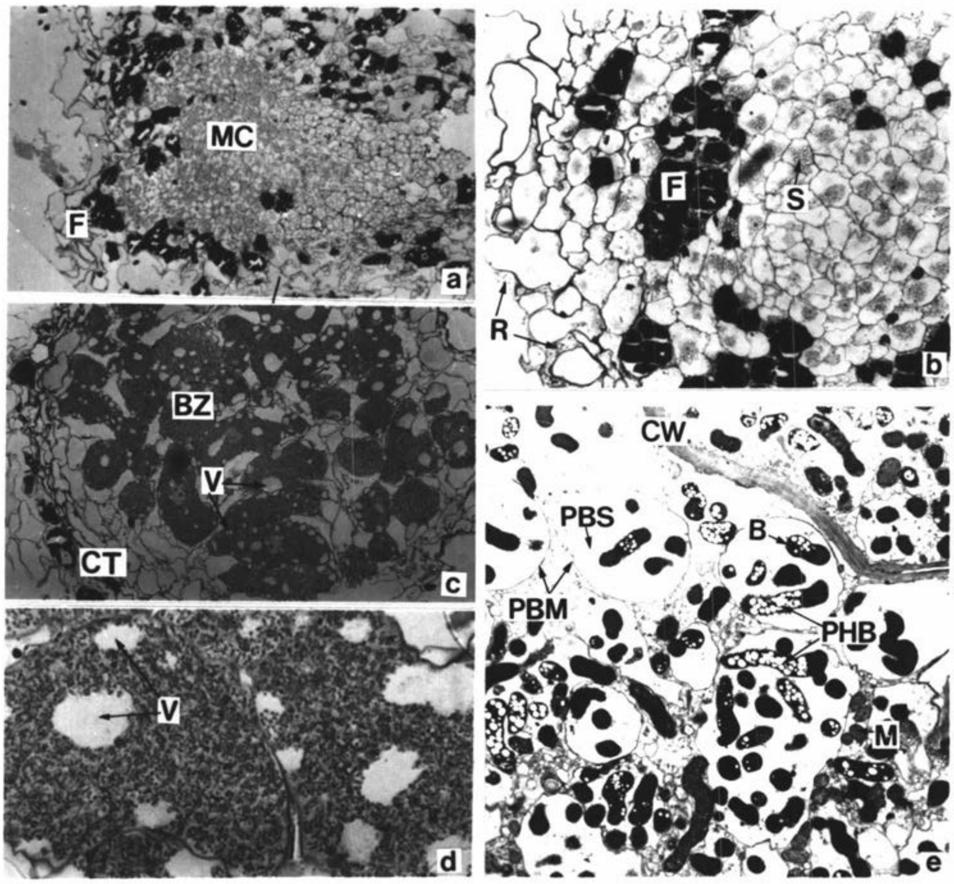
b

Approximately 40% were of a tumour-like structure and about 60% were normal looking ineffective nodules (Fig. 5.4a and b, plant 2). Bacteria isolated from these nodules were tetracycline and neomycin resistant.

5.3.5 Light and electron microscopy of nodules formed on Lotus pedunculatus by complemented derivatives of the Nod⁻ mutant strain PN233

Lotus pedunculatus plants inoculated with derivatives of the Nod⁻ mutant strain PN233 carrying either pPN305 or pPN306 formed 2 types of ineffective nodules (section 5.3.5). To determine the stage of development at which these nodules were blocked, the morphology of the nodules was examined by light and electron microscopy (section 4.2.17). The light micrographs of the cross-sections from the tumour-like structures showed that there was no evidence of rhizobial invasion (Fig. 5.5a). The cells contained numerous starch deposits and there were many flavolan-containing cortical cells (Fig. 5.5b). These features resemble those present in nodules of the Noi phenotype described in section 4.3.5.2. The morphology of the nodule cross-sections from the normal looking ineffective nodules contained abnormally large infected plant cells with numerous small vacuoles (Fig. 5.5c and d). In this respect the nodule resembles that formed by the Cof mutant PN246 (Fig. 4.10b). The bacteroids present in the infected plant cells of these nodules showed two irregular features. Firstly, while some bacteroids contained extensive poly-β-hydroxybutyrate deposits, others contained little or no poly-β-hydroxybutyrate (Fig. 5.5e). Secondly, the number of bacteroids per peribacteroid membrane and the volume of the peribacteroid space varied considerably (Fig. 5.5e).

Figure 5.5 Light and electron micrographs showing the structure of ineffective nodules and tumour-like nodules formed on Lotus pedunculatus by a derivative of the Nod$^-$ mutant PN233 carrying pPN305. B = bacteroid, BZ = bacteroid zone, CT = cortex, CW = plant cell wall, F = flavolan, M = mitochondrion, MC = meristematic cells, PBM = peribacteroid membrane, PBS = peribacteroid space, PHB = poly-β-hydroxybutyrate, R = rhizobia, S = starch deposits, V = vacuole. (a) Light micrograph of a median longitudinal section of a 28-day-old tumour-like nodule showing a distinctive mass of meristematic cells which contain extensive starch deposits. There is no evidence of rhizobial invasion into these cells. There are numerous flavolan-containing cortical cells (c.f. PN234 (Noi), Fig. 4.7) x 200. (b) Light micrograph showing meristematic cells containing starch deposits in a tumour-like nodule. Rhizobia can be seen among the epidermal cells (see arrows). x 250. (c) Light micrograph of a median transverse section of a 28-day-old nodule containing a central bacteroid zone. The infected cells contained several vacuoles (c.f. PN246 (Cof), Fig. 4.10a) x 250. (d) Light micrograph showing part of the two bacteroid-filled cells from (c). Note the numerous vacuoles within these cells (c.f. PN246 (Cof), Fig. 4.10b) x 1620. (e) Electron micrograph of bacteroids present in an infected plant cell. Several of the bacteroids contain extensive deposits of poly-β-hydroxybutyrate. The number of bacteriods per peribacteroid-membrane is seen to be larger than normal (c.f. Fig. 4.6e) and the peribacteroid space is very large. Mitochondria can be seen in the host cell cytoplasm. x 5000.


MC
F
a
F
S
R
R
b
BZ
V
CT
c
V
d
CW
PBS
B
PBM
PHB
M
e

5.3.6 DNA homology between the nod gene region of R. loti NZP2037 and other Rhizobium strains

Rhizobia capable of nodulating Lotus species include strains from R. loti and Bradyrhizobium spp. (Lotus). To identify DNA sequences in these strains homologous to the cloned nod genes of R. loti NZP2037, the 12.8 kb EcoRI fragment from pPN301 (section 5.3.1) was purified, labelled with [^{32}P], and hybridised to a Southern blot of total DNA from R. loti strains NZP2037 and NZP2213, and Bradyrhizobium spp. (Lotus) strain NZP2309 (CC814s). Strong hybridisation occurred to the 7.1 kb EcoRI fragment in NZP2037 (Fig. 5.6, lane 1); to 9.6 kb and 3.8 kb EcoRI fragments in NZP2213 (Fig. 5.6, lane 3), to 11.0 kb and 5.8 kb EcoRI fragments in NZP2309 (Fig. 5.6, lane 5). For the Hind III digests, strong hybridisation occurred to a 14.0 kb fragment in NZP2037 (Fig. 5.6, lane 2); to 23.0 kb fragment in NZP2213 (Fig. 5.6, lane 4); to 13.0 kb and 9.4 kb fragments in NZP2309 (Fig. 5.6, lane 6). Identical results were obtained when the hybridisation experiment was repeated using the 7.1 kb EcoRI fragment from the cosmid pPN305 (section 5.3.2) as a probe.

In order to determine whether the nod region of R. loti NZP2037 was homologous to DNA sequences in rhizobia of other cross inoculation groups, the 7.1 kb EcoRI fragment from pPN305 was labelled with [^{32}P] and hybridised to EcoRI digests of total DNA from R. trifolii strains NZP561 and PN100, and R. meliloti strain SU47 (Fig. 5.7a, lanes 3,4 and 5). DNA from R.loti strains NZP2037 and NZP2213 were included for comparison (Fig. 5.7a, lanes 1 and 2). When the autoradiograph of this blot was developed after 36 h, strong hybridisation was observed with R. loti strains NZP2037 and NZP2213 (Fig. 5.7a, lanes 1 and 2), but no hydridisation was observed to R. trifolii and R. meliloti total DNA

Figure 5.6 Hybridisation of the cloned *nod* gene region of NZP2037 to *R. loti* and *Bradyrhizobium spp*. (*Lotus*) total DNA. Autoradiograph showing the hybridisation pattern between the [^{32}P]-labelled *nod* gene region of NZP2037 and the total DNA digests of *R. loti* and *Bradyrhizobium spp*. (*Lotus*) strains. Lane 1, *Eco*RI digest of NZP2037 DNA; Lane 2, *Hind* III digest of NZP2037 DNA; Lane 3, *Eco*RI digest of NZP2213 DNA; Lane 4, *Hind* III digest of NZP2213 DNA; Lane 5, *Eco*RI digest of NZP2309 DNA; Lane 6, *Hind* III digest of NZP2309 DNA. Size markers are in kilobases (kb).

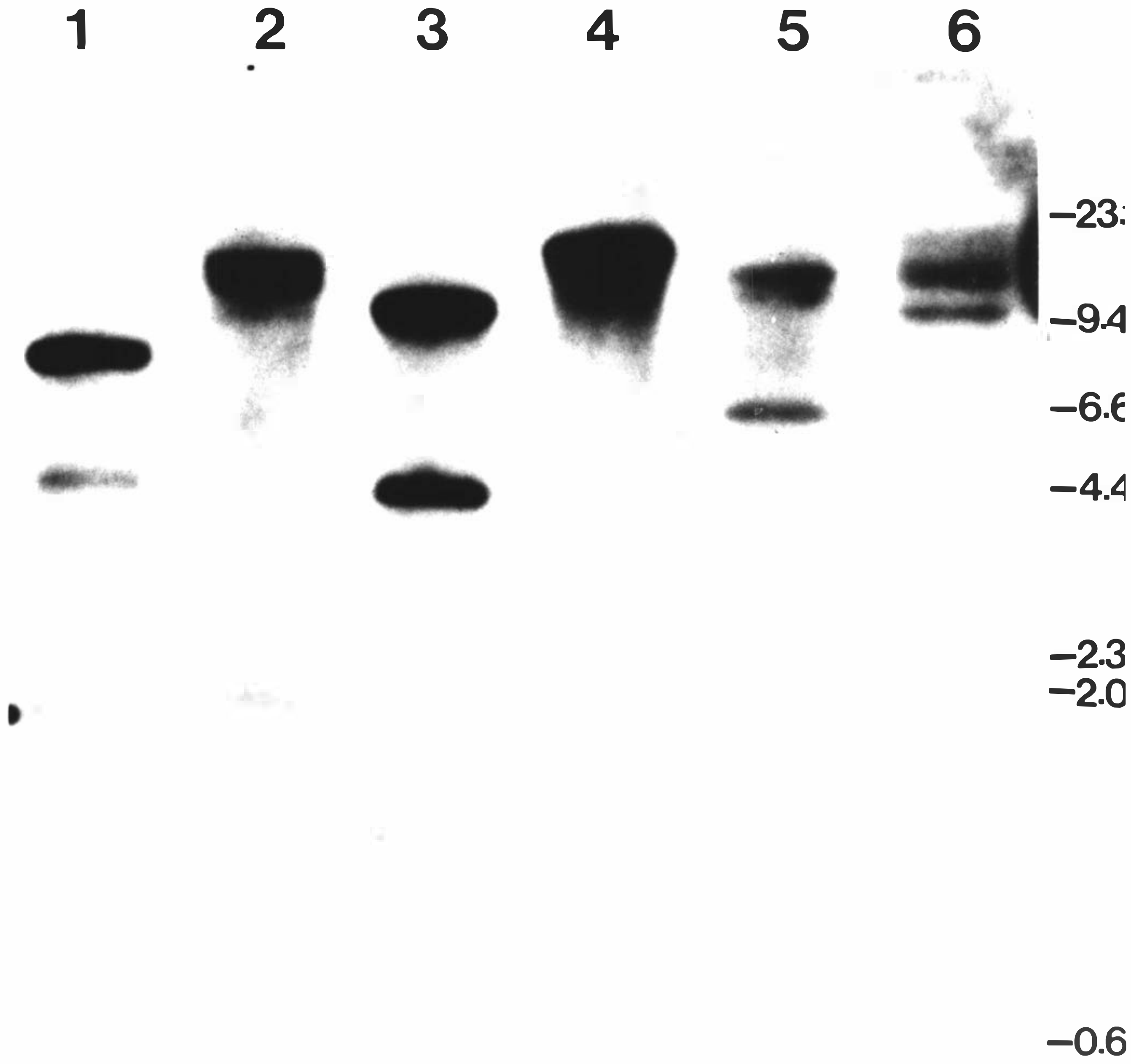
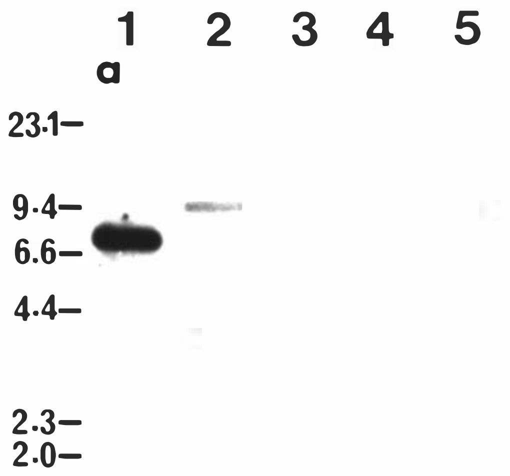
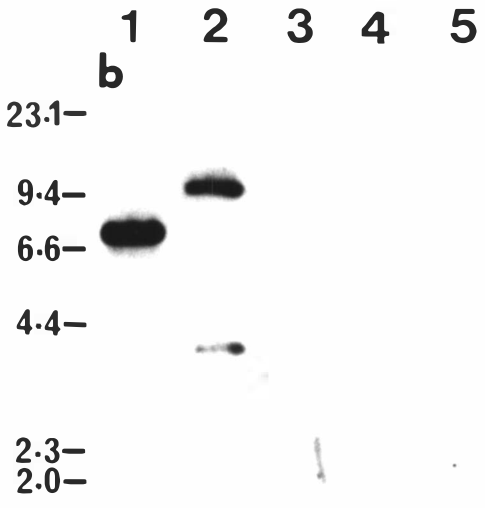

