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

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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.

1.2 Introduction - Analysis Of Field Isolates Of *Rhizobium trifolii*

The standard method for the production of new pasture is to sow cleared land with a mixture of ryegrass and clover seed. Pasture value is enhanced by the fixation of nitrogen, therefore the clover seed is coated with a proven laboratory strain of *Rhizobium trifolii*. This process ensures that a known, high level nitrogen fixing strain is available to nodulate the plant. The existence of an already adapted population of rhizobia would mean competition for the inoculant.

As a means of assessing an established population of *R. trifolii*, strains were isolated from plants obtained at a site near Tuapaka, Palmerston North. Plasmid profiles were obtained for all isolates. The stability of these profiles was examined by laboratory subculture and nodulation effectiveness as a function of plasmid content measured. A streptomycin resistant laboratory strain was inoculated into the soil, which contained an adapted population of *R. trifolii*, and attempts were made to recover it as a nodule isolate (Terzaghi, personal communication). As a result of this study, a number of different plasmid profiles were identified. A common pattern, displaying a number of similar sized plasmids, could be seen in several of the isolates. The laboratory strain appeared to be unable to compete. Strains which had been inoculated into the soil containing an established population of rhizobia could not be recovered.

It is difficult to measure genetic changes in an inoculant strain at such a site, as the presence of other strains leads inevitably to competition. Ideally, to measure adaptive changes, the soil should be initially virgin with respect to the inoculant species. A site which had been cleared from native bush and then sown for the first time would show adaptive changes clearly. Such a site exists near Taupo. A number of fields have been sown with a ryegrass-clover mixture, onto land cleared of native bush. Theoretically, this soil should be virgin with respect to

R. trifolii. A range of pasture ages exists, from one sown seven years ago to one only recently (October 1983) inoculated.

In order to compete successfully with other soil micro-organisms, adaptive changes would have to occur quickly. Such changes should already be visible in the most recently sown field. The isolation of strains was two months after sowing, at this stage the plant growth is sparse but the nodules are well formed. The second isolation was six months after sowing and reasonable plant growth had occurred. The stressful nature of the field conditions, particularly at this site, would impose a strong selective pressure on any newly arrived micro-organism. If the bacterium could acquire advantageous characteristics from other soil micro-organisms, then its chance of survival would be increased.

The inoculant strain, *R. trifolii* #2668, has a plasmid profile that is stable under laboratory conditions. The genes involved in nodulation and nitrogen fixation are located on a known plasmid. It is accepted that changes are likely to occur in laboratory strains once they enter the soil. This project aims to investigate the nature of the changes.

The identity of the strains isolated from the soil must be determined. It is crucial to know whether "changed" strains are the result of modification of the inoculant or of immigration of strains from other pastures. Because of the huge numbers of coated seeds used to sow the pasture, it is more probable that a random nodule sampling of the field would recover the inoculant strain. This assumes that any immigrant strain has no overwhelming superiority and has not been spread widely over the field. Because the distinction between "altered inoculant" and "immigrant" is so important, three identification procedures rather than just one were used. These three procedures were gel immunodiffusion, determination of patterns of intrinsic antibiotic resistance and restriction endonuclease analysis. Each has its own strengths and weaknesses, but a combination of results should allow a distinction between inoculant and immigrant to be made.

Once the identity of the isolated field strains has been established, their plasmid profiles must be examined for changes. The existence of broad host range plasmids in other soil micro-organisms suggests that conjugation may be the commonest form of genetic interaction. The standard method for the examination of large plasmids on agarose gels is that of Eckhardt (1978). Gentle lysis is required because large plasmids are fragile. Gel electrophoresis allows comparisons of plasmid size and number between the field isolate and the inoculant.

The plasmids of *R. trifolii* carry genes important in nodulation and the fixation of nitrogen as discussed previously. It is vital to determine whether or not changes have occurred in the replicon carrying these genes. The plasmid location will be determined by DNA hybridization using a *nif* probe on a Southern blot of a plasmid profile. The *nif* probe in question is the *nif HDK* genes of *R. trifolii*. This sequence contains a shorter sequence found in a number of reiterations around the *sym* plasmid (Scott *et al*, 1984). The plasmid which shows hybridization to this *nif* probe will be compared amongst the field isolates and to the inoculant strain for alterations in the size of the *sym* plasmid.

It is reasonable to expect micro-organisms which have adapted to the soil to gain functions which will enhance their survival. A common function amongst soil micro-organisms is the production of bacteriocins. Bacteriocins are small molecules which will inhibit or kill closely related strains. Soil isolated strains will be tested for their ability to produce inhibitors against related strains and some common soil inhabitants.

Acquisition of genetic material could mean the ability to transfer information also exists. Field strains of *R. trifolii* will be examined for their ability to transfer genetic information to other micro-organisms.

MATERIALS AND METHODS

2.0 Isolation of *Rhizobium trifolii* Strains From Nodules

Clover plants were obtained from the pasture and transferred to the laboratory for rhizobia isolation. The plants were examined for overall appearance and the roots for nodule size and colour. The roots were carefully washed in water to remove soil particles. A section of root containing a nodule was cut out and each nodule was surface sterilized independantly by immersion in 0.2% mercuric chloride for 30 seconds, followed by six successive washes in sterile distilled water. The nodule was aseptically transferred to a crusher and broken open. A sample of material was streaked onto YEM + actidione for rhizobia isolation and nutrient agar to test for contamination by non-rhizobia.

2.1 Bacterial Strains Used In This Investigation (Table 1)

Three laboratory strains of R. *trifolii* were used throughout this investigation as controls, including R. *trifolii* strain 2668, which was the inoculating strain employed in the study area.

Twenty-two strains of R. *trifolii* were isolated from twenty-two different plants. All were isolated from the same pasture, some two months after the field was sown (designated "first series") and some six months after sowing (designated "second series"). Gaps in enumeration are due to the initial isolation of non-rhizobial strains.

A number of non-rhizobial strains were used for various purposes throughout the study. These are listed in Table 2. M.U. denotes Massey University Culture Collection.

Isolates of soil micro-organisms were made from water washes of a white clover (*Trifolium repens* L) root mass. These were not characterized beyond a description of colony morphology and Gram staining.

TABLE 1: *Rhizobium trifolli* strains examined.

TABLE 1:

Laboratory Designation	Culture Collection Number	Time of Isolation
2668	NZP 582	-
2163	NZP 561	-
TAI	NZP 574	-
0/0	-	1st series
0/2	-	"
0/3	-	"
0/4	-	"
0/5	-	"
0/7	-	"
0/8	-	"
0/9	-	"
0/10	-	2nd series
0/11	-	"
0/12	-	"
0/13	-	"
0/14	-	"
0/15	-	"
0/18	-	"
0/19	-	"
0/20	-	"
0/22	-	"
0/23	-	"
0/24	-	"
0/25	-	"
0/26	-	"

NZP, Palmerston North Culture Collection, DSIR.

TABLE 2: Strains of non-rhizobia used.

TABLE 2:

Name	Culture Collection Number
Escherichia coli HB101	MU#672
Escherichia coli HB101(pRK290)	MU#668
Bacillus cereus	MU#2
Bacillus subtilis	MU#1
Pseudomonas aeruginosa	MU#556
Pseudomonas denitrificans	MU#288
Pseudomonas fluorescens	MU#9
Serratia marcescens	MU#81

MU, Massey Culture Collection.

2.2 Media Used For Strain Maintenance

2.2.1 Tryptone-Yeast Extract Broth (TY) (Beringer, 1974) contained (g/l): Tryptone (Difco), 5.0; Yeast extract (Difco), 3.0. The broth was sterilized by autoclaving at 121C for 15 minutes after which $5cm^3$ of sterile 1M $CaCl_2.6H_2O$ was added per litre. For solid medium; 12g agar (Davis) was added for base and 5g agar (Davis) was added for overlay, per litre of broth. This was the standard medium for growth of *R. trifolii* strains.

2.2.2 Luria Broth contained (g/litre): Tryptone (Difco), 10g; Yeast extract (Difco), 5.0g; sodium chloride, 5.0g. The pH was adjusted to 7. For solid medium, 12g of agar (Davis) was added for base and 4g of agar (Davis) was added for overlay, per litre of broth.

2.2.3 Brain Heart Infusion Broth (BHI) was made by dissolving 20.0g of Brain Heart Infusion (Difco) in 1l of distilled water. For solid medium, 15g of agar (Davis) was added per litre of broth.

2.2.4 Yeast Extract Mannitol Agar (YEM) (Vincent, 1970) contains (g/litre): mannitol (BDH), 10.0; Yeast extract (Difco), 0.4; dipotassium hydrogen phosphate, 0.5; magnesium sulfate septahydrate, 0.2; sodium chloride, 0.1; agar (Davis), 15.0. The pH was adjusted to 7.5.
YEM + actidione was obtained by adding actidione (Upjohn Corp.) to 0.002%.

