INVESTIGATION OF GENETIC CHANGES IN INOCULANT STRAINS OF RHIZOBIUM TRIFOLII ISOLATED FROM THE SOIL

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

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1985

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ACKNOWLEDGEMENTS

My thanks to the Department of Microbiology and Genetics for providing the opportunity and facilities for this investigation.

In particular, I would like to thank my supervisor, Dr Eric Terzaghi, for his help, guidance, tolerance and pecan pie.

I would like to thank the following people, without whom this thesis would never have been finished:

Dr A.N. MacGregor, for discussions and assistance.

Dr D.B. Scott (DSIR), for many gifts and much patience.

Drs Ronson and Pankhurst (DSIR), for advice and information.

Dr J.W. Tweedie, for lambda and advice.

Mrs P. McLenahan, without whom parts of this project would not "gel".

The Central Photographic Unit for photographs.

Miss Sue Campbell, for the typing and friendship.

All the nuts, grad. students and techs around the department and in particular Trish, Julie, Chris and Neville.

This thesis is dedicated to GARFIELD.

Information about the fate of plant inoculating strains of *Rhizobium trifolii* entering the soil environment is incomplete. It is known that inoculating strains must compete with existing adapted strains, if such are present. It is not known whether or not the introduced strains can adapt to soil conditions. Strains of the white clover (*Trifolium repens*) symbiont, *R. trifolii*, were isolated from plants growing as a result of sowing virgin soil with bacteria-coated seed. *Rhizobium* bacteria were isolated from one nodule on each randomly chosen plant at two and then six months after sowing. Three different methods were used to type the isolated strains because of the importance of distinguishing between derivatives of the inoculant (*R. trifolii* #2668) and adapted rhizobia immigrating from adjacent pastures. Gel diffusion identification of antigens showed that all strains reacted positively to anti-2668 serum, although the response was not identical for all strains. The determination of intrinsic antibiotic resistance patterns showed that low level resistances were accumulating in a non-random manner as time progressed. Initial isolates showed the same pattern as 2668. Restriction endonuclease analysis of the isolated strains showed them all to have a high degree of similarity to 2668, with a few being identical in pattern. This was despite alterations in numbers and sizes of plasmids (as compared to those in 2668) seen in these isolates. A *nif* gene probe of a plasmid profile showed that several strains had alterations in the size and number of bands which would hybridize, as compared to 2668. The field isolated strains had gained the ability to produce a broad range bacteriocin-like inhibitor. Conjugation experiments between *R. trifolii* #0/18 and *E. coli* HB101 showed that this inhibitor was transferrable to and expressable by the *E. coli* strain. This suggests the existence of a broad host range replicon in the field isolates which either carries or mobilizes this function.

INTRODUCTION

1.1.0 Introduction

The concept of the soil as a dynamic ecosystem was slow to develop; it's complexity giving it the status of a biological "black box". The physical properties of the soil components have been measured and quantified and the soil composition is known in some detail (McLaren and Peterson, 1967) but no integrated picture of the soil environment has been generated. One of the main difficulties in any such analysis is to apply the information obtained about any single component in the laboratory to the bulk soil environment. For example, it may be noted to what degree a certain soil type will become water-logged after heavy rain, but how will this affect the concentration of nutrients at a particle surface or the distribution of a certain species of bacteria? Information obtained about a micro-organism *in vitro* must be applied carefully to the *in vivo* situation. The response of the micro-organism in the laboratory may be unlike it's response in the soil, even to the same stimulus.

1.1.1 The Soil

The soil environment is one of austerity as useful nutrients are only available in small quantities and are competed for avidly (Grey and Parkinson, 1968). A group of micro-organisms will use those compounds it can, increasing in numbers as it does. Once all the accessable nutrient has been utilized, this group will die back and another will increase as it further uses the material. Microbial growth is seccessional and "blooms" of bacteria result. Investigations have shown that concentrations of nutrients are higher at surfaces due to absorptive forces. Because of this, bacterial numbers are high on particle surfaces. Bacteria use a number of means of attachment, many using a sugar polymer "rope" to overcome the repulsive forces between the micro-organism and the particle. This localization allows the bacteria to control its own environment, by controlling the access of external influences to the colony.