1
2
3
4
5
6
–23.
–9.4
–6.6
–4.4
–2.3
–2.0
–0.6

Figure 5.7 Hybridisation of the cloned *nod* gene region of NZP2037 to *R. loti*, *R. trifolii* and *R. meliloti* total DNA.

(a) Autoradiograph (36h exposure) showing the hybridisation pattern between the [^{32}P]-labelled NZP2037 *nod* gene region (7.1 kb *Eco*RI fragment from pPN305) and the *Eco*RI digests of total DNA from *R. loti*, *R. trifolii* and *R. meliloti* strains. Lane 1, *R. loti* NZP2037; lane 2, *R. loti* NZP2213; Lane 3, *R. trifolii* NZP561; Lane 4, *R. trifolii* PN100; Lane 5, *R. meliloti* SU47. (b) Autoradiograph (7 days exposure) from a second hybridisation experiment using the same *nod* probe and a Southern blot containing *Eco*RI digests of total DNA as listed above. Size markers are in kilobases (kb).


1 2 3 4 5
a
23·1
9·4
6.6
4·4
2·3
2·0


1 2 3 4 5
b
23·1
9·4
6·6
4·4
2·3
2·0

(Fig. 5.7a, lanes 3, 4 and 5). However, an autoradiograph developed after 7 days revealed weak homology with *R. trifolii* and *R. meliloti* DNA (Fig. 5.7b, lanes 3,4 and 5). The fragment sizes and the intensity of hybridisation observed for this blot are listed in Table 5.2. Using a 7.2 kb *Eco*RI fragment from pRt572 containing the nodulation region of *R. trifolii* (Schofield *et al*., 1983) as a probe, the reverse experiment was carried out to determine if homologous sequences were present in the *R. loti* strains. Strong hybridisation was observed to several DNA fragments from *R. trifolii* and *R. meliloti* total DNA (Fig. 5.8a, lanes 3,4 and 5), but hybridisation to *R. loti* strains NZP2037 and NZP2213 total DNA could only be observed after prolonged exposure (5 days) of the X-ray film to the blot (Fig. 5.8b, lanes 1 and 2). The size and intensity of hybridisation of the various *Eco*RI fragments that hybridised to the *R. trifolii* *nod* probe are listed in Table 5.2. As it was uncertain from the last experiment whether the *R. trifolii* *nod* probe shared homology with the *nod* region of *R. loti*, a further hybridisation experiment was carried out using a blot of the cloned *R. loti* *nod* DNA region. When the same [^{32}P]-labelled *R. trifolii* *nod* DNA was used to hybridise with *Eco*RI digests of pLAFR1 recombinant plasmids, pPN305 and pPN306, carrying the wild type nodulation gene sequences of *R. loti* NZP2037 (section 5.3.2), only the 7.1 kb *Eco*RI fragment from pPN305 and pPN306 was found to hybridise. (Fig. 5.9a and b, lanes 1 and 2).

5.3.7 'In planta' direct complementation of the R. loti NZP2037 Nod$^-$ mutant with a gene library of Bradyrhizobium spp. (Lotus) strain NZP2309

In section 5.3.6 it was shown that the *nod* region of *R. loti* NZP2037 hybridised to DNA sequences in *Bradyrhizobium* *spp*. (*Lotus*) strain NZP2309 total DNA (see Fig. 5.6) suggesting that the *nod* regions

Figure 5.8 Hybridisation of cloned *R. trifolii* *nod* DNA to *R. loti*, *R. trifolii* and *R. meliloti* total DNA.

(a) Autoradiograph (20h exposure) showing the hybridisation pattern between [^{32}P]-labelled *R. trifolii* *nod* DNA and *Eco*RI digests of total DNA. Lane 1, *R. loti* NZP2037 DNA; Lane 2, *R. loti* NZP2213 DNA; Lane 3, *R. trifolii* NZP561 DNA; Lane 4, *R. trifolii* PN100 DNA; Lane 5, *R. meliloti* SU47.

(b) Autoradiograph of the same gel after 7 days exposure. Size markers are in kilobases (kb).

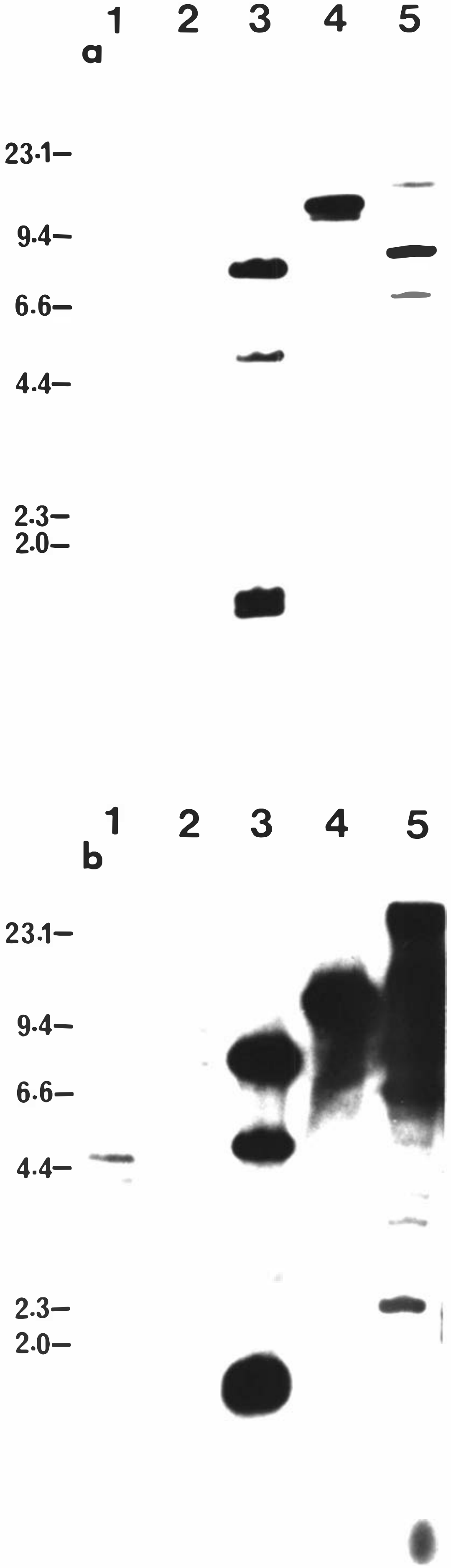

a
1
2
3
4
5
23·1
9·4
6.6
4.4
2.3
2.0
b
1
2
3
4
5
23·1
9·4
6·6
4.4
2.3
2.0

Figure 5.9 Hybridisation of *R trifolii* *nod* DNA to cloned *nod* gene sequences of *R. loti* NZP2037.

(a) Agarose gel showing *Eco*RI digests of pLAFR1 recombinant plasmids carrying *nod* gene sequences of NZP2037. Lane 1, pPN305 DNA; Lane 2, pPN306 DNA.

(b) Autoradiograph of the same gel hybridised with the $[^{32}P]$-labelled 7.2 kb *Eco*RI fragment carrying the *nod* DNA of *R. trifolii* from pRt572 (Schofield *et al.*, 1983). Size markers are in kilobases (kb).

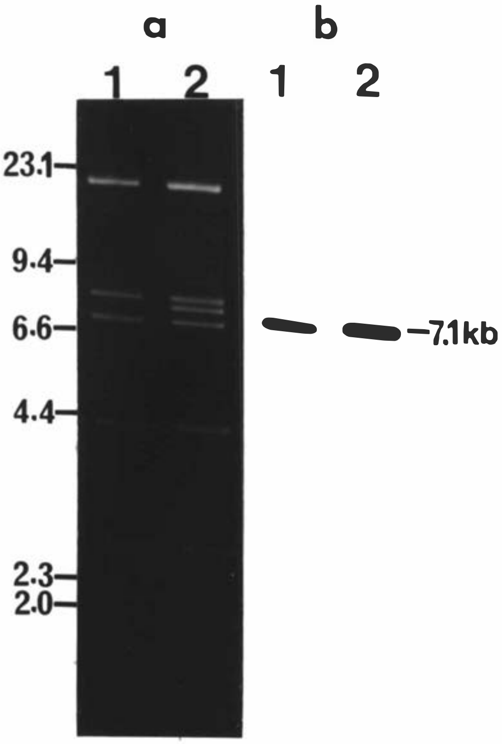

a
b
1
2
1
2
23.1
9.4
6.6
4.4
2.3
2.0
7.1kb

Table 5.2 EcoRI restriction fragments from *Rhizobium loti*, *R. trifolii* and *R. meliloti* total DNA homologous to *R. loti nod* probe and/or *R. trifolii nod* probe.