2.2.5 Nutrient agar was made by dissolving 8.0g of nutrient agar base (Difco) and 15.0g of agar (Davis) in 1l of distilled water and autoclaving at 121C for 15 minutes.

2.2.6 Tryptone Soya Agar (TSA) was made by dissolving 40g of TSA base (Oxoid) in 1l of distilled water and autoclaving at 121C for 15 minutes.

2.3 Antibiotic Containing Media

Antibiotic stock solutions were prepared as follows:

2.3.1 Ampicillin was prepared by dissolving 80mg of ampicillin (Sigma) in 20cm^3 of sterile distilled water.

2.3.2 Streptomycin was prepared by dissolving 1g of streptomycin sulfate (Glaxo) in 100cm^3 of sterile distilled water.

2.3.3 Spectinomycin was prepared by dissolving 200mg of spectinomycin dihydrochloride (Sigma) in 20cm^3 of sterile distilled water.

2.3.4 Chloramphenicol was prepared by dissolving 680mg of chloramphenicol (Sigma) in 20cm^3 of absolute ethanol.

2.3.5 Naladixic acid was prepared by dissolving 80mg of naladixic acid (Sigma) in 20cm^3 of 0.1M NaOH.

All antibiotic solutions were sterilized by passage through a 0.22μm membrane filter (Millipore). The sterile antibiotic solutions were added aseptically to the medium in question after it had been autoclaved and cooled to 50C. Concentrations of antibiotic are given (where necessary) in the text.

2.4 General Purpose Solutions

2.4.1 Tris-(hydroxymethyl)-aminomethane buffer. This buffer was made up at a number of concentrations and pHs.

2.4.2 1M Tris contained (g/litre): Trizma base (Sigma), 121.1. Two different pHs of this buffer were in general use, 7.5 and 8.0. The pH was adjusted with concentrated HCl.

2.4.3 0.1M Tris contained (g/litre): Trizma base (Sigma), 12.1. The pH was adjusted to 8.0 with concentrated HCl.

2.4.4 0.1M Tris + mercaptoethanol contained (g/litre): Trizma base, 12.1; 2-mercaptoethanol, 20 cm^3. The pH was adjusted to 8.0 with concentrated HCl.

2.4.5 Ethylene diamine tetra-acetic acid solution. This solution was used in conjunction with the Tris buffers. 0.2M EDTA (disodium salt) buffer contained (g/litre): Na_2EDTA (BDH), 67.2. The pH was adjusted to 7.2 with concentrated HCl.

2.4.6 Tris-EDTA buffer (TE) was prepared at several concentrations.

2.4.7 50/20 TE contained 50mM Tris and 20mM Na_2EDTA and was prepared by adding 50cm^3 of 1M Tris pH 8.0 and 100cm^3 of Na_2EDTA pH 7.2 to 1l distilled water. The pH was adjusted to 8.2 with concentrated HCl.

2.4.8 10/1 TE contained 10mM Tris and 1mM Na_2EDTA and was prepared by adding 10cm^3 of 1M Tris pH 8.0 and 5cm^3 of Na_2EDTA pH 7.2 to 1l of distilled water. The pH was adjusted to 8.2 with concentrated HCl.

2.4.9 50/20 TE + 0.1% sarkosyl was prepared by adding 1.0g of N-lauryl-sarcosine (Sigma) to 1l of 50/20 TE pH 8.2.

2.4.10 Acetate electrophoresis buffer contained 40mM Tris, 5mM sodium acetate and 1mM EDTA, pH 7.8. A ten times concentrated stock was prepared by dissolving 48.8g Tris, 6.8g sodium acetate-trihydrate and 3.4g EDTA in 1 litre of deionized water. The pH was adjusted to 7.8 with glacial acetic acid.

2.4.11 Borate electrophoresis buffer contained 89mM Tris, 89mM boric acid and 2.5mM EDTA, pH 8.2. A ten times concentrated stock was prepared by dissolving 108g Tris, 55g of boric acid and 9.2g of EDTA in 1l of deionized water. No pH adjustment was necessary.

2.4.12 Standard sodium citrate contained 150mM sodium chloride and 15mM sodium citrate, pH 7.0. A twenty times concentrated stock was prepared by dissolving 175.3g of NaCl and 77.4g of sodium citrate trihydrate in 1 litre of deionized water. The pH was adjusted to 7.0 with concentrated HCl.

2.4.13 5M sodium chloride was prepared by dissolving 75.5g sodium chloride in 1l deionized water.

2.4.14 Salt-Tris-EDTA (STE) contained 100mM sodium chloride 10mM Tris pH 8.0 and 1mM EDTA. It was prepared by adding $20cm^3$ of 5M sodium chloride, $10cm^3$ of 1M Tris pH 8.0 and $5cm^3$ of Na_2EDTA, pH 7.2 to 1 litre of deionized water.

2.5 Identification of *Rhizobium trifolii* strains

2.5.1 Antigen typing by gel precipitation (after Vincent, 1970).

The gel precipitation test relies on a matrix, in the form of an agar plate, in which an antigen-antisera reaction can occur. The plates contain (g/litre): sodium chloride, 8.0; Noble agar (Difco), 10.0.

Wells were cut from the plate in the shape of a six pointed star about a central well. The wells were 4mm wide and separated by 3mm from each other. A drop of agar was used to seal the bottoms of the wells once they had been cut.

A $2cm^3$ overnight culture in TY of each strain was prepared. $1cm^3$ was centrifuged in a microfuge (Eppendorf) at 12,000g for 3 minutes at room temperature. This pellet was washed once with $1cm^3$ 50/20 TE sarkosyl and once with $1cm^3$ of 50/20 TE. The pellet was resuspended in what little liquid remains between the cells and subjected to a 10 second burst of sonication (MSE Scientific) on ice. The burst cells were resuspended in 150ul of distilled water and freeze-thawed twice (-20C for 5 minutes followed by 70C for 2 minutes). Lysates are stored at -20C.

The outer wells of the rosette were filled with antigen and the one inner well with antisera. For this study antisera to *R. trifolii* #2668 (kindly donated by Dr. C. Pankhurst, DSIR) was used. The plates were incubated at room temperature for two days and then examined for lines of identity between each outer and inner wells.

All field isolates and the three laboratory strains were tested.

2.5.2 Determination of patterns of intrinsic antibiotic resistance.

A pattern of resistances to low level amounts of a number of antibiotics was determined and used to type strains.

To obtain a pattern of resistances, $0.3cm^3$ of a dense overnight culture of each strain was mixed with $3cm^3$ of TY overlay and poured onto TY agar. This overlay is incubated at room temperature for two hours.

Antibiotic sensitivity discs (Difco) containing appropriate amounts of each antibiotic were applied under aseptic conditions. No more than eight discs should be applied per plate, otherwise zones of inhibition may overlap. The plates were incubated at 30C for two days.

The following antibiotic discs were used. The amount of antibiotic per disc (in μg) is shown in brackets:

Ampicillin (10)
Chloramphenicol (50)
Erythromycin (50)
Gentamycin (10)
Kanamycin (50)
Methicillin (10)
Naladixic acid (5)
Novobiocin (30)
Penicillin (10)
Streptomycin (25)
Trimethoprim (25)

2.5.31 Restriction endonuclease analysis of *R. trifolii* DNA (after Collins *et al*, 1984).

The high sensitivity of this technique to small changes in DNA structure makes it ideal for strain identification.

Sufficient DNA was extracted from 30cm^3 of an overnight culture for this analysis. The culture was centrifuged at 3,000g for 5 minutes at 20C in an SS34 rotor (Sorval RC2B). The pellet was then washed once with 10cm^3 50/20 TE sarkosyl pH 8.0 and once with 10cm^3 of 50/20 TE pH 8.0. The final pellet was resuspended in 5cm^3 of 50/20 TE pH 8.0.

Lysozyme (Sigma) to 500ug/ml was added to the cell suspension and it was incubated at 37C for one hour. Pronase (Calbiochem) to 1mg/ml and N-lauryl-sarcosine (Sigma) to 1% were added and incubation continued overnight at 50C.

The lysed cells were decanted gently into 20cm^3 Kimax tubes and 5cm^3 of redistilled phenol (Appendix 1) was added. The lysate and phenol were gently mixed for 2 minutes and then centrifuged at 1,000g for 15 minutes at room temperature in a fixed head centrifuge (Sorvall). The clear aqueous layer was transferred to another tube with a wide bore pipette. The phenol wash was repeated twice more. The final aqueous phase was then washed twice as above in 5cm^3 of chloroform : isoamyl alcohol (24:1) to remove any remaining phenol. After these washes the aqueous phase was loaded into prepared dialysis tubing (Appendix 2). The crude DNA preparations were dialysed for two days against three changes of 10/1 TE pH 8.0 (1 litre).