1.1.2 The Rhizosphere

In a similar way, the surface of a plant offers the bacteria an area of relative protection. In fact, this area (the rhizosphere) has a number of advantages for the bacterium. The plant excretes nutrients of several types, and root movement through the soil sloughs off dead tissue which can be used as a bacterial energy source. As would be expected, bacterial numbers in the rhizosphere are large and investigation has shown that the presence of plant roots is stimulatory (Rovira and McDougall, 1967). This stimulation has been attributed to a number of factors, including the sloughing of dead tissue, the release of soluble organic compounds and a higher relative carbon dioxide concentration. A complimentary hypothesis suggests that the plant selects its rhizosphere microflora by the release of certain compounds, notably lectins, but no firm evidence is available (Miller and Bowles, 1982; Dazzo *et al*, 1976, 1981). It is interesting that the vast majority of bacteria in the rhizosphere are of a kind - the Gram negative short rods. An examination of the rhizosphere of a tobacco plant revealed that 44% of the bacteria present were Gram negative short rods, compared to 13% in the bulk soil (Alexander, 1961). The high percentage of this type of micro-organism in the rhizosphere suggests that either they out-compete other types or that the plant selects for this bacterial group.

1.1.3 The Rhizosphere Microflora

The Gram negative rods found in the rhizosphere are largely members of the genera *Pseudomonas*, *Achromobacter* and, less frequently, *Agrobacterium*. Members of these three genera, and specialized symbionts such as the rhizobia, respond most markedly to plant influences. These genera are not the only micro-organisms present, representatives of the genera *Arthrobacter*, *Mycoplana*, *Brevibacterium* *Flavobacterium*, *Serratia*, *Sarcina*, *Alginomonas*, *Bacillus* and *Mycobacterium* can be found in significant numbers. They are transitory members, their numbers will be either high or non-existant.

The numbers of bacteria in the rhizosphere mean that competition for space and nutrients is intense. As a result fast growing, biochemically flexible bacteria and those bacteria which produce inhibitors have a distinct advantage. Some bacteria survive by having the ability to utilize a "difficult" substrate, such as lignin, but these organisms are not present in high numbers as the products yield low amounts of energy. The varying types of nutritional and competitive interactions are extremely complex and may have large numbers of links.

1.1.4 Microbial Interactions

Microbial interactions are of many types and include competition for utilizable substrate or living space, the exchange of genetic information and the production of anti-microbial substances. Although bacteria are usually found localized to particle surfaces, especially those of clays such as montmorillonite (Stotzsky and Krasovsky, 1981), they can and do move quite freely through soil water (Madsen and Alexander, 1982). Bacterial interactions that involve conjugation or other exchanges of genetic infromation probably occur on particle surfaces as such contacts are of greater duration. Potential for interaction with ecosystems outside that of the soil also exists. An example is the recovery of coliform bacteria from the soil which can be traced to animal origins (Deavin, Horsgood and Rusch, 1981).

Interactions are at varying levels of intensity. Some bacteria produce substances that others require for energy. The classic example of this involves the nitrogen cycle, where *Nitrosomonas* species perform the reaction:

$$2NH_3 + 3O_2 \longrightarrow 2HNO_2 + 2H_2O + \text{energy}$$

and *Nitrobacter* species continue the oxidation:

$$2KNO_2 + O_2 \longrightarrow 2KNO_3 + \text{energy}.$$

The first reaction is more favourable energetically and, as would be expected, nitrite producers are more prevalent (Campbell and Lees, 1967).

Further types of interaction include the inhibition of competing species by bacteriocinogenic substances. A universally applicable ecological maxim states that competition is most intense between members of the same and closely related species, as all members require the same kinds of nutritional factors. Therefore anything that will inhibit other strains supplies an advantage to the producer. In the harsh and highly competitive soil environment slowing down may lead to extinction.

Some micro-organisms have developed other survival mechanisms, such as specialized symbiotic associations with plants. The most well known example of this type of interaction is found in the genus *Rhizobium*.