EcoRI DNA fragment (kb)	Homology[a] with	
	R. loti nod probe[b]	*R. trifolii nod* probe[c]
R. loti NZP2037		
10.5	0	2
7.1	5	1 [d]
4.7	0	3
4.0	2	2
3.5	0	1
1.0	0	2
R. loti NZP2213		
9.6	5	2
8.8	0	1
7.7	0	2
6.4	0	1
3.8	5	0
1.0	0	2
R. trifolii NZP561		
7.7	0	5
7.1	2	0
5.0	0	4
2.6	0	2
1.8	0	5
1.4	0	5
R. trifolii PN100		
11.7	2	5
10.5	0	4
4.5	2	0
R. meliloti SU47		
15.0	0	4
10.0	0	3
8.7	2	5
6.6	0	4
3.9	0	2
3.3	0	2
2.3	0	3

Table 5.2 Continued

a Determined by hybridisation of [^{32}P]-labelled DNA probes to Southern blots of *EcoRI* digests of total DNA. The numbers 0 to 5 indicate the intensity of hybridisation; 0 = no hybridisation detected, 5 = very strong hybridisation.

b This probe was a 7.1 kb *EcoRI* fragment purified from pPN305 (section 5.3.2) by electroelution and labelled with [^{32}P].

c This probe was a 7.2 kb *EcoRI* fragment purified from pRt572 (Schofield *et al.*, 1983) by electroelution and labelled with [^{32}P].

d The box indicates the *EcoRI* fragment carrying *nod* gene sequences in each strain and the hybridisation intensities of the fragment to the *R. loti* and *R. trifolii* [^{32}P]-labelled *nod* probes.

of these strains were homologous. To determine whether the homologous sequences from NZP2309 could complement the Nod$^-$ mutant of R. loti NZP2037, a pLAFR1 gene library was constructed (section 5.2.17) and conjugated en masse with the NZP2037 Nod$^-$ mutant, strain PN233 (section 5.2.18). The tetracycline and neomycine resistant transconjugants obtained were mass inoculated onto Lotus pedunculatus plants (section 3.2.6). Plants were examined for nodulation and growth 2 weeks and 6 weeks after inoculation respectively. All plants inoculated formed ineffective nodules. Rhizobia were isolated from these nodules and plasmid DNA isolated by the alkaline lysis procedure (section 5.2.6.2). When EcoRI digests of these cosmids were analysed on agarose gels, they were found to contain many common fragments including fragments of the sizes, 11.0 kb and 5.8 kb, found to hybridise with the NZP2037 nod probe (see Fig. 5.6, lane 5). One of the cosmids was retained and designated pPN329.

The ineffective nodules formed on Lotus pedunculatus by strain PN233 carrying pPN329 were examined by light and electron microscopy. The light micrograph of a median transverse section of a nodule showed that the central zone contained plant cells filled with a variable number of bacteroids (Fig. 5.10a). The bacteroids appeared to be packed in groups throughout the host cell cytoplasm (Fig. 5.10b). Electron microscopic examination of these cells showed bacteroids in various stages of degeneration (Fig. 5.10c). The bacteroids contained extensive deposits of poly-β-hydroxybutyrate and were not surrounded by a peribacteroid membrane (Fig. 5.10d). The host cell cytoplasm contained numerous vesicles and membranes and cellular contents of degenerating rhizobia.

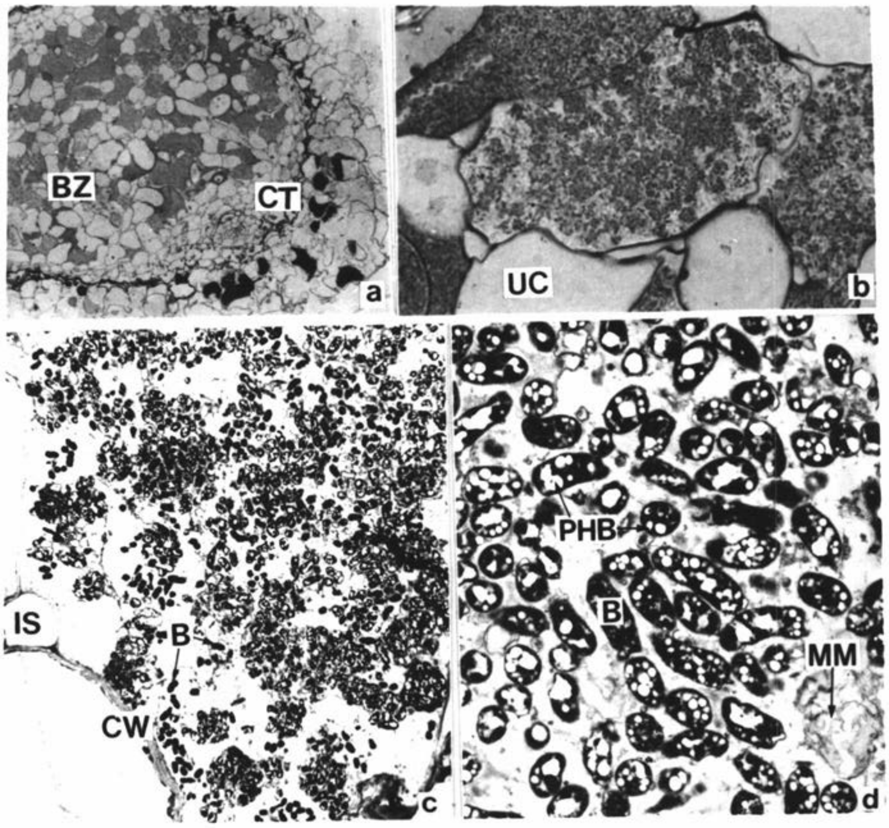
Figure 5.10 Light and electron micrographs showing the structure of root nodules formed on Lotus pedunculatus by a derivative of the Nod^- mutant PN233 containing pPN329. B = bacteroid, BZ = bacteroid zone, CT = cortex, CW = plant cell wall, IS = intercellular space, MM = membrane material, PHB = poly-β-hydroxybutyrate, UC = uninfected cell.

(a) Light micrograph of a median transverse section of a 28-day-old nodule showing a disorganised bacteroid zone containing many enlarged infected and uninfected cells. x 250.

(b) Light micrograph of an infected plant cell. The bacteroids present in this cell appear to be clumped into groups. This cell is surrounded by several uninfected cells. x 250.

(c) Electron micrograph of part of the infected cell showing large numbers of bacteroids, often clumped together, in the cell cytoplasm. The bacteroids are not enclosed within peribacteroid membranes. x 2,600.

(d) Electron micrograph showing bacteroids within the cytoplasm of an infected plant cell. They are enclosed within peribacteroid membranes and they contain many granules of poly-β-hydroxybutyrate. The host cell cytoplasm contains debris of membrane materials. x 10,500.


BZ
CT
a
UC
b
IS
B
CW
c
PHB
B
MM
d

5.3.8 Complementation of Rhizobium nodulation function using heterologous nod regions

As only weak DNA homology was observed between the R. loti and R. trifolii nod regions (section 5.3.6), a series of complementation experiments was carried out to determine if the two nod regions shared common functions. A pLAFR1 recombinant plasmid carrying a 11.7 kb EcoRI fragment from the nod region of the R. trifolii Sym plasmid pRtr514a (Scott et al., 1984) was transferred into the Nod$^-$ mutant of R. loti, strain PN233, in a triparental cross. The tetracycline and neomycin resistant transconjugants were single colony purified and inoculated onto Lotus pedunculatus and red clover (Trifolium pratense) plants. Numerous tumour-like nodules formed on Lotus pedunculatus 4 weeks after inoculation, a few normal looking ineffective nodules formed after 6 weeks. However, there were no nodules formed on red clover. Similar results were obtained when the Sym plasmid cointegrate pPN1 (pRtr514a::R68.45) from R. trifolii PN100 (Scott and Ronson, 1982) was mobilised into the R. loti Nod$^-$ mutant PN233 by conjugation.

To test whether the nod gene sequences of R. loti NZP2037 were capable of restoring the nodulation ability of a Sym plasmid cured derivative of R. trifolii, strain PN104, the R. loti pLAFR1 nod cosmid, pPN306, was introduced in a triparental cross. The tetracycline resistant transconjugants were single colony purified and inoculated onto red clover and Lotus pedunculatus plants. No nodules were formed on either red clover or Lotus pedunculatus.

5.4 DISCUSSION

The 12.8 kb EcoRI DNA fragment carrying the Tn5::nod DNA sequences from the R. loti Nod$^-$ mutant strain PN233 was successfully cloned into pBR328 (section 5.3.1). The resultant recombinant plasmid, pPN301, was used to isolate the wild type sequences from a pLAFR1 gene library of R. loti (section 5.3.2). Two positive cosmid clones were isolated and shown to span a region of approximately 30 kb in the R. loti genome. These cosmids (pPN305 and pPN306) had 4 common EcoRI DNA fragments (total 22 kb) and when each was hybridised with the 12.8 kb EcoRI fragment from pPN301, only the 7.1 kb EcoRI fragment hybridised (Fig. 5.2b, lanes 1 and 3) confirming that this was the fragment into which Tn5 had inserted.

When the cosmids pPN305 and pPN306 were transferred into the Nod$^-$ mutant strain PN233, complementation of the Nod$^-$ phenotype occurred but only 3/16 of the plants were Fix$^+$ (Fig. 5.4 a and b, plant 3) and the others were Fix$^-$ (Fig. 5.4a and b, plant 2). The three Fix$^+$ plants contained both Fix$^-$ and late appearing Fix$^+$ nodules and showed delayed nitrogen fixation. The Fix$^-$ plants contained Fix$^-$ nodules of two types: about 40% of the nodules resembled the tumour-like Noi phenotype (Fig. 4.7) and 60% were of the Cof phenotype (Fig. 4.9 and Fig. 4.10). Similar results were obtained by C.E. Pankhurst (personal communication) in an alternative attempt to isolate nod genes from the NZP2037 pLAFR1 gene library by direct 'in planta' complementation (Long et al., 1982) of the Nod$^-$ mutant PN233. Cosmids identical to pPN305 and pPN306 were isolated by this alternative approach. These observations suggest that PN233 was complemented to varying degrees by cosmids pPN305 or pPN306.

The fact that only 3/16 of the plants were Fix$^+$ could be attributed to the need for recombination to occur for complementation of the Fix$^+$ phenotype in this mutant. There are three observations that support this explanation. Firstly, the Rhizobium recipient was not a recombination-deficient strain. Secondly, bacteria recovered from the effective nodules were tetracycline and neomycin sensitive indicating that both the pLAFR1 cosmid (pPN305 or pPN306) and the Tn5 DNA sequences were lost presumably by a site specific recombination event, assuming no reversion occurred. Thirdly, it is unlikely the Nod$^+$ Fix$^+$ phenotype arose as a result of reversion as repeated plant tests with PN233 showed that it is a stable, non-reverting mutant. The inability of this *R. loti* Nod$^-$ mutant to be complemented *in trans* for fixation (Fix) is in contrast to complementation tests that have been carried out with Nod$^-$ mutants of *R. meliloti* (Long *et al*., 1982) *R. trifolii* (Schofield *et al*., 1983) and *R. leguminosarum* (Downie *et al*., 1983a). However, Scott *et al*. (1982) also observed a delay in nitrogen fixation when complementing a Nod$^+$Fix$^-$ mutant of *R. trifolii* with a RP4 recombinant plasmid carrying the wild type genes. They attributed this result to a requirement for site specific recombination before complementation occurred. The correction of the Nod$^-$ to Nod$^+$ phenotype by cosmids containing the wild type 7.1 kb *Eco*RI fragment indirectly demonstrates that the *nod* gene mutation was caused by a Tn5 insertion into this fragment. This was recently comfirmed by reverse mutagenesis using site directed exchange of the cloned Tn5 containing fragment for wild type sequences (D.B. Scott, personal communication). The fact that the development of most nodules in these experiments were not restored to Fix$^+$ would suggest that the Tn5-induced *nod* mutation has some effect, possibly polar (Berg *et al*., 1980), on a *fix* gene and that this lesion cannot be complemented *in trans*.

As *Lotus* species can be nodulated by strains from *Rhizobium loti* and *Bradyrhizobium spp*. (*Lotus*), the cloned *nod* gene region from *R. loti* strain NZP2037 was used as a hybridisation probe to test if these other

Lotus strains carried homologous sequences. Strong hybridisation was observed in both the R. loti strains NZP2037 and NZP2213 (Fig. 5.6, lanes 1,2,3 and 4). In Bradyrhizobium spp. (Lotus) strain NZP2309, the DNA sequences homologous to the NZP2037 nod probe did not hybridise as strongly as those in NZP2213 (c.f. Fig. 5.6, lanes 3 and 4 and Fig. 5.6, lanes 5 and 6), but were more highly conserved with the R. loti nod gene sequences than the sequences that hybridised from R. trifolii and R. meliloti (Fig. 5.7 a and b, lanes 3,4 and 5). Additional hybridising bands were found in all three Lotus strains suggesting that part of the 7.1 kb EcoRI fragment from NZP2037 is reiterated elsewhere on the genome. These results indicate that the nod gene regions in the fast-growing and slow-growing Lotus are conserved, despite their lack of total DNA homology (Chapter 2).