The dialysed DNA was removed from the sac and precipitated by the addition of one-tenth volume of

3M sodium acetate and two volumes of absolute ethanol, followed by precipitation at -20C overnight. This was followed by centrifugation at 17,000g for 15 minutes at -10C in an SS34 rotor (Sorvall RC-2B). The supernatant was decanted gently and the pellets resuspended in 1.2cm^3 of 10/1 TE pH 8.0.

2.5.32 DNA concentrations of the samples were determined by spectrophotometry. A 0.2cm^3 sample was diluted to 2cm^3 in 10/1 TE pH 8.0 and examined in a spectrophotometer (Cecil Instruments) with 10/1 TE pH 8.0 as a blank. Readings of optical density were recorded at 10nm intervals over the range of 230-280nm. Calculation of nucleic acid concentration used the following formula:

Amount of sample (μl) required for 2μg DNA = 2 x 2/DNA concentration at O.D.(260) x 1,000.

Previous experience had shown that approximately half the O.D.(260) was RNA and this was allowed for in the equation.

2.5.33 The DNA preparation should be of sufficient concentration to allow the overall digest to be performed in a total volume of 100μl. 20μl of five times concentration of restriction endonuclease buffer (Appendix 3) was added per digest and the volume adjusted to 100μl with water. To the DNA sample, 0.5μl of restriction endonuclease was added. Initially, a number of restriction endonucleases were tested to find the one which gave reproducible and distinctive patterns. Restriction endonucleases (Amersham) tested were: Alu I, Bam HI, Bgl II, Bst EII, Eco RI, Hind III, Kpn I, Pst I and Sal I. All digests were incubated at 37C for two hours followed by 10 minutes at 65C except Bst EII which was incubated at 60C for two hours.

Cultures were centrifuged at 4,000g for 10 minutes at 4C in a GSA rotor (Sorvall). The pellets were washed in 50cm^3 of ice cold STE pH 7.8, followed by a wash in 50cm^3 of ice cold STE pH 8.0. The pellets were resuspended in 5cm^3 of STE pH 8.0, combined and transferred to a 200cm^3 flask. 1cm^3 of lysozyme (Sigma) (at 10mg/cm^3 in 10mM Tris pH 8.0) was added per 10cm^3 of culture. The liquid was brought to the point of boiling and immersed in boiling water for 40 seconds. The cells were cooled on ice for 5 minutes and centrifuged at 45,000g for 25 minutes at 0C in a SW41 rotor (Beckman). The supernatant was transferred to a 100cm^3 flask. Two volumes of ethanol were added and the crude DNA preparation precipitated at -20C overnight.

The mixture was centrifuged at 12,000g for 15 minutes at 0C in an SS34 rotor (Sorvall). The pellet was resuspended in 3.8cm^3 of 50/20 TE pH 8.0. Cesium chloride (Sigma) was dissolved to a concentration of 1g/cm^3. The volume was measured and 0.8cm^3 of 10mg/cm^3 ethidium bromide added per 10cm^3 of cesium chloride gradient. This solution was centrifuged at 10,000g for 10 minutes at 0C, and debris removed from the top of the tube. The gradient was centrifuged at 400,000g for 5 hours at 20C in a TV865 vertical rotor (Beckman). The gradients were examined using a hand held UV source (UV Products). The upper layers of linear DNA were removed and discarded. The band of covalent closed circular (CCC) DNA was transferred to a fresh tube and washed with 20 x SSC saturated iso-propanol until no ethidium bromide remained. Two volumes of distilled water were added followed by a tenth volume of 2.5M sodium acetate pH 5.2 and two volumes of ice cold ethanol. The plasmid DNA was precipitated at -20C overnight and then centrifuged at 20,000g for 15 minutes at 0C in a SS34 rotor (Sorvall). The pellet was dried in a vacuum desiccator and resuspended in 0.25cm^3 of 50/20 TE pH 8.0.

One twentieth volume of 5M sodium chloride and 2 volumes of cold 95% ethanol were added, followed by incubation at -70C for two hours to precipitate the DNA. The digests were centrifuged at 12,000g for 5 minutes at 4C in a microfuge (Eppendorf). The supernatant was discarded and the pellet dried at 37C for 15 minutes. 30μl of TE-glycerol plus SDS was added and the pellet resuspended. TE-glycerol plus SDS consists of 10mM Tris pH 8.0, 1mM EDTA, 20% glycerol and 0.5% sodium dodecyl sulfate. The TE-glycerol plus SDS was loaded into the well of a 0.7% agarose (BioRad) gel and sealed with agarose. The gel was submerged approximately 2mm in 1 times concentration borate buffer. The DNA was electrophoresed for one hour at 20 volts and then at 80 volts for six hours.

The gel was stained in 0.5$\mu g/cm^3$ ethidium bromide (Sigma) for one hour. The gel was then examined by ultra-violet light on a transilluminator (UV Products) and photographed on Kodak Tri-X film through a Wratten 23A (red) filter.

2.6 Preparation and Electrophoresis of Plasmids

Large plasmids are generally fragile and difficult to recover in quantity. A gentle preparation was required and *in situ* lysis reduces the possibility of breakage. The method chosen was that of Eckhardt (1978).

2.6.1 The solutions used for this method were as follows:

Solution 1 contained (g/10cm^3 of 1 x borate buffer): Ficoll 400 (Sigma), 1.0; Bromophenol blue (Sigma), 0.005; RNase, 10μl. The RNase (Amersham) solution was at a concentration of 10mg/cm^3 in 50/20 TE pH 8.0 and was treated at 98C for 2 minutes to remove DNase activity. Immediately prior to use 100μl of a 2mg/cm^3 solution of lysozyme (Sigma) was added per 1cm^3 of solution 1.

Solution 2 contained (g/10cm^3 of 1 x borate buffer): Ficoll 400 (Sigma), 1.0; sodium dodecyl sulfate (Sigma), 0.2.

Solution 3 contained (g/10cm^3 of 1 x borate buffer): Ficoll 400 (Sigma), 0.5; sodium dodecyl sulfate (Sigma), 0.2.

2.6.2 Cells were initially removed from plates and inoculated into 5cm^3 of TY. This culture was incubated at 30C for two days with shaking to acheive saturation. A subculture at a concentration of 1/100 in TY was prepared and incubated sixteen hours with shaking.

1cm^3 of culture was centrifuged at 12,000g for 3 minutes at room temperature in a microfuge (Eppendorf). The cells were washed once with 1cm^3 of 50/20 TE-1% sarkosyl pH 8.0 and once with 1cm^3 of 50/20 TE pH 8.0. 20μl of solution 1 was added to the cell pellet. The pellet was briefly resuspended with the pipette tip and transferred to the gel. Agar percentage was

variable, the most common was 0.7% agarose (BioRad) dissolved in 1 x borate buffer. The resuspended cells were incubated for 15 minutes, then 20μl of solution 2 was added, followed by 20μl of solution 3. The gel was flooded with 1 x borate buffer to a depth of approximately 2mm. The plasmids were electophoresed at 20 volts for one hour then 120 volts for ten hours.

Visualization and photography was as discussed in section 2.5.33

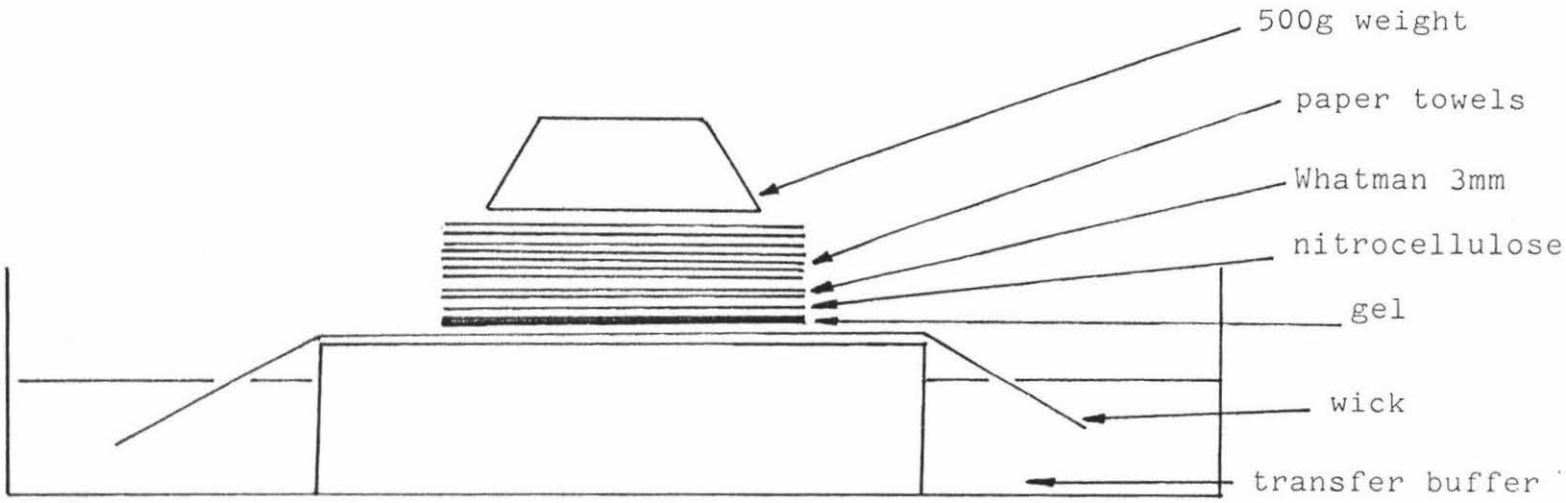
2.7 Southern Transfer of DNA to Nitrocellulose

The transfer of DNA from agarose gels to nitrocellulose (the Southern blot) and the subsequent examination by DNA hybridization is a cornerstone of molecular biology. Several variants of the procedure exist, the one used in this study was that of Maniatis *et al* (1982).