1.1.5 Identifying *Rhizobium* Species

The genus *Rhizobium* is large and diverse, and the members are able to nodulate leguminous plants. Identification of species is generally on the basis of what plants the bacterium is able to nodulate (Jordan and Allen, 1974), but exceptions to this general scheme are common. *Rhizobium trifolii* is identified as the species which nodulates white clover (*Trifolium repens*), but some *R. trifolii* strains will also nodulate peas (*Pisum sativum*). Other methods which have been proposed include numerical taxonomy, phage typing and DNA hybridization (Ward, 1982).

One method that is commonly used on a laboratory basis as a means of determining identity is the use of strain and species specific antisera. Although identification is normally unequivocable at the species level, cross reaction between *R. meliloti* and some *Agrobacterium* species has been shown (Vincent, 1970). It is possible to obtain antisera to only one epitope (antigenic determinant) by either absorption or hybridoma technology, but both processes are expensive and

difficult. Another consideration is the initial choice of epitope, it must be something that will be characteristic of this strain. For routine purposes gel immunodiffusion analyses using unabsorbed antisera have been shown to be sufficient to identify more than 90% of *R. trifolii* strains, but other *Rhizobium* species with fewer predominant bands may require absorbed antisera (Vincent, 1970). The use of antisera for identification remains a quick and specific procedure.

Antibiotics have been commonly used to type bacteria on the basis of their sensitivities and resistances. These resistances often relate to the presence of R factors (antibiotic resistance carrying plasmids) within a cell. Low level resistances to antibiotics can be detected and if a sufficiently large number of antibiotics are used, then a specific pattern of "intrinsic" antibiotic resistance may be established. This property has been applied to the identification of *R. leguminosarum* strains (Josey *et al*, 1979). The pattern of resistance was a stable property of *R. leguminosarum*, but the same antibiotic tests gave more varied results in *R. phaseoli*, however useful results could still be obtained (Beynon *et al*, 1980). This procedure has been shown to be useful for *R. trifolii* (Ronson, personal communication).

Techniques are available which examine organisms at the DNA level. Such procedures have been used on the genus *Rhizobium* and an identification scheme has been developed for *R. trifolii* using colony hybridization. This technique allows the total genomic DNA from a bacterial colony to be examined. The colonies are lysed on a nitrocellulose sheet and the DNA is bound in place by vacuum baking. Because of the large number of colonies which may fit on a single sheet of nitrocellulose, many strains may be screened for a particular sequence at the same time (Hodgson and Roberts, 1983).

Restriction endonuclease fingerprinting is becoming a common method for analysing a micro-organisms genome. The total genomic DNA of a strain is extracted, digested with a restriction endonuclease and subjected to agarose electrophoresis. The resulting patterns are very stable and highly reproducible. These patterns can be used to distinguish species, for example *R. trifolii*, *R. japonicum* and *R. meliloti* can be clearly shown to be different by this process (Mielenz *et al*, 1979). This procedure has been used for identification in many genera, including *Leptospira* (Marshall *et al*, 1981), *Mycobacterium* (Collins *et al*, 1984) and *Brucella* (O'Hara *et al*, in press). The process has been shown to be very sensitive to genomic differences.

The large number of methods should allow conclusive identification of *Rhizobium* species, especially if more than one test is used. Fortunately, because of the symbiotic nature of the rhizobia, obtaining specimens is easy. Extracts of nodules from leguminous plants can be prepared and *Rhizobium* cells recovered. Studies of strains isolated in this way have yielded some interesting results. An Otago study tested field isolated strains against laboratory inoculant cultures for serotype and ability to form nodules. Fluorescent antibodies were used to type field strains; from the results a minimum of ten different serotypes were identified. It was also noted that only 22% of the field strains were of the same antigenic type as the reference strain (Gaur and Lowther, 1980). A laboratory test showed that the field strains generated a range of effectivity of nodule production from 2 to 138% of that of the reference strain.

The range of effectiveness and antigenic response raises the question of the fate of an inoculant introduced to the field environment. If there is no indigent species of rhizobia which could nodulate the plant in question, then the inoculant will face no competition for nodule formation. However, an existing adapted population of rhizobia would undoubtedly compete with any newly introduced strain.

1.1.6 Competition Between Rhizobia

The introduction of a strain of rhizobia into a soil already harbouring other strains of that species must inevitably lead to competition for nutrients, living space and plant nodulation sites if such factors would be limiting. When a *Rhizobium* coated clover seed is introduced into the soil, as occurs in the sowing of a field for pasture, the existing population will compete with the bacteria introduced on the seed (Hale, 1981).