Confirmation that the sequences that hybridised in the slow-growing strain NZP2309, carried genes involved in nodulation, was obtained by isolation of these sequences by 'in planta' complementation. The NZP2309 cosmids that complemented the nodulation function of the R. loti NZP2037 Nod$^-$ mutant contained fragments of identical sizes to those that hybridised with the R. loti NZP2037 nod gene probe (Fig. 5.6, lanes 5 and 6). However, as found in the complementation experiments with the NZP2037 gene library, the nodules formed with strains of PN233 containing NZP2309 cosmids were again Fix$^-$. This result would again suggest that recombination is necessary for restoration of the Fix$^+$ phenotype. It is interesting to note that in contrast to the mixed morphologies displayed by nodules induced by PN233 carrying pPN305 or pPN306 (section 5.3.4), those formed by strain PN233 carrying NZP2309 nod cosmids were of a single morphology. In addition, although the external morphology of the nodules was normal, they were smaller than the nodules induced by the *R. loti* wild type strain NZP2037 on *Lotus pedunculatus*, and the nodule sections were found to contain extensive deposits of poly-β-hydroxybutyrate. The size of the nodules and the numerous poly-β-hydroxybutyrate granules suggested that they strongly resembled those induced by the wild type strain NZP2309 on *Lotus pedunculatus* (Wood *et al.*, 1984). The fact that the *R. loti* Nod$^-$ mutant, PN233, when complemented with *nod* cosmids from NZP2037 or NZP2309 gene libraries, induced different nodule morphologies would suggest that there are some differences between these two conserved *nod* gene regions.

Nodulation genes have recently been isolated from another slow-growing strain of *Bradyrhizobium spp.* (*Parasponia*) by direct complementation of a Nod$^-$ mutant of *Rhizobium meliloti* (Marvel *et al.*, 1984) and by hybridisation with a *nod* ABC gene sequence fragment from *Rhizobium trifolii* (K.F. Scott, personal communication). The 11.0 kb and 5.8 kb *Eco*RI fragments of the NZP2309 *nod* cosmid isolated in this study were also found to show weak DNA homology with the *R. trifoli nod* ABC gene sequence (C.E. Pankhurst, personal communication) suggesting that 'common' *nod* genes are carried on these fragments.

It has been shown by comparative physical and genetical studies that some early nodulation gene sequences are highly conserved physically and functionally among the *Rhizobium* species (Rolfe *et al.*, 1984; Kondorosi *et al.*, 1984). However, this study showed that the cloned *R. loti* NZP2037 *nod* gene probe showed only weak DNA homology with DNA sequences known to carry *nod* gene sequences from *R. trifolii* and *R. meliloti* (Table 5.2). Using a 7.2 kb *Eco*RI fragment from the *R. trifolii nod* gene region (Schofield *et al.*, 1983) as a hybridisation probe a reverse experiment was carried out and again very weak DNA homology was observed with sequences from *R. loti* strains (Table 5.2). Precise interpretation of these hybridisation results was difficult as multiple bands hybridised in *R. trifolii*, *R. meliloti* and *R. loti* DNA digests presumably due to the presence of reiterated sequences. Therefore it was not possible to confirm that the DNA homology between the *nod* gene regions of *R. trifolii* and *R. loti* was due to *nod* gene homology *per se* despite the fact that the *R. trifolii* *nod* ABC gene region was found to hybridise with the cloned *R. loti* *nod* gene region (C.E. Pankhurst, personal communication). Therefore, genetic experiments were carried out to determine if there were functional similarities between the two *nod* regions. When a 11.7 kb *Eco*RI fragment spanning the *nod* gene region of *R. trifolii* strain PN100 (Scott *et al*., 1984) was transferred into the *R. loti* NZP2037 Nod$^-$ mutant, strain PN233, the resulting transconjugants were found to form numerous tumour-like nodules and a few late appearing Fix$^-$ nodules on *Lotus pedunculatus* suggesting that some gene functions were conserved in the *R. loti* and *R. trifolii* *nod* gene regions. However these strains were unable to induce any nodule-like structures at all on red clover. Similar results were obtained when the Sym plasmid cointegrate of *R. trifolii*, pPN1 (Scott and Ronson, 1982) was mobilised into the *R. loti* Nod$^-$ mutant. However, Ronson and Scott (1983) reported that when pPN1 was introduced into the *R. loti* wild type strain NZP2037, the resulting transconjugants formed Fix$^-$ nodules on the normal host plant *Lotus pedunculatus*. They suggested that the defective symbiotic phenotype was due to interference of pPN1 with the expression of the *R. loti* symbiotic genes. Hence it is difficult to interpret the complementation shown by pPN1 in the NZP2037 Nod$^-$ mutant. In another complementation experiment, the *nod* cosmid of *R. loti* NZP2037, pPN306, was transferred into a *R. trifolii* Sym plasmid-cured Nod$^-$ derivative, strain PN104, but there was no correction of the Nod$^-$ phenotype when tested either on red clover or *Lotus pedunculatus*. The fact that there was no nodulation observed on *Lotus pedunculatus* suggests that either this *R. loti nod* gene region does not carry sufficient genetic information for *Lotus* specific nodulation or that the genes are not expressed in the heterologous chromosomal background. The inability of these strains to nodulate red clover was not surprising considering that the entire Sym plasmid was absent from these strains. In contrast, Schofield *et al*. (1984) reported that a cloned 14 kb *Hind* III fragment spanning the *nod* region of a *R. trifolii* strain, when introduced into a *R. trifolii* Sym plasmid-cured Nod$^-$ derivative was able to restore normal nodulation functions to this strain. In addition, the same cloned fragment was found to confer clover specific nodulation ability to *Agrobacterium* and unrelated 'cowpea' rhizobia. Downie *et al*. (1983a) also reported that a 10 kb region of *nod* DNA from *R. leguminosarum* was able to restore the nodulation ability of a *R. leguminosarum* Sym plasmid-cured Nod$^-$ derivative and also conferred pea specific nodulation ability to a Sym plasmid-cured Nod$^-$ derivative of *R. phaseoli*.

The physical and genetical analysis carried out here on the *R. loti* NZP2037 *nod* gene region demonstrates that at least some of the *nod* gene sequences carried on the *R. loti* 7.1 kb *Eco*RI fragment (Fig. 5.3) belong to the 'common' *nod* gene category. However, in contrast to the finding that the 'common' *nod* gene sequences were highly conserved between *R. trifolii* and *R. meliloti* (Rolfe *et al*., 1984; Kondorosi *et al*., 1984), the *R. loti nod* gene region showed only weak DNA homology with the *nod* regions from other *Rhizobium* species. It is most likely that the *R. loti nod* region isolated here also contains *nod* gene sequences unique to

Lotus rhizobia as there was a high degree of DNA homology shown by the *Lotus* strains from two different genera. However, whether these gene sequences correspond to genetic determinants for host specificity remains to be resolved.

5.5 SUMMARY

The 12.8 kb *Eco*RI DNA fragment containing the Tn5::*nod* DNA sequences from the *R. loti* NZP2037 Nod$^-$ mutant has been cloned and used as a hybridisation probe to identify the wild type sequences from a pLAFR1 gene library of *R. loti* wild type strain NZP2037. Two cosmids which showed homology to the probe contained approximately 22 kb of DNA in common, including a 7.1 kb *Eco*RI fragment which corresponded to the fragment into which Tn5 had inserted. Hybridisation and complementation experiments confirmed that the 7.1 kb *Eco*RI fragment contained *nod* gene sequences of *R. loti* NZP2037 and that these sequences were more highly conserved between the two genera of strains that nodulate *Lotus* than with *R. trifolii* or *R. meliloti*. It was also shown that despite the weak DNA homology between the *nod* region of *R. loti* and that of *R. trifolii* or *R. meliloti*, genetic studies confirmed that at least some of the *nod* gene sequences carried on the 7.1 kb *Eco*RI fragment belong to the highly conserved 'common' *nod* gene sequence category.

CHAPTER SIX

GENERAL DISCUSSION

Earlier studies (Jensen, 1967; Jensen and Hansen, 1968) demonstrated that unlike most legumes nodulated by named *Rhizobium* species, *Lotus* plant species could be nodulated by both fast- and slow-growing strains of rhizobia. The fast-growing *Lotus* rhizobia have many of the cultural characteristics of fast-growing *Rhizobium* species and have recently been classified as a new species, *Rhizobium loti* (Jarvis *et al*., 1982). Little is known about the slow-growing *Lotus* rhizobia but it has been suggested that they are genetically related to slow-growing strains from soybean and *Lupinus* (Vincent, 1974). Slow-growing *Lotus* rhizobia are now classified within the genus *Bradyrhizobium*. Studies carried out using the important pasture legume *Lotus pedunculatus* Cav. have shown that fast-growing *R. loti* strains such as NZP2037 and slow-growing *Bradyrhizobium spp*. (*Lotus*) strains such as NZP2309 can both nodulate *Lotus pedunculatus* effectively (Pankhurst *et al*., 1977; Pankhurst *et al*., 1979), but the genetic basis for these symbiotic characteristics is unknown.

In the first part of this study, the total DNA homology between fast- and slow-growing *Lotus* rhizobia was compared. Results showed that the fast- and slow-growing *Lotus* rhizobia shared less than 10% relative DNA homology even though they shared the capacity to nodulate the same plant species. An analogous system has also been reported for strains that nodulate soybean. It has now been established that soybean rhizobia include strains from fast-growing *Rhizobium fredii* (Scholla and Elkan, 1984) and slow-growing *Bradyrhizobium japonicum* (Jordan, 1982). As with the *Lotus* strains these 2 groups of rhizobia share little total

DNA homology (Scholla *et al*., 1984). Diversity within *R. loti* exists but there is insufficient data to support any further subdivision of these strains. In contrast, the slow-growing *Lotus* rhizobia could be divided into 2 distinct DNA homology groups. Heterogeneity based upon DNA homology studies has also been shown with *B. japonicum* (Hollis *et al*., 1981). Most slow-growing strains studied here shared up to 40% relative DNA homology with each other. It appears that while heterogeneity exists within groups of slow-growing rhizobia, they still share up to 40% of relative DNA homology between groups.

Analysis of the plasmids in *Lotus* rhizobia indicated that like many other groups of rhizobia studied to date, the presence of large indigenous plasmids is a common feature of the *Lotus* rhizobia. However, unlike other *Rhizobium* species studied to date, *R. loti* strains carry a single large indigenous plasmid and these plasmids do not carry nodulation (*nod*) and nitrogen fixation (*nif*) genes. It is unknown whether the multiple plasmids identified in the slow-growing *Bradyrhizobium spp*. (*Lotus*) strains carry any symbiotic determinants. Such a possibility cannot be ruled out but there is no evidence to date for *nod* and *nif* genes being located on large indigenous plasmids present in a number of slow-growing strains studied (Haugland and Verma, 1981; Masterson *et al*., 1982). The role of the plasmids in *Lotus* rhizobia is still unknown.

Using Tn5 mutagenesis coupled with molecular cloning techniques (Meade *et al*., 1982; Long *et al*., 1982; Scott *et al*., 1982; Schofield *et al*., 1983; Downie *et al*., 1983a), a *nod* gene region of *R. loti* NZP2037 was cloned. The Tn5-induced Nod$^-$ mutant of *R. loti* NZP2037 was isolated by random Tn5 mutagenesis using pSUP1011 as a Tn5 donor. This mutant was defective in root hair curling (Hac). The wild type *nod* gene sequences were isolated from a pLAFR1 gene library of NZP2037 using the cloned Tn5-containing fragment from the Nod$^-$ mutant as a probe. In addition, a range of Tn5-induced Fix$^-$ mutants of *R. loti* NZP2037 have also been isolated. These mutants included strains that were blocked in nodule initiation (Noi), bacterial release (Bar) and nitrogen fixation (Cof). Suprisingly, only the cosmids that complemented the Fix$^-$ mutants PN236 and PN237 shared common DNA fragments (C.E. Pankhurst, personal communication), suggesting that these symbiotically important gene regions are not closely linked in the *R. loti* genome. Widely scattered *fix* alleles have recently been identified on the chromosome of *R. meliloti* (Forrai *et al*., 1983) and *R. phaseoli* (Noel *et al*., 1984).