2.7.1 Three solutions were used in succession to prepare the DNA in the gel for transfer. These solutions were formulated as follows:
The depurinating solution consisted of 0.25M HCl.
The denaturing solution contained (g/litre): sodium chloride, 87.6; sodium hydroxide, 20.0.
The neutralizing solution contained (g/litre): sodium chloride, 87.6; Tris base (Sigma), 121.1. The pH was adjusted to 8.0.

The gel was washed for 15 minutes in 200cm^3 of depurinating solution. This was repeated and followed by a rinse with 200cm^3 of distilled water. The gel was agitated gently for one hour in 200cm^3 of denaturing solution, and then for one hour in 200cm^3 of neutralizing solution. It was then ready for transfer.

DIAGRAM 1: Assembly of the apparatus for a Southern blot.


500g weight
paper towels
Whatman 3mm
nitrocellulose
gel
wick
transfer buffer

The nitrocellulose was cut to a size that would overlap the gel slightly and four pieces of Whatman 3mm were cut slightly smaller than the gel. Two of the pieces of Whatman 3mm and the nitrocellulose were soaked in 2 x SSC, the filter paper briefly and the nitrocellulose for 5 minutes. The blot was assembled as shown in diagram 1. The reservoirs were filled with 10 x SSC. Transfer of the plasmid DNA was allowed to proceed overnight.

The blot was dismantled and the nitrocellulose filters washed in 6 x SSC for 5 minutes. The filter was blotted dry, placed between two sheets of Whatman 3mm and baked at 80C for 2 hours under vacuum.

To ensure the DNA had transferred, all agarose gels were restained in 0.5ug/ml ethidium bromide (Sigma) for one hour and examined on the transilluminator.

2.8 Isolation of Plasmid DNA Suitable for DNA Hybridization

A sequence of DNA which was to be used as a probe was maintained on an amplifiable vector so that a large quantity was available. The method used for isolation of the plasmid pPN435, which carries the *R. trifolii nif HDK* operon as a 5.2kb EcoRI fragment inserted into the EcoRI site of pBR328, was that of Maniatis *et al* (1982). The pPN435 was a gift from Dr D.B. Scott, DSIR.

2.8.1 A $5cm^3$ saturated culture of the bacterium carrying the plasmid of interest was inoculated into $500cm^3$ of Luria broth containing ampicillin at $50ug/cm^3$ to maintain selection for the plasmid. Chloramphenicol to $170ug/cm^3$ was added to amplify the plasmid, once an O.D.(600) of approximately 0.4 was reached. Incubation was continued overnight at 37C with shaking.

Spectrophotometric determination of DNA concentration was by the same method described in section 2.5.32

2.8.2 Plasmid purity was determined by agarose gel electrophoresis. 2μg of plasmid DNA in TE-glycerol plus SDS were electrophoresed for one hour in a 10cm^3 volume minigel. Examination and photography have been described in section 2.5.33.

Restriction endonuclease analysis of the plasmid was carried out. 2μg of pPN435 DNA were digested with EcoRI and electrophoresed as described in section 2.5.33. The expected result for pPN435 was a 5.2 and a 4.9 fragment.

2.9 Production of Radioactively Labelled Probe

There are two main methods available for producing radioactively labelled probes; nick translation and random priming. The latter procedure was chosen for this study. The method of random priming was that of Whitfield (1982).

2.9.1 The probe DNA was digested at 37C for 45 minutes using the restriction endonuclease EcoRI, in Hae III buffer. The enzyme will cut at the centre 4 base pairs of its recognition sequence. 100μg of random primer DNA (Appendix 4) was added and the mix boiled for 2 minutes, then quickly cooled on ice. The ingredients for the radiolabelling reaction were as follows: distilled water, 2.5μl; 5 x AluI buffer, 1.5μl; 20mM dATP, dTTP and dGTP (Amersham), 1.0μl; (^{32}P) dCTP (10mCi/cm^3, Amersham), 3.0μl; Klenow fragment of DNA polymerase (Amersham), 2 units. The reaction was allowed to proceed at 37C for one hour and then stopped by the addition of 3μl of 200mM Na_2EDTA pH 7.2. 50μl of redistilled phenol and 50μl of chloroform : isoamyl alcohol (24:1) were added and mixed with the labelled DNA. The preparation was

centrifuged at 12,000g for 2 minutes at room temperature in a microfuge (Eppendorf). The aqueous phase was transferred to a second tube. The phenol : chloroform : isoamyl alcohol (25:24:1) mix was washed a further three times with 50μl of distilled water (as above) and the fractions combined.

The washed probe was loaded onto a Sephadex G-50 fine column (Pharmacia) which had been equilibrated in STE pH 8.0. The probe was washed through the column and collected as 200μl aliquots. Approximately 7.5cm^3 of STE pH 8.0 was required to remove any significant radioactivity from the column. The number of counts per second for each tube was determined with a hand held counter (Mini Instruments) and the first peak of radioactivity kept. One twentieth volume of 5M sodium chloride and two volumes of absolute ethanol were added to each aliquot of the peak and all were precipitated at -20C overnight. They were then centrifuged at 12,000g for 5 minutes at room temperature and the pellets resuspended in sterile distilled water such that the total volume in all tubes was 100μl. The probe was bulked and stored at 4C.

2.9.2 The level of radioactivity was determined with a scintillation counter (L-7500, Beckman). A 2μl sample of the 100μl of probe was added to 10cm^3 of distilled water. The radioactivity, in counts per minute, of this sample was taken as a proportional measure of the total activity of the probe.

2.10 Hybridization of Radioactively Labelled Probe to Nitrocellulose Bound DNA

The detection of homologous DNA sequences with a radioactively labelled probe is the standard method of examining a genome for a specific sequence. The method used for this study was that of Scott *et al* (1984).

2.10.1 10 x Denhardts' solution was used to prepare the filters for hybridization and contained (cm^3/500cm^3): 1M Hepes (Sigma) pH 7.0, 25.0; 20 x SSC, 75.0; 3mg/cm^3 herring sperm DNA (kindly donated by Dr D.B. Scott, DSIR), 3.0; 10mg/cm^3 *E. coli* tRNA (Amersham), 1.0; 20% sodium dodecyl sulfate (Sigma), 2.5; Ficoll 70 (Sigma), 1.0g; bovine serum albumin (Sigma), 1.0g; polyvinylpyrrolidine-10 (Sigma), 1.0g.

2.10.2 Nitrocellulose filters were prehybridized for two hours in 25cm^3 of 10 x Denhardts' at 65C in a sealed plastic bag. At the end of this time, all but 1-2cm^3 of the 10 x Denhardts' was removed and 1,500-2,000 counts per second of freshly boiled probe was added. This was evenly distributed over the filter and the hybridization was allowed to proceed overnight at 65C.

The probe mix was decanted in a waste container for radioactive material and the filter was removed and washed three times in 200cm^3 of 2 x SSC. These wash mixtures were monitored for activity and discarded into special containers until the counts were no higher than background. The filter was blotted dry and a small amount of radioactive (^{14}C-valine) ink was used to unambiguously record orientation. The filter was wrapped in gladwrap and placed in a cassette (DuPont) with a piece of Kodak X-ray film and an intensifying screen (DuPont). The cassette was wrapped in brown paper and stored overnight at -70C. The film was developed in an automatic X-ray film developing machine (Kodak). If this length of

exposure was insufficient a new piece of film was inserted and the process extended as long as deemed necessary.