A study of competitivity has revealed that a quantitative relationship exists between the numbers of nodules formed by the applied inoculum and the number formed by soil strains. From this relationship a "competitive parameter" has been derived (Amarger and Lobreau, 1982). It must be remembered that although soil strains may nodulate the plant with similar or greater efficiency than the introduced strain, they may not fix nitrogen at anything like the same level. The mode of action of this competition is reflected in the fate of the inoculant strains.

Field tests have shown that introduced strains can be reisolated in higher numbers in the first year after inoculation and in lower numbers in the second (Brockwell *et al*, 1982). By the second year isolated strains had the pattern of the native strain, which seemed to indicate that the inoculant had succumbed. Little is known about the mechanism of competition between rhizobia for nodulation "sites" (if such exist), nor about the persistence of strains in the soil.

An alternate explanation for the disappearance of the inoculant strain is its rapid remodeling by the acquisition of genetic information. Such information is contained within already adapted rhizobia strains and other soil sources. Acquisition leads to survival and the alteration of genetic structure to one which resembles that of the native rhizobial species. Such integration into an already existing population is not inconceivable, considering the large number of broad

host range plasmids that exist in soil micro-organisms.

Whichever of these explanations is correct, there is no doubt that competition does occur. There are a number of factors that can affect the degree of competition. These include the host plant, the soil type and soil temperature. There are undoubtedly others, some may be rhizobially derived. These might include the production of inhibitory substances, such as bacteriocins (Hodgson *et al*, 1984). It is conceivable that abilities which would enhance the survivability and competitivity of rhizobial strains are carried on plasmids. Whether or not this is the case, there is no doubt that functions important in nodulation and nitrogen fixation are carried on plasmids in *Rhizobium trifolii*.

1.1.7 Rhizobial Plasmids

The plasmids of the genus *Rhizobium* are of vital importance to the nitrogen fixation (*nif*) and nodulation (*nod*) capacity of the bacteria. The physical attributes of these replicons is variable, with a wide variety of plasmid sizes reported within and between species. *R. meliloti* has a resident transferrable plasmid of 90 kilobases (kb) and a megaplasmid of 600kb (Bedmar and Olivares, 1980). *R. leguminosarum* also shows this range of sizes, with some at 150kb and a megaplasmid of 900kb (Tichy and Lotz, 1981). It is a moot point whether these very large replicons should be considered as plasmids or as mini-chromosomes, knowing that the *E. coli* genome is approximately 4,000kb in length.

Despite the wide range in size, some conservation of plasmid structure has been demonstrated. Restriction endonuclease analysis of a *R. meliloti* plasmid generated a highly reproducible band profile. Comparison of band profiles of plasmids from a variety of sources showed there was geographical sequence conservation. Plasmids from the same geographical area were more similar to each other than to plasmids from other geographical areas (Huguet *et al*, 1980). Some structural similarity might be expected, considering that at least one plasmid per cell must carry the genes

involved in nodulation and nitrogen fixation.

There is evidence that the larger plasmids carry the genes involved in nitrogen fixation and nodulation. Radio-labelled probes consisting of the *R. meliloti nif* region, when hybridized to plasmid profiles of *R. leguminosarum* strains immobilized on nitrocellulose, indicate that a plasmid ranging from 195 to 825kb carries this function (Krol *et al*, 1983). *R. trifolii* plasmids also carry nodulation and nitrogen fixation genes (Schofield *et al*, 1983). Physical and genetic maps of the *R. trifolii nif* and *nod* regions are being prepared (Scott *et al*, 1984) but are not yet complete.

Further proof of the plasmid-borne nature of these functions has been gained from complementation studies. Transfer of plasmids from a nod^+ *R. leguminosarum* strain to a non-nodulating field derivative resulted in restoration of the nodulating and nitrogen fixing capacity. The number of nodules produced on the previously non-nodulating strain varied with the donor, but no significant differences in nitrogen fixing levels were detected (DeJong *et al*, 1981). In some cases, the introduction of further plasmids resulted in a drop in the nitrogen fixing ability of the strain relative to that of the nodulating parent strain.