Physical and genetical studies have shown that a 7.1 kb *Eco*RI fragment from NZP2037 *nod* cosmids pPN305 and pPN306 carried gene sequences involved in nodulation. These *nod* gene sequences were shown to be conserved among *Lotus* rhizobia from two different genera. The *nod* gene region of *Bradyrhizobium spp*. (*Lotus*) strain NZP2309 was isolated by direct complementation (Long *et al*., 1982) of the *R. loti* NZP2037 Nod$^-$ mutant. It was noted that the *R. loti* NZP2037 Nod$^-$ mutant when complemented with *nod* cosmids from NZP2037 or NZP2309 gene libraries, induced nodules of different morphologies on *Lotus pedunculatus* suggesting that there are some differences between these two conserved *nod* gene regions. It has been observed that *Lotus pedunculatus* plants inoculated by NZP2309 fix more nitrogen than *Lotus pedunculatus* inoculated with NZP2037 (Pankhurst, 1977). The possibility exists that the differences shown in the symbiotic genes of these two different *Lotus* strains may account for this variation in nodule effectiveness.

Recently DNA sequences encoding some early *nod* functions have been isolated from *B. japonicum* (Sutton *et al.*, 1984), but to date there is no report on the comparative studies of the *nod* regions from fast- and slow-growing soybean strains. Nodulation gene sequences have also been isolated from *Bradyrhizobium spp.* (*Parasponia*) strains (Marvel *et al.*, 1984; K.F. Scott, personal communication). The homology between these *Bradyrhizobium nod* gene regions isolated has not yet been studied.

The presence of conserved 'common' *nod* gene sequences (*nod* ABC) has been reported in a number of *Rhizobium* species and in some strains of *Bradyrhizobium* species (Rolfe *et al.*, 1984; Kondorosi *et al.*, 1984; K.F. Scott, personal communication). These 'common' *nod* gene sequences are highly conserved physically and functionally in *Rhizobium* species. In contrast, this work showed that the *R. loti nod* gene region isolated only showed very weak DNA homology with the 'common' *nod* gene sequences from *Rhizobium trifolii*. However genetical studies showed that these two *nod* gene regions shared some functional similarities despite the weak DNA homology between them. Weak DNA homology was also observed between the *nod* gene region of *Bradyrhizobium spp.* (*Lotus*) strain NZP2309 and the *nod* ABC gene sequences from *R. trifolii*. These results further support the suggestion that a group of conserved bacterial *nod* gene sequences involved in early nodule formation are present in most if not all rhizobia. Host specific nodulation gene sequences have also been identified in *R. trifolii*, *R. meliloti* and *R. leguminosarum*, but such gene sequences have not yet been identified in *R. loti*. It has been shown that the *nod* gene regions isolated from *R. trifolii* and *R. leguminosarum* are confined to a region less than 10 kb of DNA (Schofield *et al.*, 1983; Downie *et al.*, 1983a). It is unknown whether the *R. loti* NZP2037 *nod* gene region is confined to the 7.1 kb *Eco*RI fragment as the boundaries of the *nod* gene region in NZP2037 have not been defined.

In conclusion, this study has provided a detailed molecular and genetical analysis of some of the symbiotic genes from *Lotus* rhizobia. The isolation of a range of Tn5-induced symbiotic mutants will facilitate further molecular and genetical studies on the symbiotic genes of *Lotus* rhizobia. Furthermore the isolation of two *nod* gene regions from *Lotus* strains with heterogeneous genetical backgrounds may provide a unique system for studies aimed at the elucidation of the molecular mechanisms underlying host specificity of the legume-rhizobia symbioses.

REFERENCES

ABDEL-GHAFFER, A.S. & JENSEN, H.L. (1966). The rhizobia of Lupinus densiflorus Benth., with some remarks on the classification of root nodule bacteria. Archiv fur Mikrobiologie 54, 393-405.

ALLEN, O.N. (1957). Family II. Rhizobiaceae Conn, 1938. In: Bergey's manual of determinative bacteriology 7th edition pp. 285-296. Edited by R.S. BREED, E.G.D. MURRAY & N.R. SMITH. The Williams and Wilkins Co., Balitmore.

AMEMURA, M., SHINAGAWA, H., MAKINO, K., OTSUJI, N. & NAKATA, A. (1982). Cloning of and complementation tests with alkaline phosphatase regulatory genes (phoS ans phoT) of Escherichia coli. Journal of Bacteriology 152, 692-701.

ANDERSEN, K., TAIT, R.C. & KING, W.R. (1981). Plasmid required for utilization of molecular hydrogen by Alcaligenes eutrophus. Archives of Microbiology 129, 384-390.

BANFALVI, Z., SAKANYAN, V., KONCZ, C., KISS, A., DUSHA, I. & KONDOROSI, A. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of R. melitoli. Molecular and General Genetics 184, 318-325.

BARRATT, B.I.P. & JOHNSTONE, P.D. (1984). Effects of insects, seeding rate, and insecticide seed dressing on white clover and Maku lotus in tussock grassland. New Zealand Journal of Agricultural Research 27, 13-18.

BAUER. W.D. (1977). Lectins as determinants of specificity in legume-Rhizobium symbiosis. Basic Life Science 9, 283-297.

BAUER, W.D. (1981). Infection of legumes by rhizobia. Annual Review of Plant Physiology 32, 407-449.

MASSEY UNIVERSITY
LIBRARY

BERG, D.E., WEISS, A. & CROSSLAND, L. (1980). Polarity of Tn5 insertions in *Escherichia coli*. Journal of Bacteriology 142, 439-446.

BERINGER, J.E. (1974). R factor transfer in *Rhizobium leguminosarum*. Journal of General Microbiology 84, 188-198.

BERINGER, J.E., BEYNON, J.L., BUCHANAN-WOLLASTON, A.V. & JOHNSTON, A.W.B. (1978). Transfer of drug resistance transposon Tn5 to *Rhizobium*. Nature 276, 633-634.

BERINGER, J.E., BREWIN, N.J. & JOHNSTON, A.W.B. (1982). Genetics. In: Nitrogen fixation volume 2: *Rhizobium*, pp. 167-181. Edited by W.J. BROUGHTON. Clarendon Press, Oxford.

BEYNON, J.L., BERINGER, J.E. & JOHNSTON, A.W.B. (1980). Plasmids and host range in *Rhizobium leguminosarum* and *Rhizobium phaseoli*. Journal of General Microbiology 120, 421-429.

BOLIVAR, F., RODRIGUEZ, R.L., GREENE, P.J., BETLACH, M.C., HEYNEKER, H.L., BOYER, H.W., CROSA, J. & FALKOW, S. (1977). Construction and characterisation of new cloning vehicles. II. A multi-purpose cloning system. Gene 2, 95-113.

BONNER, T.I., BRENNER, D.J., NEUFELD, B.R. & BRITTEN, R.J. (1973). Reduction in the rate of DNA reassociation by sequence divergence. Journal of Molecular Biology 81, 123-135.

BOYER, H.W. & ROULLAND-DUSSOIX, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. Journal of Molecular Biology 41, 459-472.

BRENNER, D.J., FANNING, G.R., RAKE, A.V. & JOHNSON, K.A. (1969a). Batch procedure for thermal elution of DNA from hydroxyapatite Analytical Biochemistry 28, 447-459.

BRENNER, D.J., FANNING, G.R., JOHNSON, K.E., CITERELLA, R.V. & FALKOW, S. (1969b). Polynucleotide sequence relationships among members of Enterobacteriaceae. Journal of Bacteriology 98, 637-650.

BRENNER, D.J. & FALKOW, S. (1971). Molecular relationships among members of the Enterobacteriaceae. Advances in Genetics 16, 81-118.

BRENNER, D.J., FANNING, G.R., SKERMAN, F.J. & FALKOW, S. (1972). Polynucleotide sequence divergence among strains of Escherichia coli and closely related organisms. Journal of Bacteriology 109, 953-965.

BREWIN, N.J., BERINGER, J.E., BUCHANAN-WOLLASTON, A.V., JOHNSTON, A.W.B. & HIRSCH, P.R. (1980). Transfer of symbiotic genes with bacteriocigenic plasmids in Rhizobium leguminosarum. Journal of General Microbiology 116, 261-270.

BREWIN, N.J., DIBB, N.J., & HOMBRECHER, G. (1983). Natural variation in Rhizobium plasmids. In: Molecular genetics of bacteria-plant interaction pp 113-120. Edited by A. PUHLER, Springer-Verlog, Berlin, Heidelberg.

BRITTEN, R.J., PAVICH, M. & SMITH, J.A. (1970). A new method for DNA purification. Carnegie Institute, Washington Year-book 68, 400-402.

BROCK, J.L. (1973). Growth and nitrogen fixation of pure stands of three pasture legumes with high/low phosphate. New Zealand Journal of Agricultural Research 16, 483-491.

BROCKWELL, J. & HELY, F.W. (1966). Symbiotic characteristics of Rhizobium meliloti: an appraisal of the systematic treatment of nodulation and nitrogen fixation interactions between hosts and rhizobia of diverse origins. Australian Journal of Agricultural Research 17, 885-899.

BROUGHTON, W.J. (1978). A review: Control of specificity in legume-Rhizobium associations. Journal of Applied Bacteriology 45, 165-194.

BUCHANAN-WOLLASTON, A.V., BERINGER, J.E., BREWIN, N.J., HIRSCH,P.R. & JOHNSTON, A.W.B. (1980). Isolation of symbiotically-defective mutants in Rhizobium leguminosarum by insertion of the transposon Tn5 into transmissible plasmid. Molecular and General Genetics 178, 185-190.

BUIKEMA, W.J., LONG, S.R., BROWN, S.E., VAN DEN BOS, R.C., EARL, C. & AUSUBEL, F.M. (1983). Physical and genetic characterisation of Rhizobium meliloti symbiotic mutants. Journal of Molecular and Applied Genetics 2, 249-260.

BURRIS, R.H. (1977). Overview of nitrogen fixation. Basic Life Sciences 9, 9-18.

BURRIS, R.H. (1980). The global nitrogen budget - science or seance? In: Nitrogen fixation volume I: Free-living systems and chemical models. pp 7-16. Edited by W.E. NEWTON & W.E. ORME-JOHNSON. University Park Press, Baltimore.

BURKARDT, B. & BURKARDT, H.J. (1984). Visualisation and exact molecular weight determination of a Rhizobium meliloti megaplasmid. Journal of molecular Biology 175, 213-218.

BURNS, R.C. & HARDY, R.W.F. (1975). Nitrogen fixation in bacteria and higher plants. Springer-Verlag, New York.

CANNON, F.C., RIEDEL, G.E. & AUSUBEL, F.M. (1977). Recombinant plasmid that carries part of the nitrogen fixation (nif) gene cluster of Klebsiella pneumoniae. Proceedings of the National Academy of Sciences of the United States of America 74, 2963-2967.

CANNON, F.C., RIEDEL, G.E. & AUSUBEL, F.M. (1979). Overlapping sequences of Klebsiella pneumoniae nif DNA cloned and characterised. Molecular and General Genetics 174, 59-66.

CANTRELL, M.A., HICKOK, R.E. & EVANS, H.J. (1982). Identification and characterisation of plasmids in hydrogen uptake positive and hydrogen uptake negative strains of Rhizobium japonicum. Archives of Microbiology 131, 102-106.

CASSE, F., BOUCHER, C., JULLIOT, J.S., MICHEL, M. & DENARIE, J. (1979). Identification and characterisation of large plasmids in Rhizobium meliloti using agarose gel electrophoresis. Journal of General Microbiology 113, 229-242.

CEN, Y., BENDER, G.L., TRINICK, M.J., MORRISON, N.A., SCOTT, K.F., GRESSHOFF, P.M., SHINE, J. & ROLFE, B.G. (1982). Transposon mutagenesis in rhizobia which can nodulate both legumes and non-legume Parasponia. Applied and Environmental Microbiology 43, 233-236.

CHANG, A.C.Y. & COHEN, S.N. (1978). Construction and characterisation of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. Journal of Bacteriology 134, 1141-1156.

CLAUSEN, T. (1968). Measurement of ^{32}P activity in a liquid scintillation counter without the use of scintillator. Analytical Biochemistry 22, 70-73.