2.10.3 On some occasions the filters were reprobed. The material already hybridized was removed by washing the filter for 20 minutes in 20mM sodium hydroxide. The filter was then washed for 15 minutes in 0.5M Tris pH 7.4, 2.0M NaCl and finally for 15 minutes in 2 x SSC. The filter was then re-used for DNA hybridization as described above.

2.11 Transfer of a Pattern of Intrinsic Antibiotic Resistance

Theoretically if "new" intrinsic antibiotic resistances have been expressed by the cell, then they may have been acquired from an external source. If so, it may prove possible to transfer these "new" resistances.

A standard mating procedure was used to cross several of the *R. trifolii* strains with *E. coli* HB101 ($RecA^-$, rm^-, Sm^r). The cross was carried out on TY plates; $0.1cm^3$ of a dense culture of each parent and $0.1cm^3$ of a mixture of the parents were spotted on the plate. The *R. trifolii* strain was added at a higher cell density due to its' lower growth rate. The plate was incubated at 30C for two days.

After two days growth the cross mixture was sub-cultured into Luria broth containing $500\mu g/cm^3$ of streptomycin. The broth was incubated at 30C overnight with shaking. To test for transfer of intrinsic antibiotic resistances, $0.3cm^3$ of this culture was mixed with $3.0cm^3$ of Luria top agar and poured onto Luria base plates. Antibiotic discs were placed on the overlay as discussed previously. Control plates containing both parents were also tested. All plates were incubated at 30C for two days. The exconjugant was compared to the parents for response and scored accordingly.

2.12 Production of Bacteriocins by *Rhizobium trifolii* Strains

The production of bacteriocins has been demonstrated for other *Rhizobium* species, notably *R. leguminosarum*. *R. trifolii* strains were examined for the production of bacteriocins against other strains and some soil micro-organisms. The property of bacteriocin production would be of adaptive importance to a soil dwelling micro-organism and may be transmissable to aid dissemination. The ability of *R. trifolii* strains to transfer a bacteriocinogenic factor was investigated. The mating procedure was the same as that discussed in section 2.11.

2.12.1 The initial examination for bacteriocin production was by stabbing a small amount of test culture into an indicator strain overlay. All *R. trifolii* field isolates and the three laboratory strains (TA1, 2163 and 2668) were used as tester cultures. The indicator bacteria were *R. trifolii* strains TA1, 2163, 2668, 0/0, 0/9, 0/10, 0/11, 0/18, 0/20 and 0/23 as well as strains of a number of common soil micro-organisms namely *Bacillus cereus*, *B. subtilis*, *Pseudomonas aeruginosa*, *Ps. fluorescens*, *Ps. denitrificans* and *Serratia marcescens*.

The test procedure was as follows: $0.3cm^3$ of a dense overnight culture of the indicator was mixed thoroughly with $3.0cm^3$ of TY soft agar onto a TY agar plate. The plates were incubated at room temperature for two hours. A small amount of culture was stabbed into the overlay with a tooth-pick in a known pattern. The plates were incubated at 30C for two days and scored for presence or absence of a zone of inhibition.

2.12.2 Selection for a strains of *E. coli* containing a bacteriocinogenic factor was as follows: a heavy loopful of the cross was resuspended in $1.0cm^3$ of water and a dilution series from 10^0 to 10^{-5} prepared.

$0.2cm^3$ of each dilution was mixed with $0.2cm^3$ of a dense culture of indicator bacteria and resuspended thoroughly in $3.5cm^3$ of Luria soft agar. Luria overlay plates were poured and incubated at 30C for two days once set.

Any colonies showing a zone of inhibition were picked off and inoculated into $5cm^3$ of Luria containing $500ug/cm^3$ streptomycin and streaked onto LSm plates. The cultures were incubated at 37C until well grown.

The liquid cultures were sub-cultured into a further $5cm^3$ of LSm and grown at 37C overnight. $1cm^3$ of culture was removed and subjected to the Eckhardt plasmid analysis as discussed in section 2.6.

Cultures showing evidence of a plasmid were tested for bacteriocin production as previously discussed.

2.12.3 Plasmids may be cured from a strain of bacteria by heat treatment (Morrison *et al*, 1983). A heavy culture of *R. trifolii* #0/18 was inoculated onto two TY agar plates. These plates were incubated for five days, one at 37C and one at 40C. The plates were then incubated at 30C for three days. The colonies that grew were inoculated into $5cm^3$ of TY broth and incubated at 30C for two days with shaking. These cultures were diluted and analysed by the Eckhardt plasmid procedure (section 2.6). The presumptive cured strains were tested for the production of bacteriocins as previously discussed.

RESULTS AND DISCUSSION

3.1 Isolation and Maintenance of Bacterial Strains

Plants from which nodules were taken were chosen at random. One nodule was taken from each plant to ensure the sample base was as broad as possible. More isolates were taken in the second series because of the greater density of plant growth at this stage. Contamination of the nodule isolates was noticed occassionally. Further sub-culturing on YEM agar was sufficient to purify rhizobial cultures. Identification of rhizobia could easily be made on the basis of colony morphology.

3.1.1 Soil micro-organisms were isolated by washing the root mass of nodulated plants in sterile distilled water. These rhizosphere "washes" were plated on TSA and nutrient agar. The micro-organisms obtained showed a wide range of colonial morphologies on agar plates. Of the fifty-five colonies which were Gram stained, thirty were Gram negative pleomorphic rods, twelve Gram negative cocci, three Gram positive rods and ten fungi. The high proportion of Gram negative rods was as expected from other work (Alexander, 1961).

3.2 Antigenic Characterization of *Rhizobium trifolii* Strains

All *R. trifolii* field isolates and the three laboratory strains were tested for their response to anti-2668 serum. Rhizobial cells were prepared to release antigenic proteins and polysaccharides. Gel diffusion plates were examined after two days. All strains of rhizobia, except 0/3, showed at least one line of precipitation with the anti-2668 serum (Plates 1 and 2). There was a degree of similarity in the lines of precipitation between the strains. A decision on the similarity could only be made when strains were in adjacent wells. A number of combinations must be tested in order to compare all the reacting antigens. From the tests carried out, the strains can be grouped as follows:

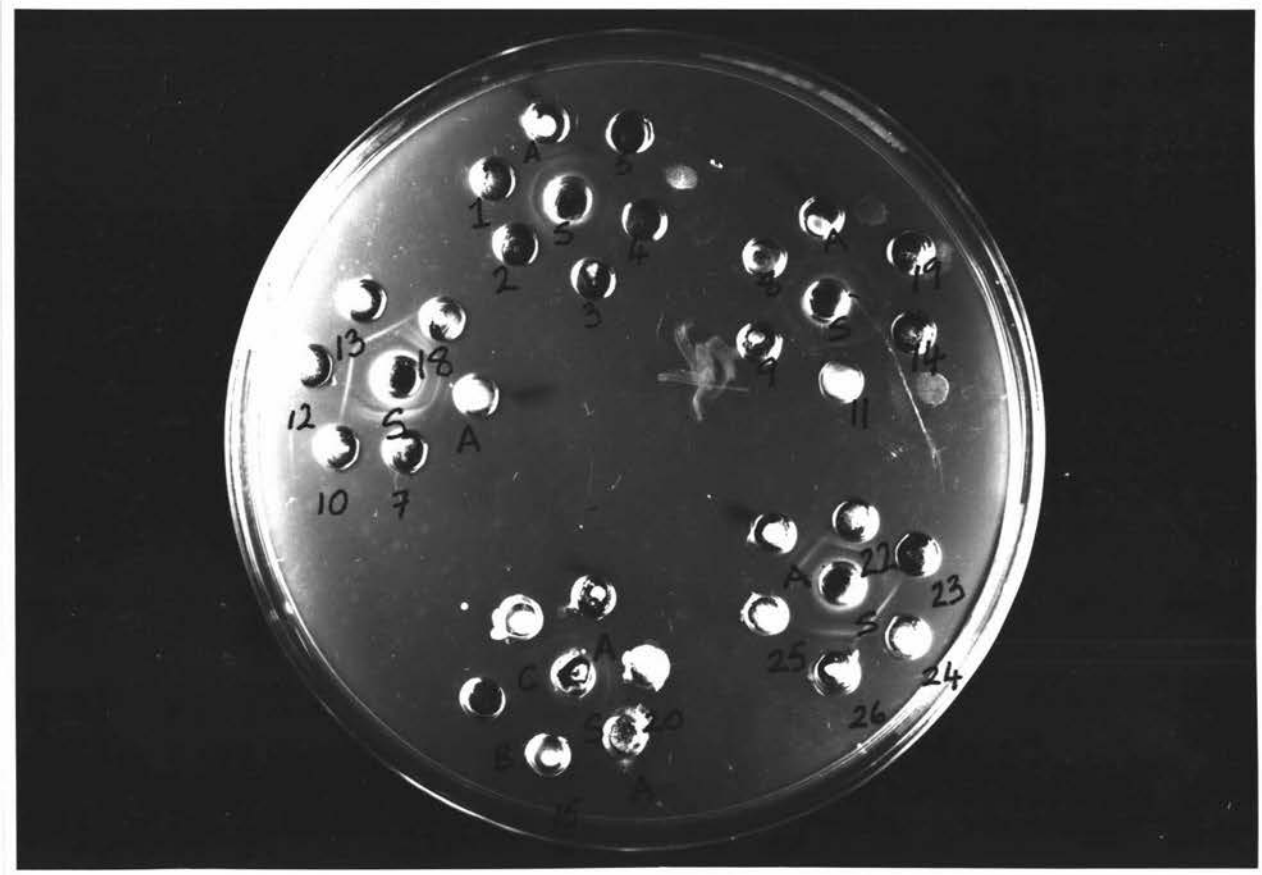


Plate 1. Antigenic reaction of R. trifolii strains to anti-2668 serum. S = serum. A = 2668. B = 2163. C = TAI. The numbers are the field isolates.