Although plasmid sizes and numbers, including that of the symbiotic plasmid (pSym), are variable a likelihood exists that sequences important in nodulation and nitrogen fixation would be conserved. Sequencing of promoter regions in the *nif* genes of *Klebsiella pneumoniae*, *R. meliloti*, *R. japonicum* and *R. parasponiae* shows that a consensus sequence exists for these organisms at sites ten and twenty-six base pairs upstream from the transcription initiation site. The success of the *K. pneumoniae nif KDH* genes as a *nif* region probe for similar operons in rhizobial species suggests evolutionary similarities exist (Ausubel, 1984). Such discoveries are not unexpected, as the processes of nodulation and nitrogen fixation are substantially similar amongst the rhizobia. It seems unlikely that two systems

for nitrogen fixation could have arisen independently, therefore the similarities between the *Klebsiella* and *Rhizobium* operons is predictable. However although physical mapping of the symbiotic plasmid is proceeding rapidly, information on the actual gene products is less substantial.

The range of sizes of pSym is large and so is the amount of DNA of unknown function on each replicon. A few gene products have been characterized, the pSym of *R. leguminosarum* codes for a twenty-four kilodalton (kdal) protein which is present in large amounts in the rhizosphere. The locus for this protein maps between the *nod* and *nif* genes, but strains mutant in this protein will still undergo nodulation. No function has yet been assigned to the protein; there is speculation it may enhance competitivity (Dibb *et al*, 1984). The protein coding regions of the *R. meliloti* nodulation genes have been examined in some detail. An 8.5kb EcoRI fragment containing the nodulation genes expresses at least eight proteins. Three were subsequently mapped to a 3.3kb *nod* gene cluster and insertion mutagenesis suggests they may function in the early stages of nodulation (Schmidt *et al*, 1984). With further subcloning of the *nif* and *nod* genes, further functions may be elucidated. It should be noted that, although some *in vitro* information is available on these genes, the action of pSym and other rhizobial plasmids in the endosymbiotic state is poorly understood.

Rhizobium bacteria change their structure quite markedly once they become localized into nodules. Total RNA from the bacteroids has been isolated and used as a probe against a plasmid profile of *R. leguminosarum*. This hybridization showed that only pSym was strongly expressed in the endo-symbiotic state (Krol *et al*, 1982). No selective amplification of these plasmids had occurred. Expression of the nitrogenase regulatory and structural genes must occur, but whether the overall regulation is plant or bacteroid controlled is not known. Most of the DNA of the symbiotic plasmid has no function associated with it. One fuction demonstrated by a

number of plasmids (not necessarily pSym) is that of self-transmissability. Therefore genetic information can be exchanged between strains, possibly even species of rhizobia.

1.1.8 Transfer of Plasmids

Plasmid transfer occurs widely as a natural phenomenon and has been used as an experimental tool. The first and best characterized of the conjugative plasmids is pF (fertility) of *E. coli*. This plasmid is self-transmissable, meaning that it carries all the information necessary for conjugation. Some plasmids only carry a mobilization site and require a self-transmissable plasmid to transfer. Plasmids exist which have the ability to transfer between diverse micro-organisms, the so called "broad host range" plasmids. A number of examples of this type of replicon can be found in soil bacteria.

Several cases of plasmid transfer in the genus *Rhizobium* have been reported. The incompatability group 1 plasmids, pR68.45 and pRP4, have been transferred between serologically distinguishable strains of *R. japonicum* (Pilacinski and Schmidt, 1981). Strains of *R. leguminosarum* also exhibit conjugation. Four field strains were examined and each exhibited a different plasmid profile, some members of which were larger than 150kb. One of the plasmids (pRL3JI), which carries the gene for a *medium* bacteriocin, demonstrated a high level of transmissability and some mobilization of chromosomal markers. Co-integration between pRL3JI and resident plasmids has also been shown (Hirsch, 1979). Therefore, even if the incoming plasmid was incompatable, the ability to co-integrate means that any acquired genetic information can be maintained. Some homology must exist between the resident plasmid and the transferring factor for this integration to occur.