CLEWELL, D.B. & HELINSKI, D.R. (1969). Supercoiled DNA-protein complex in Escherichia coli: purification and induced conversion to an open circular form. Proceedings of the National Academy of Sciences of the United States of America 62, 1159-1166.

COHEN, S.N., CHANG, A.C.Y. & HSU, L. (1972). Nonchromosomal antibiotic resistance in bacteria:genetic transformation of Escherichia coli by R-factor DNA. Proceedings of the National Academy of Sciences of the United States of America 69, 2110-2114.

COLE, M.A. & ELKAN, G.H. (1973). Transmissible resistance to penicillin G, neomycin, and chloramphenicol in Rhizobium japonicum. Antimicrobial Agents and Chemotherapy 4, 248-253.

CORBIN, D., DITTA, G. & HELINSKI, D.R. (1982). Clustering of nitrogen fixation (nif) genes in Rhizobium meliloti. Journal of Bacteriology 149, 221-228.

CRAIG, A.S. & WILLIAMSON, K.I. (1972). Three inclusions of rhizobial bacteroids and their cytochemical character. Archiv fuer Mikrobiologie 87, 165-171.

CROW, V.L., JARVIS, B.D.W. & GREENWOOD, R.M. (1981). Deoxyribonucleic acid homologies among acid-producing strains of Rhizobium. International Journal of Systematic Bacteriology 31, 152-172.

DANIELS, D.L., DE WET, J.R. & BLATTNER, F.R. (1980). New map of bacteriophage lambda DNA. Journal of Virology 33, 390-400.

DART, P.J. (1977). Infection and development of leguminous nodules. In: A treatise on dinitrogen fixation. Section III, Biology pp367-472. Edited by R.W.F. HARDY & W.S. SILVER. John Wiley, New York.

DAVIS, M.R. (1981a). Growth and nutrition of legumes on a high country yellow-brown earth subsoil. I. Phosphate response of Lotus, Trifolium, Lupinus, Astragalus, and Coronilla species and cultivars. New Zealand Journal of Agricultural Research 24, 321-332.

DAVIS, M.R. (1981b). Growth and nutrition of legumes on a high country yellow-brown earth subsoil. II. A comparison of tropical and temperate species. New Zealand Journal of Agricultural Research 24, 333-337.

DAVIS, M.R. (1981c). Growth and nutrition of legumes on a high country yellow-brown earth subsoil. III. The effect of lime. New Zealand Journal of Agricultural Research 24, 339-348.

DAZZO, F.B. (1980a). Adsorption of microorganisms to roots and other plant surfaces. In: Adsorption of microorganisms to surfaces. pp253-316. Edited by G. BITTON & K. MARSHALL. John Wiley, New York.

DAZZO, F.B. (1980b). Determinants of host specificity in the Rhizobium-clover symbiosis. In: Nitrogen fixation II. pp165-187. Edited by ORME-JOHNSON & W.E. NEWTON. University Park Press, Maryland.

DAZZO, F.B. & HUBBELL, D.H. (1982). Control of root hair infection. In: Nitrogen fixation volume II: Rhizobium. pp275-309. Edited by W.J. BROUGHTON. University Press, Oxford.

DELEY, J. & RASSEL, A. (1965). DNA base composition, flagellation and taxonomy of the genus Rhizobium. Journal of General Microbiology 41, 85-91.

DENARIE, J., BOISTARD, P., CASSE-DELBART, F., ATHERLY, A.G., BERRY, J.O. & RUSSELL, P. (1981). Indigenous plasmids of Rhizobium. In: International Review of Cytology Supplement 13. Biology of the Rhizobiaceae. pp225-246. Edited by K.L. GILES & A.G. ATHERLY. Academic Press, London, New York.

DENHARDT, D.T. (1966). A membrane-filter technique for the detection of complementary DNA. Biochemical Biophysical Research Communications 23, 641-646.

DITTA, G., STANFIELD, S., CORBIN, D. & HELINSKI, D.R. (1980). Broad host range DNA cloning system for gram-negative bacteria:construction of a gene bank of Rhizobium meliloti. Proceedings of the National Academy of Sciences of the United States of America 77, 7347-7351.

DIXON, R.O.D. (1969). Rhizobia with particular reference to relationships with host plants. Annual Review of Microbiology 23, 137-158.

DJORDJEVIC, M.A., ZURKOWSKI, W., SHINE, J. & ROLFE, B.G. (1983). Sym plasmid transfer to various symbiotic mutants of *Rhizobium trifolii*, *R.leguminosarum* and *R.meliloti*. Journal of Bacteriology 156, 1035-1045.

DOWNIE, J.A., HOMBRECHER, G., MA, Q-S., KNIGHT, C.D., WELLS, B. & JOHNSTON, A.W.B. (1983a). Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. Molecular and General Genetics 190, 359-365.

DOWNIE, J.A., MA, Q-S., KNIGHT, C.D., HOMBRECHER, G. & JOHNSTON, A.W.B. (1983b). Cloning of the symbiotic region of *Rhizobium leguminosarum*: The nodulation genes are between the nitrogenase genes and a *nif* A-like gene. EMBO Journal 2, 947-952.

DUNICAN, L.K. & CANNON, F.C. (1971). The genetic control of symbiotic properties in *Rhizobium*: evidence of plasmid control. Plant and Soil Special Volume, 73-79.

DUNICAN, L.K. & TIERNEY, A.B. (1974). Genetic transfer of nitrogen fixation from *Rhizobium trifolii* to *Klebsiella aerogenes*. Biochemical and Biophysical Research Communications 57, 62-72.

DUSHA, I., RENALIER, M.H., TERZAGHI, B., GARNERONE, A.M., BOISTARD, P. & JULLIOT, J.S. (1983). Studies on *Rhizobium meliloti* plasmids and on their role in the control of nodule formation and nitrogen fixation: a strategy for region specific transposon mutagenesis of pSym megaplasmid. In: Molecular genetics of the bacteria-plant interaction. pp46-54. Edited by A. PUHLER, Springer-Verlag, Berlin, Heidelberg.

ECKHARDT, T. (1978). A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1, 584-588.

ELKAN, G.H. & USANIS, R.A. (1971). Theoretical deoxyribonucleic acid homology between strains of Rhizobium japonicum. International Journal of Systematic Bacteriology 21, 295-298.

ELKAN, G.H. (1981). The taxonomy of the Rhizobiaceae. In: International Review of Cytology, Supplement 13. Biology of Rhizobiqceae. pp1-12. Edited by K.L. GILES & A.G. ATHERLY. Academic Press, London, New York.

FAHRAEUS, G. (1957). The infection of clover root hair by nodule bacteria, studied by a simple glass slide technique. Journal of General Microbiology 16, 374-381.

FIGURSKI, D.H. & HELINSKI, D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proceedings of the National Academy of Sciences of the United States of America 76, 1648-1652.

FISCHER, S.G. & LERMAN, L.S. (1979). Length-independent separation of DNA restriction fragments in two dimension gel electrophoresis. Cell 16, 191-200.

FORRAI, T., VINCZE, E., BANFALVI, Z., KISS, G.B., RANDHAWA, G.S. & KONDOROSI, A. (1983). Localisation of symbiotic mutations in Rhizobium meliloti. Journal of Bacteriology 153, 635-643.

FRED, E.B., BALDWIN, I.L. & McCOY, E. (1932). Root nodule bacteria and leguminous plants. University of Wisconsin Studies of Science No.5. University of Wisconsin, Madison.

FRIEDMAN, A.M., LONG, S.R., BROWN, S.E., BUIKEMA, W.J. & AUSUBEL, F.M. (1982). Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of Rhizobium mutants. Gene 18, 289-296.

FUHRMANN, M. & HENNECKE, H. (1982). Coding properties of cloned nitrogenase structural genes from *Rhizonium japonicum*. Molecular and General Genetics *187*, 417-425.

GIBBINS, A.M. & GREGORY, K.F. (1972). Relatedness among *Rhizobium* and *Agrobacterium* species determined by three methods of nucleic acid hybridisation. Journal of Bacteriology *111*, 129-141.

GIBSON, D.I., HAYES, P. & LAIDLAW, A.S. (1975). The influence of phosphate and lime on the growth and nitrogen fixation of *Lotus uliginosus* and *Trifolium repens* under greenhouse conditions. Journal of the British Grassland Society *30*, 295-301.

GOODCHILD, D.J. (1977). The ultrastructure of root nodules in relation to nitrogen fixation. International Review of Cytology Supplement 6, 235-288.

GRAHAM, P.H. (1964). The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. Journal of General Microbiology *35*, 511-517.

GROSS, D.C., VIDAVER, A.K. & KLUCAS, R.V. (1979). Plasmids, biological properties and efficacy of nitrogen fixation in *Rhizobium japonicum* strains indigenous to alkaline soils. Journal of General Microbiology *114*, 257-266.

GRUNSTEIN, M. & HOGNESS, D.S. (1975). Colony hybridisation: A method for the isolation of cloned DNAs that contain a specific gene. Proceedings of the National Academy of Sciences of the United States of America *72*, 3961-3965.

HAAS, D. & HOLLOWAY, B.W. (1976). R. factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Molecular and General Genetics *144*, 243-251.

HAHN, M. & HENNECKE, H. (1984). Localised mutagenesis in *Rhizobium japonicum*. Molecular and General Genetics *193*, 46-52.

HART, A.L., HALLIGAN, G. & HASELMORE, R.M. (1981). Analysis of the response to phosphorus by pasture legumes in a controlled environment. New Zealand Journal of Agricultural Research *24*, 197-201.

HAUGLAND, R. & VERMA, D.P.S. (1981). Interspecific plasmid and genomic DNA sequence homologies and localization of *nif* genes in effective and ineffective strains of *Rhizobium japonicum*. Journal of Molecular and Applied Genetics *1*, 205-217.

HELMERHORST, E. & STOKES, G.B. (1980). Microcentrifuge desalting: A rapid quantitative method for desalting small amounts of protein. Analytical Biochemistry *104*, 130-135.

HENNECKE, H. (1981). Recombinant plasmids carrying nitrogen fixation genes from *Rhizobium japonicum*. Nature *291*, 354-355.

HERBERLEIN, G.T., DE LEY, J. & TIJTGAT, R. (1967). Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium* and *Chromobacterium*. Journal of Bacteriology *94*, 116-124.

HIGASHI, S. (1967). Transfer of clover infectivity of *R.trifolii* to *R.phaseoli* as mediated by an episomic factor. Journal of General and Applied Microbiology *13*, 391-403.

HIRSCH, P.R., VAN MONTAGU, M., JOHNSTON, A.W.B., BREWIN, N.J. & SCHELL, J. (1980). Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. Journal of General Microbiology *120*, 403-412.

HIRSCH, A.M., LONG, S.R., BANG, M., HASKINS, N. & AUSUBEL, F.M. (1982). Structural studies of alfalfa roots infected with nodulation mutants of _Rhizobium meliloti_. Journal of Bacteriology _151_, 411-419.

HOHN, B. (1979). _In vitro_ packaging of λ and cosmid DNA. In: Methods in Enzymology _68_, 299-309. Edited by R. WU. Academic Press, New York.

HOLLIS, A.B., KLOOS, W.E. & ELKAN, G.H. (1981). DNA:DNA hybridisation studies of _Rhizobium japonicum_ and related _Rhizobiaceae_. Journal of General Microboilogy _123_, 215-222.

HOLMES, D.S. & QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Analytical Biochemistry _114_, 193-197.

HOLSTERS, M., SILVA, B., GENETELLO, C., ENGLER, G., VAN VLIET, F., DE BLOCK, M., VILLARROEL, R., VAN MONTAGU, M. & SCHELL, J. (1978). Spontaneous formation of cointegrates of the oncogenic Ti-plasmid and the wide-host-range P-plasmid RP4. Plasmid _1_, 456-467.

HOMBRECHER, G., BREWIN, N.J. & JOHNSTON, A.W.B. (1981). Linkage of genes for nitrogenase and nodulation ability on plasmids in _Rhizobium leguminosarum_ and _R.phaseoli_. Molecular and General Genetics _182_, 133-136.