Plate 2. Antigenic reaction of R. trifolii strains to anti-2668 serum. S = serum. A = 2668. B = 2163. C = TAI. The numbers are the field isolates.

Group 1: 0/0, 0/2, 0/4, and 0/5.
Group 2: 0/7, 0/8, 0/9, 0/10, 0/11, 0/14, 0/15, 0/18, 0/19, 0/20 and 0/22.
Group 3: 0/12 and 0/13.
Group 4: 0/24, 0/25 and 0/26.

These groupings can only be regarded as tentative until further combinations of strains are examined. The field isolated rhizobia strains all showed a line of precipitation to anti-2668 serum, indicating some identity. 2668 can be regarded as a member of all groups. Rhizobial strains possess more than one soluble antigen and give multiple bands in gel diffusion. The number actually obtained depends on, among other things, the strain, the density of the suspension and the age of the cells. Some antigens are highly strain specific and these are the ones important for strain identification (Vincent, 1970). Confirmation of strain identity can be ensured by the use of absorbed antisera. The anti-2668 serum used is sufficiently non-specific that significant cross-reaction to 2163 and TA1 occurs. This is not totally unexpected, all three are strains of *R. trifolii* and the antigen used to prepare the serum was whole cells. Species specific antigens do exist and would allow this cross-reactivity. To counteract this tendency either a more stringent test, such as countercurrent electrophoresis, or absorbed antisera must be used.

3.3 Intrinsic Antibiotic Resistance Patterns of *R. trifolii* Strains

The intrinsic antibiotic resistance patterns were determined for the field isolates and laboratory strains using eleven antibiotics. The results are shown in Table 3. The concentrations of antibiotic in the discs are shown in $\mu g/cm^3$ and are listed below (brackets):

TABLE 3: Responses of *R. trifolii* strains to low levels of antibiotics.

TABLE 3:

Strain	Amp	Chl	Ery	Gnt	Kan	Mth	Nal	Nov	Pen	Stm	Trm
TA1	-	-	+	-	-	+	-	-	+	+	-
2163	-	-	+	-	-	+	-	-	+	+	-
2668	-	-	-	-	-	+	-	-	+	+	-
0/0	-	-	-	-	-	+	-	-	+	+	-
0/2	-	-	-	-	-	+	-	-	+	+	-
0/3	-	-	-	-	-	+	-	-	+	+	-
0/4	-	-	-	-	-	+	-	-	+	+	-
0/5	-	-	-	-	-	+	-	-	+	+	-
0/7	-	+	+	-	-	+	-	-	+	+	-

TABLE 3 (continued):

Strain	Amp	Chl	Ery	Gnt	Kan	Mth	Nal	Nov	Pen	Stm	Trm
0/8	-	-	+	-	-	+	-	-	+	+	-
0/9	-	-	+	-	-	+	-	-	+	+	-
0/10	-	-	+	-	-	+	+	-	+	+	-
0/11	+	-	+	-	-	+	+	-	+	+	-
0/12	+	+	+	-	-	+	+	-	+	+	-
0/13	+	-	+	-	-	+	+	-	+	+	-
0/14	+	+	+	-	-	+	+	-	+	+	-
0/15	+	-	+	-	-	+	+	-	+	+	-
0/18	-	+	+	-	-	+	+	-	+	+	-
0/19	+	+	+	-	-	+	+	-	+	+	-

TABLE 3 (continued):

Strain	Amp	Chl	Ery	Gnt	Kan	Mth	Nal	Nov	Pen	Stm	Trm
0/20	+	+	+	-	-	+	+	-	+	+	-
0/22	+	+	+	-	-	+	+	-	+	+	-
0/23	+	+	+	-	-	+	+	-	+	+	-
0/24	-	+	+	-	-	+	+	-	+	+	-
0/25	+	+	+	-	-	+	+	-	+	+	-
0/26	+	+	+	-	-	+	+	-	+	+	-

+, growth of bacterial strain. -, inhibition of bacterial strain.

Ampicillin	(10)	Amp
Chloramphenicol	(50)	Chl
Erythromycin	(50)	Ery
Gentamycin	(10)	Gnt
Kanamycin	(50)	Kan
Methicillin	(10)	Mth
Naladixic acid	(5)	Nal
Novobiocin	(30)	Nov
Penicillin	(10)	Pen
Streptomycin	(25)	Stm
Trimethoprim	(25)	Trm

A number of differences between the pattern of the laboratory strains and the field isolated strains were noted. The differences were especially pronounced in the six month isolates.

Several of the strains (2668, 2163, 0/0, 0/7, 0/10,0/18) were tested for their level of antibiotic resistance. The results are shown in Table 4. The alterations in level are minor, except for naladixic acid which shows a significant increase.

Examination of a natural population of *Rhizobium phaseoli* by determining intrinsic antibiotic resistance showed a degree of heterogeneity (Beynon and Josey, 1980). However most of the strains isolated had a greater number of sensitivities than the inoculating strain. Wild type strains of *R. leguminosarum* were isolated during another study and genetically marked derivatives made from them (Josey *et al*, 1979). The marked strains were found to have patterns of resistance related to those of their wild-type parents but with modifications which could be attributed to mutagenic treatments. All *R. leguminosarum* strains were used to inoculate plants and, when recovered as single colony isolates, were found to exhibit the same resistance pattern as the inoculant.

TABLE 4: Levels of antibiotic resistance (ug/cm^3) shown by some *R. trifolii* field isolated strains.

TABLE 4:

Antibiotics	Strains					
	2668	2163	0/0	0/7	0/10	0/18
Amp	5	5	10	10	5	10
Chl	25-	25-	25-	25	25	ND
Ery	25-	25	25	25	25	25
Nal	2.5-	20	2.5-	20	10	20
Nov	15-	ND	15-	ND	ND	ND
Pen	40	20	40	40	40	40
Stm	12.5-	12.5	12.5-	12.5-	12.5	12.5

-, level of resistance is lower than concentrations tested.
ND, not done.

The *R. trifolii* fixed isolates exhibited an increase in the numbers of intrinsic antibiotic resistance. These acquisitions occurred in a non-random manner. Once a resistance was obtained, all subsequent isolates would also exhibit the same resistance. This could be interpreted as a series of changes in the genome occurring in a particular order in all strains. The mechanism of resistance in these field isolates is not necessarily the same as occurs when a bacterial strain gains a high level resistance. These changed resistances could be attributed to immigrant 2163 strains which have gained low level resistances; the basic patterns shown by the two laboratory strains are very similar. Further antibiotics must be used, to attempt to find a series of antibiotics which will distinguish between 2163, 2668 and any derivatives of the inoculant. This test is insufficiently discriminating as it stands.

3.4 Restriction Endonuclease Analysis of *R. trifolii* Strains

Nine different restriction endonucleases were tested for their ability to digest *R. trifolii* DNA (Plates 3 and 4). The enzymes BglII, BamHI, HindIII and KpnI did not totally digest *R. trifolii* total genomic DNA. The enzyme AluI, the only restriction endonuclease of the nine with a four base pair recognition sequence, overdigested the DNA. The enzymes BstEII, EcoRI, PstI and SalI all totally digested *R. trifolii* DNA. The restriction endonuclease of choice for this type of study must generate a clear and repeatable series of fragments on an agarose gel. Using this criteria, BstEII was the enzyme of choice. Both SalI and PstI produced clear patterns, but repeatability was dependant on DNA purity. BstEII did not show this dependancy, possibly because of the higher temperature of incubation.