Host microbes may take advantage of the regions of portable homology offered by the insertion sequences they contain. The discovery of a *Rhizobium* specific insertion

element suggests another pathway for the acquisition of "extra" genetic information. Insertion sequences are notorious as sites of co-integration, the classic example being the insertion of pF into the *E. coli* genome at delta-gamma. The rhizobial element (ISR1) could act as a recognition region for incoming plasmids from other rhizobial species. This element also shows a strong affinity for the broad host range episome pRP4, which can transfer to *Rhizobium* species from a number of sources. ISR1 will cause mutations in its recipient at high frequencies (Priefer *et al*, 1980) and could be used as a mutagenic treatment. The ability of *Rhizobium* species to exchange plasmids within its own genus as well as with other genera raises the possibility of acquisition of genetic information form other soil micro-organisms.

1.1.9 Natural Evolution In The Soil

The recombination of procaryote genetic material is much more common than was originally believed; the genome is capable of integrating large scale change. Much of the recombination that occurs is of a type previously described as "illegitimate", as it was believed to involve no homology between the two recombining sequences. Such events are so common they are no longer regarded as illegitimate and do involve homologous sequences, albeit of a shorter length (Ikeda *et al*, 1984). The term "site specific" recombination is now applied to such events; plasmid co-integration may involve this type of recombination.

Means exist for bacterial strains to exchange ancillary genetic information, as typified by conjugation. Such information could prove vital to the cell when a change in extracellular conditions demands a degree of flexibility. The information, which may have been maintained in only a few cells in the population, can be quickly disseminated if selective pressures are encountered. This concept is encompassed in the process of coupled evolution proposed by Reanney (1978).

Other factors may contribute to this genetic pool. Viruses, proteins and nucleic acids can become adsorbed to clay particles and thereby avoid microbial degradation. If naked DNA persists in natural habitats, it is possible that its genetic information could be transmitted to any suitable host that gains access to it (Stotzky and Krasovsky, 1983). Thererfore, even dead micro-organisms could affect the content of the gene pool, and contribute to the transfer of genetic information.

1.1.10 Coupled Evolution in the Soil

The theory of coupled evolution postulates that all members of the bacterial microflora are linked by the transfer of genetic information. Not all members of a population of micro-organisms could adapt to a changed environment, but as long as some do, the survival of the species is ensured (Reanney, 1983).

Pseudomonas species are known rhizosphere inhabitants and are capable of maintaining a number of potential genetic vectors. Bacteriophage able to infect *Pseudomonas* species are present in the soil and although no cases of special transduction have been reported, general transduction is not uncommon. Plasmid transduction occurs, but at low frequencies. Conjugation between pseudomonads and other genera has been reported; for example, the plasmid pRP4 has been shown to enter *Rhizobium* cells.

Genetic exchange occurs in the genus *Bacillus* in the soil. Strains of *Bacillus subtilis* can exchange linked blocks of genes, which leads to extensive reorganisation of the genome structure and to the appearance and eventual dominance of a single phenotype (Graham and Istock, 1979).

Conjugation has been demonstrated in sterile soil between *E. coli* strains. This process is stimulated by the clay mineral montmorillonite (Weinberg and Stotzky, 1972). Should transfer occur in the normal soil environment, the

potential for the introduction of genetic information from diverse sources need hardly be stressed.

Conjugative, broad host range and R factor plasmids are associated with many rhizosphere inhabitants. Considering the large numbers of bacteria present in the rhizosphere, the potential recipients of a broad host range factor are inumerable. Only one or a few members need maintain, for example, a certain gene/operon/plasmid coding for a novel degradative pathway. The existence of a self-transmissable replicon in the cell could cause this function to be spread throughout the population, should substrate become available. This pathway may have little function except under special circumstances, but if it only existed within a few cells there would be only a small "genetic load". The pathway may mutate to perform a new function in a few cases and further extend the phenotype.

In this way, with a large number of potential participants, deleterious mutations could be selected against and advantageous or neutral mutations maintained. If evolution does proceed by "tinkering" (Jacob, 1977), gene pools with large numbers of diverse members will be able to test more gene combinations than those with small and more homogenous memberships. Introduced strains can provide new sources of information for the population, but may need to acquire certain genes in order to compete successfully and transfer this new information.