HOOYKAAS, P.J.J., VAN BRUSSELL, A.A.N., DEN DULK-RAS, H., VAN SLOGTEREN, G.M.S. & SCHILPEROORT, R.A. (1981). Sym-plasmid of _Rhizobium trifolii_ expressed in different rhizobial species and in _Agrobacterium tumefaciens_. Nature _291_, 351-353.

HUGUET, T., ROSENBERG, C., CASSE-DELBART, F., DE LAJUDIE, P., JOUANIN, L., BATUT, J., BOISTARD, P., JULLIOT, J.S. & DENARIE, J. (1983). Studies on _Rhizobium meliloti_ plasmids and on their role in the control of nodule formation and nitrogen fixation: the pSym megaplasmids and other large plasmids. In: Molecular genetics of the bacteria-plant interaction. pp35-45. Edited by A. PUHLER. Springer-Verlag

ISH-HOROWITZ, D. & BURKE, J.F. (1981). Rapid and efficient cosmid cloning. Nucleic Acids Research 9, 2989-2998.

JACOB, A.E., CRESSWELL, J.M., HEDGES, R.W., COETZEE, J.N. & BERINGER, J.E. (1976). Properties of plasmids constructed by the *in vitro* insertion of DNA from *Rhizobium leguminosarum* or *Proteus mirabilis* into RP4. Molecular and General Genetics 147, 315-323.

JARVIS, B.D.W., MACLEAN, T.S., ROBERTSON, I.G.C. & FANNING, G.R. (1977). Phenetic similarity and DNA base sequence homology of root nodule bacteria from New Zealand native legumes and *Rhizobium* strains from agricultural plants. New Zealand Journal of Agricultural Research 20, 235-248.

JARVIS, B.D.W., DICK, A.G. & GREENWOOD, R.M. (1980). Deoxyribonucleic acid homology among strains of *Rhizobium trifolii* and related species. Journal of Systematic Bacteriology 30, 42-52.

JARVIS, B.D.W., PANKHURST, C.E. & PATEL, J.J. (1982). *Rhizobium loti*, a new species of legume root nodule bacteria. International Journal of Systematic Bacteriology 32, 378-380.

JENSEN, H.L. (1967). Mutual host plant relationships in two groups of legume root nodule bacteria (*Rhizobium* spp.). Archiv fur Mikrobiologie 59, 174-179.

JENSEN, H.L. & HANSEN, A.L. (1968). Observations on host plant relation in root nodule bacteria of the *Lotus-Anthyllis* and the *Lupinus-Ornithopus* groups. Acta Agriculturae Scandinavica 18, 135-142.

JOHNSTON, A.W.B., BEYNON, J.L., BUCHANAN-WOLLASTON, A.V., SETCHELL, S.M., HIRSCH, P.R. & BERINGER, J.E. (1978). High frequency transfer of nodulation ability between strains and species of *Rhizobium*. Nature 276, 634-636.

JONES, W.T., LYTTLETON, J.W. & CLARKE, R.T.J. (1970). Bloat in cattle XXXIII. The soluble proteins of legume forages in New Zealand, and their relationship to bloat. New Zealand Journal of Agricultural Research 13, 149-156.

JONES, W.T., BROADHURST, R.B. & GURNSEY, M.P. (1982). Partial characterisation of bovine salivary proteins by electrophoretic methods. Biochemica et Biophysics Acta 701, 382-388.

JORDAN, D.C. & ALLEN, O.N. (1974). Family III. Rhizobiaceae Conn, 1938. In: Bergey's manual of determinative bacteriology, 8th edition pp261-264. Edited by R.D. BUCHANAN & N.E. GIBBONS. The Williams and Wilkins Co., Baltimore.

JORDAN, D.C. (1982). Transfer of Rhizobium japonicum Buchanan 1980 to Bradyrhizobium japonicum gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. International Journal of Systematic Bacteriology 32, 136-139.

JORDAN, D.C. (1984). Family III. Rhizobiaceae Conn, 1938. In: Bergey's manual of systematic bacteriology, 9th edition, volume 1 pp234-244. Edited by N.R. KREIG and HOLT, J.G.. The Williams and Wilkins Co., Balitmore.

JORGENSEN, R.A., ROTHSTEIN, S.J. & REZNIKOFF, W.S. (1979). A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Molecular and General Genetics 177, 65-72.

KADO, C.I. & LIU, S.T. (1981). Rapid procedure for detection and isolation of large and small plasmids. Journal of Bacteriology 145, 1365-1373.

KAIN, W.M. & ATKINSON, D.J. (1975). The development of resistant pasture and methods of pasture management for grass grub control. Proceedings of the New Zealand Grassland Association 37, 132-137.

KLECKNER, N., ROTH, J & BOTSTEIN, D. (1977). Genetic engineering *in vivo* using translocatable drug-resistance elements: New methods in bacterial genetics. Journal of Molecular Biology *116*, 125-159.

KONDOROSI, A. & JOHNSTON, A.W.B. (1981). The genetics of *Rhizobium*. In: International Review of Cytology Supplement 13. Biology of the *Rhizobiaceae*, pp191-219 Edited by K.L. GILES & A.G. ATHERLY. Academic Press, London, New York.

KONDOROSI, A., KONDOROSI, E., PANKHURST, C.E., BROUGHTON, W.J. & BANFALVI, Z. (1982). Mobilization of a *Rhizobium meliloti* megaplasmid carrying nodultion and nitrogen fixation genes into other rhizobia and *Agrobacterium*. Molecular and General Genetics *188*, 433-439.

KONDOROSI, E., BANFALVI, Z. & KONDOROSI, A. (1984). Physical and genetic analysis of a symbiotic region of *Rhizobium meliloti*: Identification of nodulation genes. Molecular and General Genetics *193*, 445-452.

KROLL, J. (1973). Tandem-crossed immunoelectrophoresis. In: Scandinavian Journal of Immunology Supplement, volume 2 (No. 1). A manual of quantitative immunoelectrophoresis, methods and applications pp57-59. Edited by N.H. AXELSON, J. KROLL & B. WEEKE Universitetsforlaget, Oslo.

LAMB, J., HOMBRECHER, G. & JOHNSTON, A.W.B. (1982). Transmissible nodulation plasmid in a strain of *Rhizobium phaseoli*. Molecular and General Genetics *186*, 449-452.

LANCASHIRE, J.A., GOMEZ, J.S. & McKELLAR, A. (1980) 'Grasslands Maku' *Lotus* seed production research and practice. Herbage Seed Production: pp80-86. New Zealand Grassland Association.

LANGE, R.T. (1961). Nodule bacteria associated with the indigenous leguminosae of South-Western Australia. Journal of General Microbiology *26*, 351-359.

LEVY, E.B. (1918). The birdsfoot trefoils. New Zealand Journal of Agriculture 17, 347-351.

LIBBENGA, K.R. & BOGERS, R.L. (1974). Root-nodule morphogenesis. In: The biology of nitrogen fixation pp430-471. Edited by A. QUIPEL. North Holland, Amsterdam.

LOHNIS, F. & HANSEN, R. (1921). Nodule bacteria of leguminous plants. Journal of Agricultural Research 20, 543-546.

LONG, S.R., BUIKEMA, W.J. & AUSUBEL, F.M. (1982). Cloning of Rhizobium meliloti nodulation genes by direct complementation of Nod$^-$ mutants. Nature 298, 485-488.

LOWTHER, W.L. (1977). Comparison of Huia white clover and Maku lotus oversown separately or as a mixture. Proceedings of the New Zealand Grassland Association 38, 133-139.

MA, Q-S., JOHNSTON, A.W.B., HOMBRECHER, G. & DOWNIE, J.A. (1982). Molecular genetics of mutants of Rhizobium leguminosarum which fail to fix nitrogen. Molecular and General Genetics 187, 166-171.

MAIER, R.J. & BRILL, W.J. (1976). Ineffective and non-nodulating mutant strains of Rhizobium japonicum. Journal of Bacteriology 127, 763-769.

MARVEL, D.J., KULDAU, G., HIRSCH, A.M., PARK, J., TORREY, J.G. & AUSUBEL, F.M. (1984). Cloning and characterisation of a nodulation locus from Rhizobium parasponium. In: Advances in nitrogen fixation research pp691. Edited by C. VEEGER & W.E. NEWTON. Nijhoff/Junk Pudoc, Wageningen.

MASTERSON, R.V., RUSSELL, P.R. & ATHERLY, A.G. (1982). Nitrogen fixation (nif) genes and large plasmids of Rhizobium japonicum. Journal of Bacteriology 152, 928-931.

MAZUR, B.J., RICE, D. & HASELKORN, R. (1980). Identification of blue-green algal nitrogen fixation genes by using heterologous DNA hybridisation probes. Proceedings of the National Academy of Sciences of the United States of America 77, 186-190.

MEADE, H.M., LONG, S.R., RUVKUN, G.B., BROWN, S.E. & AUSUBEL, F.M. (1982). Physical and genetic characterisation of symbiotic and auxotrophic mutants of R.meliloti induced by transposon Tn5 mutagenesis. Journal of Bacteriology 149, 114-122.

MEIJER, E.G.M. (1982). Development of leguminous nodules. In: Nitrogen fixation volume 2: Rhizobium pp311-331. Edited by W.J. BROUGHTON, Clarendon Press, Oxford.

MILLER, J.H. (1972). Ultraviolet light mutagenesis. In: Experiments in molecular genetics pp121-124. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

MILLER, J.H. (1972). Formulae and Recipes. In: Experiments in molecular genetics pp433. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

MOFFETT, M.L. & COLWELL, R.R. (1968). Adansonian analysis of the Rhizobiaceae. Journal of General Microbiology 51, 245-266.

NAPOLI, C. & ALBERSHEIM, P. (1980). Infection and nodulation of clover by nonmotile Rhizobium trifolii. Journal of Bacteriology 141, 979-980.

NEWCOMB, W. (1981). Nodule morphogenesis and differentiation. In: International Review of Cytology Supplemnt 13. Biology of the Rhizobiaceae pp247-298. Edited by K.L. GILES & A.G. ATHERLY. Academic Press, London, New York.

NOEL, K.L., SANCHEZ, A., FERNANDEZ, L., LEEMANS, J. & CEVALLOS, M.A. (1984). Rhizobium phaseoli symbiotic mutants with transposon Tn5 insertions. Journal of Bacteriology 158, 148-155.

NORDMEYER, A.H. & DAVIS, M.R. (1977). Legumes in high-country development. Proceedings of the New Zealand Grassland Association, 38, 119-125.

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

NUTI, M.P., LEDEBOER, A.M., LEPIDI, A.A. & SCHILPEROORT, R.A. (1977). Large plasmids in different *Rhizobium* species. Journal of General Microbiology 100, 241-248.

NUTI, M.P., LEPIDI, A.A., PRAKASH, R.K., SCHILPEROORT, R.A. & CANNON, F.C. (1979). Evidence for nitrogen fixation (*nif*) genes on indigenous *Rhizobium* plasmids. Nature 282, 533-535.

PANKHURST, C.E. (1977). Symbiotic effectiveness of antibiotic resistant mutants of fast-and slow-growing strains of *Rhizobium* nodulating *Lotus*species. Canadian Journal of Microbiology 23, 1026-1033.

PANKHURST, C.E., CRAIG, A.S. & JONES, W.T. (1979). Effectiveness of *Lotus* root nodules I: morphology and flavolan content of nodules formed on *Lotus pedunculatus* by fast-growing *Lotus* rhizobia. Journal of Experimental Botany 30, 1085-1093.

PANKHURST, C.E., BROUGHTON, W.J. & WIENEKE, U. (1983). Transfer of an indigenous plasmid of *Rhizobium loti* to other rhizobia and *Agrobacterium tumefaciens*. Journal of General Microbiology 129, 2535-2543.

PARHAM, R.A. & KAUSTINEN, H.M. (1977). On the site of tannin synthesis in plant cells. Botanical Gazette 138, 465-467.

PATEL, J.J. (1976). Morphology and host range of virulent phages of *Lotus* rhizobia. Canadian Journal of Microbiology 22, 204-212.