3.4.1 The *R. trifolii* field isolates and the three laboratory strains were subjected to restriction endonuclease analysis with BstEII (Plates 5-8). The resulting DNA fragments were electrophoresed on agarose gels. The patterns produced were compared

PLATE 3: Comparison of electrophoretic patterns formed by lambda and *R. trifolii* #2668 DNA using various restriction endonucleases. The enzymes used were (right to left): AluI, BamHI, BglII, BstEII and EcoRI.

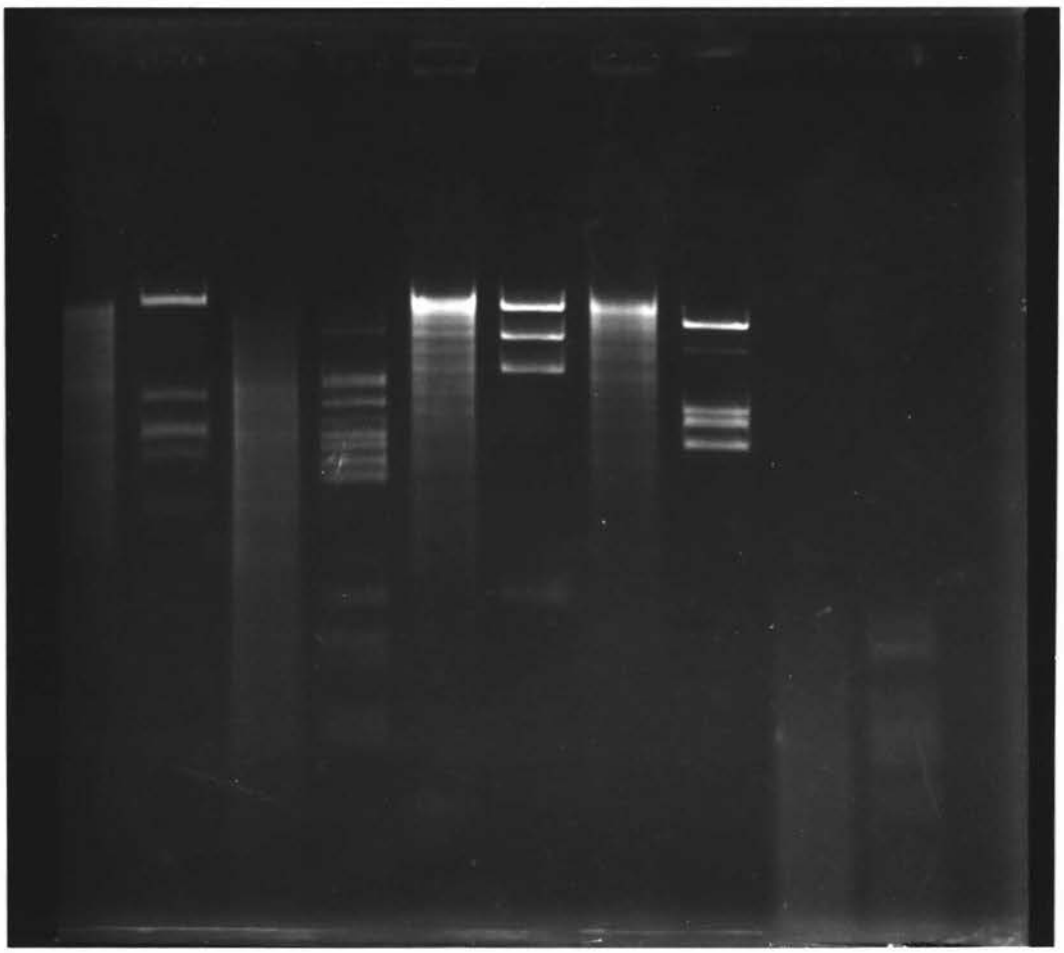


PLATE 4: Comparison of electrophoretic patterns formed by lambda and *R. trifolii* #2668 DNA using various restriction endonucleases. The enzymes used were (right to left): HindIII, KpnI, PstI and SalI.

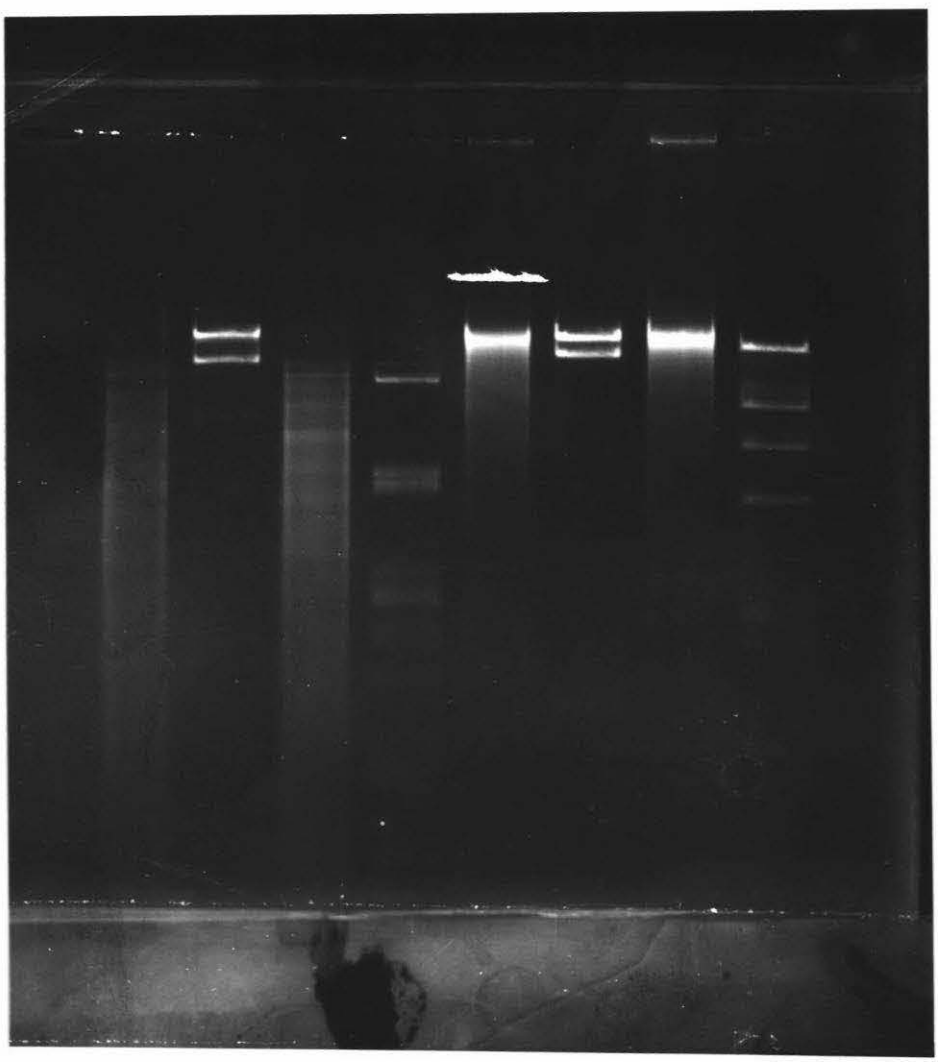


PLATE 5: Restriction endonuclease analysis of *R. trifolii* strains with the enzyme BstEII. Order (right to left): lambda, 2668, 0/0, 0/2, 0/3, 0/4, 0/5, and 0/7.

