Characterisation of a *Rhizobium loti* nodulation mutant.

A thesis presented in partial fulfilment of the requirements
for the degree of
Doctor of Philosophy
in molecular genetics at
Massey University

Julie Marie Collins-Emerson

1991

ABSTRACT.

The aim of the project was to characterise the *Rhizobium loti* Nod$^-$ Tn*5* mutant strain, PN233. The Tn*5* insertion had been previously localised to a 7.1 kb *Eco* RI chromosomal fragment. This fragment was sub-cloned and a *Bam* HI/*Sal* I endonuclease restriction map for the region was determined. *Hind* III digests were utilised to identify the approximate location of the Tn*5* 233 insertion and those of four other Tn*5* insertions (4016, 4019, 4047 and 4053) in the 7.1 kb region. The 233 mutation was found to map to a 1.45 kb *Sal* I fragment and that of an overlapping 2.8 kb *Bam* HI fragment.

The 7.1 kb *Eco* RI fragment and a larger 22.7 kb fragment that encompassed this region, had been cloned into pLAFR1. The construct carrying the 22.7 kb fragment (pPN305) was crossed into four *R.l.* bv. *trifolii* strains, each mutant in one of the four common *nod* genes, A,B,C, and D. The construct was able to complement the *nod*C mutation indicating the presence of a *nod*C gene somewhere on the 22.7 kb region.

The mutations 4047 and 4053 had been found to map to either side of the 233 Tn*5* insertion. Both insertions affected nodule formation and were thus included in further plant complementation tests. These experiments involved crossing both the pPN305 and a construct bearing the smaller 7.1 kb *Eco* RI fragment (pPN25) into the *R. loti* and *R.l.* bv. *trifolii* Tn5 mutants. What was unusual about the results was that, while the 7.1 kb fragment was able to complement the mutations, the larger 22.7 kb fragment which encompasses that region could complement PN4047 and PN4053 but was unable to complement the PN233 mutant.

The 2.8 kb *Bam* HI and 1.45 kb *Sal* I fragments, to which the 233 insertion was mapped, and that of an adjacent 1.2 kb *Sal* I fragment, were sub-cloned and then *Bal* 31 digested in both orientations to create a series of overlapping fragments. These fragments were then sequenced. The data revealed that the 233 Tn*5* had inserted into the *R. loti nod*C gene. It was determined that the 4047 Tn*5* was also located in this gene, slightly upstream of 233, while 4053 had inserted into

the 5'-region of *nod*I which is downstream of *nod*C. *Nod*A was identified upstream of *nod*C indicating an arrangement of common *nod* genes different from the conventional *nod*ABCIJ found in other rhizobia. The promoter for these *nod* genes, the *nod* box, was located upstream of the *nod*A gene.

A particularly puzzling aspect of the results is that, while PN4047 is complemented by both pPN305 and pPN25, PN233, which has an insertion in the same gene, could only be complemented by the smaller fragment carried by the pPN25 construct. To explain this result, it is proposed that PN233 is producing a mutant NodC protein and that this, in combination with doubled copies of a gene or genes present elsewhere on the 22.7 kb fragment, is responsible for interfering with complementation in this mutant. Alternatively, it may be that the imbalance of doubled copies of downstream, co-transcribed genes in the presence of one copy of a functional *nod*C gene causes complementation failure.

ACKNOWLEDGEMENTS.

Sincere thanks goes to my chief supervisor Assoc. Prof. Eric Terzaghi for his guidance, patience, enthusiasm and friendship and to Prof. Barry Scott for his advice and assistance and for providing the research topic.

I wish to express my gratitude to the Department of Microbiology and Genetics for their support. Also, many staff members have been generous in their friendship and given of their time in either a technical or advisory capacity, in particular Robert C., Carolyn, Ron, Dawn, Shirley and Trish, while Laura Medhurst and Steven Thomas worked on the computer diagrams. This has been much appreciated.

The encouragement and friendship shown by Mike, Trish, Carolyn, David, Wil, Robert H., Grant, Mark and Chris and many members of the MGU past and present is most valued.

My parents are thanked for their encouragement and support throughout my education (and Dad for his proof reading).

Finally, sincere thanks go to my husband Stephen for his friendship, support and love - a vital ingredient in the success and completion of the project.

TABLE OF CONTENTS. page no.

LIST OF FIGURES.

LIST OF TABLES.

1.0 INTRODUCTION.

Microbes play an important role in the biological cycles of carbon, oxygen, nitrogen and sulphur which are fundamental to life on this planet. While nitrogen is abundant on Earth, most of it is not readily utilisable by plants. Consequently, one of the factors that most limits global agricultural productivity is the availability of fixed nitrogen. The dinitrogen bond is very strong. Consequently, industrial processes directed towards fixing nitrogen require specialised conditions and are energy-intensive. However, many microbes in either a free-living state and/or in a symbiotic relationship with a plant are capable of fixing atmospheric nitrogen by converting it to ammonia via the following chemical conversions.

$$N_2 + 6e^- + 6H^+ + 12ATP \xrightarrow[\text{NITROGENASE}]{Mg^{2+}} 2NH_3 + 12ADP + 12Pi$$

At present, a vast amount of money and effort is expended on applying industrial nitrogenous fertiliser, which is mainly a product of the Haber process, to agricultural crops. The impact of industrial fertilisers on the environment and the economic reality that nitrogen fertiliser is beyond the reach of many countries, is of worldwide concern. Research into biological nitrogen fixation is therefore of considerable interest, not only academically, but because of the potential applications it may have for crop improvement and productivity.