PRAKASH, R.K., HOOYKAAS, P.J.J., LEDEBOER, A.M., KIJNE, J.W., SCHILPEROORT, R.A., NUTI, M.P., LEPIDI, A.A., CASSE, F., BOUCHER, C., JULLIOT, J.S. & DENARIE, J. (1980). Detection, Isolation, and characterisation of large plasmids in Rhizobium. Nitrogen Fixation, Volume II pp139-163. Edited by W.E. Newton and W.H. Orme-Johnson. University Park Press. Baltimore pp139-163.

PRAKASH, R.K., SCHILPEROORT, R.A. & NUTI, M.P. (1981). Large plasmids of fast-growing rhizobia: homology studies and location of structural nitrogen fixation (nif) genes. Journal of Bacteriology 145, 1129-1136.

RALEIGH, E.A. & SIGNER, E.R. (1982). Positive selection of nodulation deficient Rhizobium phaseoli. Journal of Bacteriology 151, 83-88.

ROBERTS, G.P., WALTER, T.L., SILVER, L.E. & BRILL, W.J. (1980). Use of two dimensional polyacrylamide gel electrophoresis to identify and classify Rhizobium strains. Applied and Environmental Microbiology 39, 414-422.

ROBERTSON, J.G., LYTTLETON, P., BULLIVANT, S., GRAYSTON, G.F. (1978). Membranes in lupin root nodules. I. The role of golgi bodies in the biogenesis of infection threads and peribacteroid membranes. Journal of Cell Science 30, 129-149.

ROBERTSON, J.G. & FARNDEN, K.J.F. (1980). Ultrastructure and metabolism of the developing legume root nodule. In: The biochemistry of plants: a comprehensive treatise. Edited by P.K. STUMPF and E.E. COHN. Volume 5. Amino acids: their synthesis and metabolism pp65-113. Edited by B.J. MIFLIN. Academic Press, New York.

ROLFE, B.G., GRESSHOFF, P.M. & SHINE, J. (1980). Rapid screening for symbiotic mutants of Rhizobium and white clover. Plant Science Letter 19, 277-284.

ROLFE, B.G., DJORDJEVIC, M., SCOTT, K.F., HUGHES, J.E., BADENOCH-JONES, J., GRESSHOFF, P.M., CEN, Y., DUDMAN, W.F., ZURKOWSKI, W. & SHINE, J. (1981). Analysis of the nodule forming ability of fast-growing Rhizobium strains. In: Current perspectives in nitrogen fixation. pp142-145. Edited by A.H. GIBSON and W.E. NEWTON. Australian Academy of Science, Canberra.

ROLFE, B.G., RIDGE, R.W., MORRISON, N.A., SCHOFIELD, P.R., BASSAM, B.J., WATSON, J.M., SHINE, J. & DJORDJEVIC, M.A. (1984). Conservation of root hair curling (Hac) functions in fast-growing rhizobia. In: Advances in nitrogen fixation research pp719. Edited by C. VEEGER & W.E. NEWTON. Nijhoff/Junk, PUDOC, Wageningen.

RONSON, C.W. & SCOTT, D.B. (1983). Identification, broad host range mobilization and mutagenesis of a Rhizobium trifolii Sym::R68.45 cointegrate plasmid. In: Molecular genetics of the bacteria-plant interaction pp177-187. Edited by A. PUHLER. Springer-Verlag, Berlin, Heidelberg.

RONSON, C.W., ASTWOOD, P.M. & DOWNIE, J.A. (1984). Molecular cloning and genetic organisation of C_4-dicarboxylate transport genes from Rhizobium leguminosarum. (In press).

ROSENBERG, C., BOISTARD, P., DENARIE, J. & CASSE-DELBART, F. (1981). Genes controlling early and late functions in symbiosis are located on a megaplasmid in Rhizobium meliloti. Molecular and General Genetics 184, 326-333.

RUSSELL, P.E. & JONES, D.G. (1973). The effect of ultra violet light on the symbiotic effectiveness and some physiological characteristics of four strains of Rhizobium trifolii. Journal of Applied Bacteriology 36, 567-573.

RUVKUN, G.B. & AUSUBEL, F.M. (1980). Interspecies homology of nitrogenase genes. Proceedings of the National Academy of Sciences of the United States of America 77, 191-195.

RUVKUN, G.B. & AUSUBEL, F.M. (1981). A general method for site-directed mutagenesis in prokaryotes. Nature 289, 85-88.

RUVKUN, G.B., SUNDARESAN, V. & AUSUBEL, F.M. (1982). Site-directed transposon Tn5 mutagenesis and complementation analysis of the Rhizobium meliloti symbiotic nitrogen fixation (nif) genes. Cell 29, 551-559.

SANGER, F., COULSON, A.R., HONG, G.F., HILL, D.F. & PETERSON, G.B. (1982). Nucleotide sequence of bacteriophage λ DNA. Journal of Molecular Biology 162, 729-773.

SCHOFIELD, P.R., DJORDJEVIC, M.A., ROLFE, B.G., SHINE, J. & WATSON, J.M. (1983). A molecular linkage map of nitrogenase and nodulation genes in Rhizobium trifolii. Molecular and General Genetics 192, 459-465.

SCHOFIELD, P.R., RIDGE, R.W., DJORDJEVIC, M.A., ROLFE, B.G., SHINE, J. & WATSON, J.M. (1984). Host-specific nodulation is encoded on a 14kb DNA fragment in Rhizobium trifolii. Plant Molecular Biology 3, 3-11.

SCHOLLA, M.H. & ELKAN, G.H. (1984). Rhizobium fredii sp. nov., a fast-growing species that effectively nodulates soybeans. (In press).

SCHOLLA, M.H., MOOREFIELD, J.A. & ELKAN, G.H. (1984). DNA homology between fast-growing soybean nodulating bacteria and other rhizobia. (In press).

SCHWINGHAMER, E.A. (1960). A study of induced variation in rhizobia. I. Defined media and nodulation test techniques. Applied Microbiology 8, 349-352.

SCHWINGHAMER, E.A. (1980). A method for improved lysis of some Gram-negative bacteria. Federation of European Microbiological Societies Microbiology Letters 7, 157-162.

SCOTT, R.S. (1979). Lotus pedunculatus 'Grassland Maku', on acid tussock grassland soils. Aglink, 1/2000/2/79 FPP204. New Zealand.

SCOTT, D.B. & TAIT, R.C. (1980). Detection of Plasmids in Rhizobium japonicum. Basic Life Sciences 17, 137-140.

SCOTT, D.B. & RONSON, C.W. (1982). Identification and mobilization by cointegrate formation of a nodulation plasmid in Rhizobium trifolii. Journal of Bacteriology 151, 36-43.

SCOTT, D.B., COURT, C.B., RONSON, C.W., SCOTT, K.F., WATSON, J.M., SCHOFIELD, P.R. & SHINE, J. (1984). Organisation of nodulation and nitrogen fixation genes on a Rhizobium trifolii symbiotic plasmid. Archives of Microbiology (In press).

SCOTT, K.F., HUGHES, J.E., GRESSHOFF, P.M., BERINGER, J.E., ROLFE, B.G. & SHINE, J. (1982). Molecular cloning of Rhizobium trifolii genes involved in symbiotic nitrogen fixation. Journal of Molecular and Applied Genetics 1, 315-326.

SIMON, R., PRIEFER, U. & PUHLER, A. (1983). Vector plasmids for in vivo and in vitro manipulation of Gram-negative bacteria. In: Molecular genetics of the bacteria-plant interaction, pp98-106. Edited by A. PUHLER. Berlin, Springer-Verlag.

SIMON, R., PRIEFER, U. & PUHLER, A. (1984). A broad host range mobilisation system for in vivo genetic engineering: random and site-specific transposon mutagensis in Gram-negative bacteria. Biotechnology 1, 784-791.

SOUTHERN, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98, 503-518.

STACEY, G., PAAU, A.S., NOEL, K.D., MAIER, R.J., SILVER, L.E. & BRILL, W.J. (1982). Mutants of Rhizobium japonicum defective in nodulation. Archives of Microbiology 132, 219-224.

SUTTON, W.D. (1974). Some features of the DNA of Rhizobium bacteroids and bacteria. Biochimica et Biophysica Acta 366, 1-10.

SUTTON, W.D., PANKHURST, C.E. & CRAIG, A.S. (1981). The Rhizobium bacteroid state. In: International Review of Cytology Supplement 13. Biology of the Rhizobiacene pp149-171. Edited by K.L. GILES & A.G. ATHERLEY. Academic Press, London, New York.

SUTTON, W.D. (1982). Nodule development and senescence. In: Nitrogen fixation Volume 3: Legumes pp144-212. Edited by W.J. BROUGHTON. University Press. Oxford.

SUTTON, B.C.S., STANLEY, J., ZELECHOWSKA, M.G. & VERMA, D.P.S. (1984). Isolation and expression of Rhizobium japonicum cloned DNA encoding an early soybean nodulation function. Journal of Bacteriology 158, 920-927.

TAYLOR, J.M., ILLMENSEE, R. & SUMMERS, J.S. (1976). Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochemical Biophysical Acta 442, 324-330.

THORNTON, H.G. (1930). The early development of the root-nodule of lucerne (Medicago sativa L.). Annals of Botany 44, 385-392.

TMANNETJE, L. (1967). A reexamination of the taxonomy of the genus Rhizobium and related genera using numerical analysis. Antonie van Leeuwenhoek 33, 477-491.

VAN VLIET, F., SILVA, B., VAN MONTAGU, M. & SCHELL, J. (1978). Transfer of RP4 :: Mu plasmids to Agrobacterium tumefaciens. Plasmid 1, 446-455.

VENABLE, J.H. & COGGESHALL, R. (1965). A simplified lead citrate stain for use in electron microscopy. Journal of Cell Biology 25, 407-408.

VINCENT, J.M. (1970). The cultivation, isolation and maintenance of rhizobia. In: A manual for the practical study of root-nodule bacteria pp3. (IPB Handbook no 15). Oxford, Blackwell Scientific Publications.

VINCENT, J.M. (1974). Root nodule symbiosis with Rhizobium. In: The biology of nitrogen fixation pp266-341. Edited by A. QUIPEL. North Holland Pub. Co. Amsterdam. The Netherlands.

VINCENT, J.M. (1980). Factors controlling the legume-Rhizobium symbiosis. In: Nitrogen fixation, volume II, pp 103-129. Edited by W.E. NEWTON and W.H. ORME-JOHNSON. University Park Press, Baltimore, Maryland.

WAHL, G.M., STERN, M. & STARK, G.R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridisation by using dextran sulfate. Proceedings of the National Academy of Sciences of the United States of America 76, 3683-3687.

WHITE, L.D. (1972). The taxonomy of the crown-gall organism Agrobacterium tumefaciens and its relationship to rhizobia and other agrobacteria. Journal of General Microbioology 72, 565-574.

WHITFELD, P.L., SEEBURG, P.H. & SHINE, J. (1982). The human pro-opiomelanocortin gene: organisation, sequence, and interpersion with repetitive DNA. DNA 1, 133-143.

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

WITKIN, E.M. (1976). Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacteriological Review 40, 869-907.

WOOD, S.M., LAYZELL, D.B., NEWCOMB, W. & PANKHURST, C.E. (1984). A morphometric study of effective nodules induced by Rhizobium loti and Bradyrhizobium spp. (Lotus) on Lotus pedunculatus. (In press).

YAO, P.Y. & VINCENT, J.M. (1968). Host specificity in the root hair 'curling factor' of *Rhizobium* spp. Australian Journal of Biological Science *22*, 413-423.

ZAENEN, J., VAN LAREBEKE, N., TEUCHY, H., VAN MONTAGU, M. & SCHELL, J. (1974). Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. Journal of Molecular Biology *86*, 109-127.

ZURKOWSKI, W., HOFFMAN, M. & LORKIEWICZ, Z. (1973). Effect of acriflavine and sodium dodecyl sulphate on infectiveness of *R.trifolii*. Acta Microbiologia Polonica, Series A*5*, 55-60.

ZURKOWSKI, W. & LORKIEWICZ, Z. (1978). Effective method for the isolation of non-nodulating mutants of *Rhizobium trifolii*. Genetic Research *32*, 311-314.

ZURKOWSKI, W. & LORKIEWICZ, Z. (1979). Plasmid-mediated control of nodulation in *Rhizobium trifolii*. Archives of Microbiology *123*, 195-201.