1.1 DINITROGEN-FIXING PLANT - MICROSYMBIONT ASSOCIATIONS.

1.1.1 NON-LEGUMINOUS ASSOCIATIONS.

A number of dicotyledonous, non-leguminous plants from phylogenetically unrelated families and genera, form nitrogen-fixing root nodules in symbiotic relationships with endophytes that mainly belong to the order Actinomycetales and to the genus *Rhizobium*. The actinomycete endophytes of non-legumes can be placed in one family Frankiaceae, with a single genus *Frankia* (Becking, 1975). Examples of

some plant genera that have members which bear non-leguminous nodules formed by actinomycete-host symbioses are *Casuarina*, *Myrica*, *Alnus*, *Cerocarpus*, *Coriaria*, *Comptonia*, and *Colletia* (Becking, 1975; Bowes *et al.*, 1977; Callaham *et al.*, 1979). Some non-leguminous, nodule-bearing dicotyledonous hosts capable of symbioses with *Rhizobium* or supposed *Rhizobium* species are *Trema*, *Parasponia*, *Zygophyllum*, *Fagonia*, *Viola* and *Opuntia* (Becking, 1975).

Two morphological types of actinomycetous nodules are known: 1) *Alnus*-type and 2) *Myrica-Casuarina*-type (Torrey and Callaham, 1978). *Alnus*-type nodules are found in representatives of Belutaceae, Elaegnaceae, Rhamnaceae, Coriariaceae and Rosaceae. The nodules comprise modified, often dicotomously-branched roots of arrested growth which usually have a coralloid appearance (Becking, 1975). *Myrica/Casuarina*-type nodule lobes give rise to a normal root which is negatively geotropic (Torrey and Callaham, 1978).

Actinomycetes enter the host plant via root hair infection. These hairs curl on invasion by the actinomycete (Torrey and Callaham, 1978; Callaham *et al.*, 1979; Lalonde, 1980). The hyphae perforate the root cortical cells by local degradation of the cell walls and penetrate the host cell cytoplasm where it is then surrounded by host plasma membrane and a thick polysaccharide material termed the capsule. The hyphae branch extensively in specific layers of the cortex, penetrating most of the host cytoplasm (Newcomb *et al.*, 1978; Lalonde, 1980). These nodules are highly modified lateral branches, both in their origin and in their development (Becking, 1975; Torrey and Callaham, 1978).

1.1.2 LEGUMINOSEAE AND *RHIZOBIUM*.

Much research effort is focused on nitrogen fixation in leguminous plants, many of which are of immense agricultural significance both as pasture and as food crops, examples being clover, lucerne (alfalfa), peas, beans, soya beans and peanuts. This symbiotic relationship enables them to grow in nitrogen-deficient soils and hence they are also of ecological importance, in that they can be used

to reclaim poor and nitrogen-deficient land. There are about 18,000 species in the family Leguminoseae and over 90 percent of plants in the sub-families Mimosodeae and Papilionoideae bear highly specialised root nodules which provide the appropriate microaerobic conditions necessary for the nitrogen-fixing bacterial symbiont, *Rhizobium* (Vincent, 1982).

Members of the genus *Rhizobium* characteristically invade the roots of leguminous plants and produce root nodules. Taxonomic classification of *Rhizobium* tends to be based on the plant affinity (cross-inoculation group) concept. Until recently, fast (generation time of under 6 hours) and slow (generation time greater than 6 hours) -growing rhizobia were grouped in the same genus *Rhizobium*. However studies of numerical taxonomy, RNA cistron similarities, DNA base ratio determination, nucleic acid hybridisation, immunology, composition of extracellular polysaccharides, carbohydrate utilisation and metabolism, bacteriophage and antibiotic susceptibilities, protein composition and types of intracellular inclusion bodies in bacteroids (Vincent, 1977; Elkan, 1981; Jordan, 1982; Trinick, 1980) have supported a major division of the genus into the fast-growing *Rhizobium* and the slow-growing *Bradyrhizobium* sp. (Buchanan-Wollaston *et al.*, 1980; Jordan, 1982).

Fast-growing *R. japonicum* isolated from Asian-type soya beans have physiological characteristics similar to other fast-growing rhizobia, but their symbiotic properties are similar to the cowpea miscellany (Stowers and Eaglesham, 1984). These types of rhizobia have been grouped into a new species, *R. fredii* (Scholla and Elkan, 1984; Sadowsky *et al.*, 1987). Likewise, the fast-growing strains that nodulate *Lotus* sp. show low DNA:DNA homology (10-15%) both with other *Rhizobium* sp. and with slow-growing strains able to form symbiotic relationships with *Lotus* (Crow *et al.*, 1981). These fast-growers have been grouped into a new *Rhizobium* species, *R. loti* (Jarvis *et al.*, 1982). *R. leguminosarum*, *R. trifolii* and *R. phaseoli* are now categorised as different *R. leguminosarum* biovars, i.e. *R.l.* bv. *viciae*, *R.l.* bv. *trifolii* and *R.l.* bv. *phaseoli*. Table 1.1 lists host plants and their corresponding microsymbionts.

Table 1.1: Microsymbionts and their respective host plants .

Bacterium	host plant
Rhizobium meliloti	lucerne (alfalfa)
R. leguminosarum bv. *viciae*	pea, vetch
bv. *trifolii*	clover
bv. *phaseoli*	bean
Rhizobium fredii	soya bean
Bradyrhizobium japonicum	soya bean
Rhizobium loti	*Lotus*
Azorhizobium caulinodans	*Sesbania* (stem nodules)
Rhizobium NG234	*Parasponia* (a non-legume)
Bradyrhizobium sp. Parasponia	*Parasponia* (a non-legume)