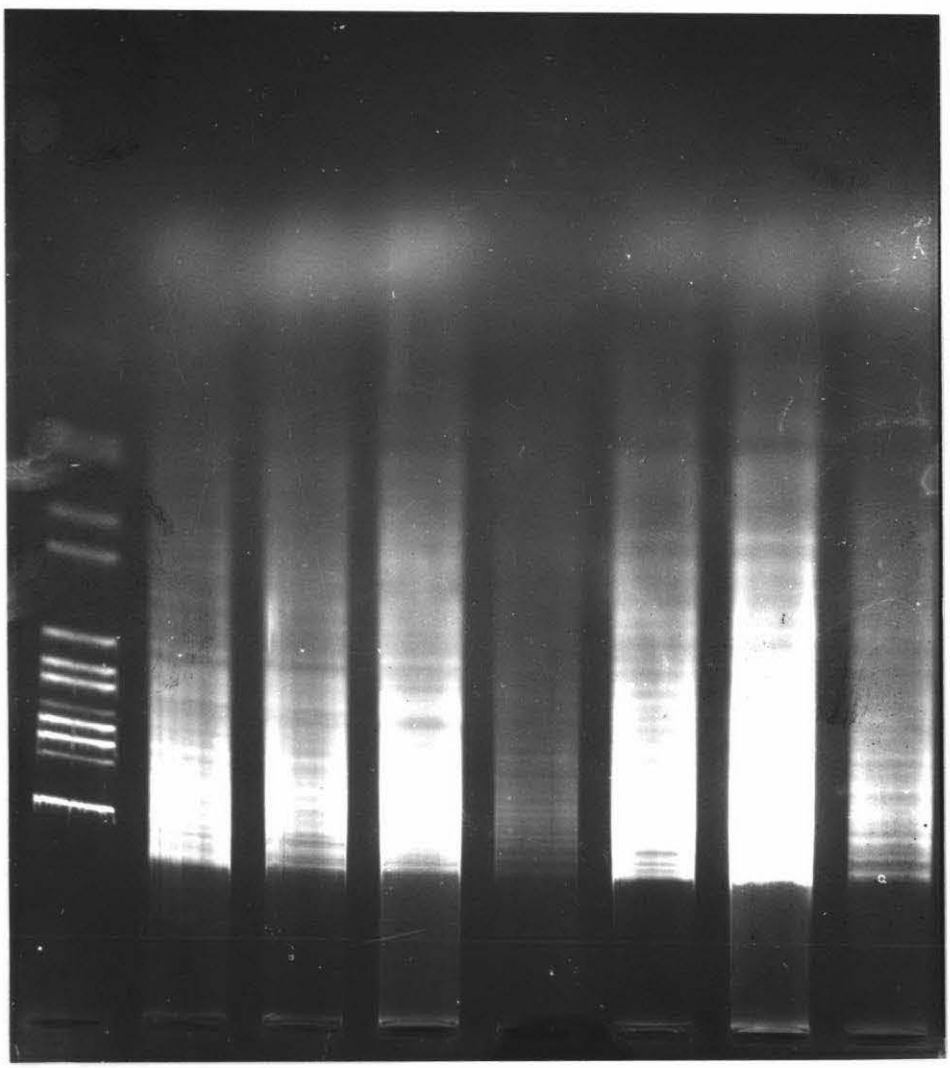


PLATE 6: Restriction endonuclease analysis of *R. trifolii* strains with the enzyme BstEII. Order (right to left): lambda, 2668, 0/8, 0/9, 0/10, 0/11, 0/12 and 0/13.

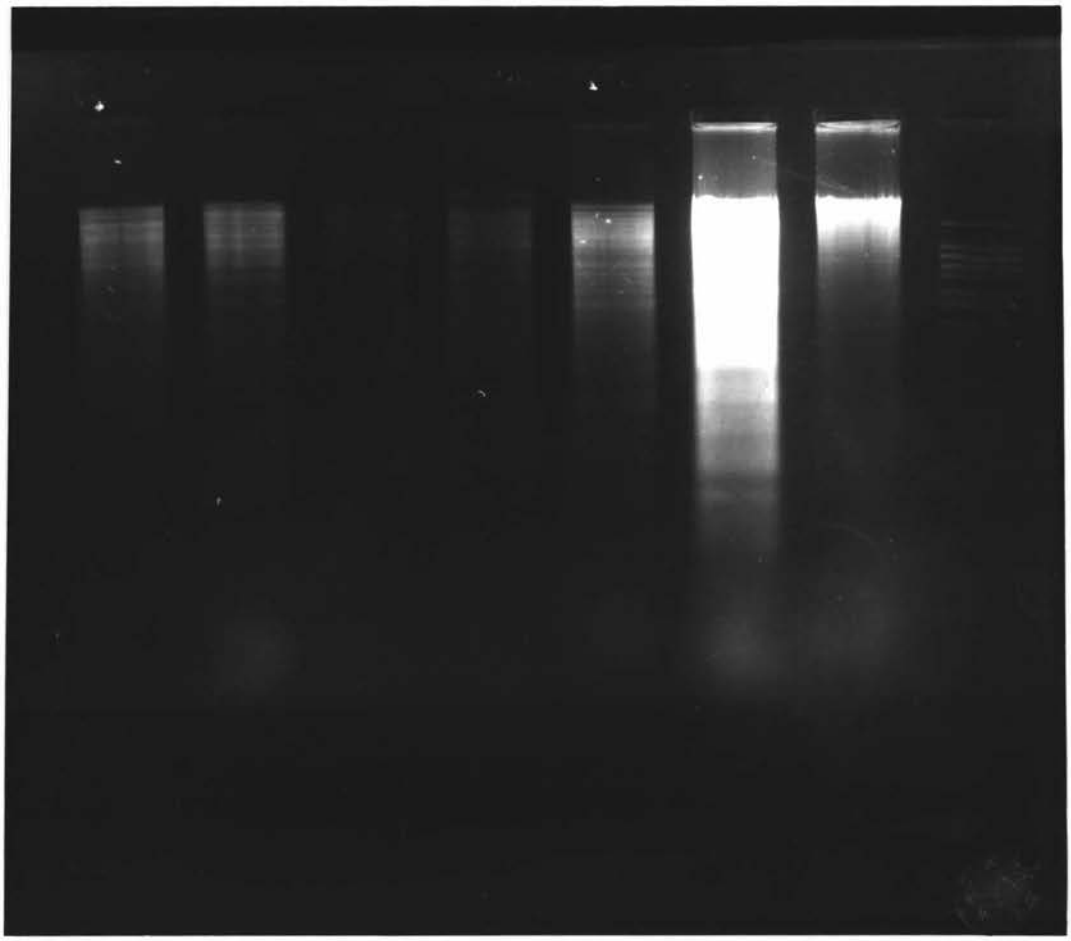


PLATE 7: Restriction endonuclease analysis of *R. trifolii* strains with the enzyme BstEII. Order (right to left): 2668, 0/8, 0/14, 0/15, 0/18, 0/26, TA1 and 2163.

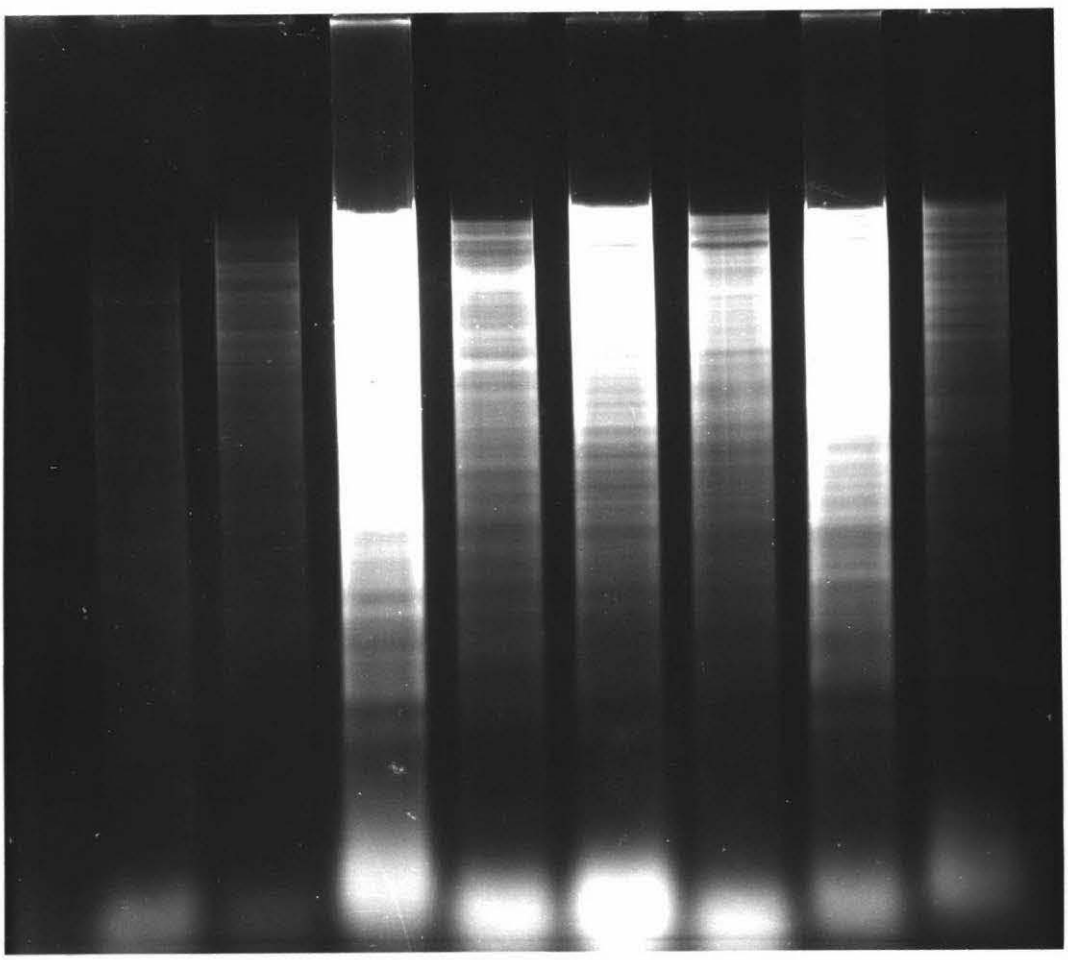
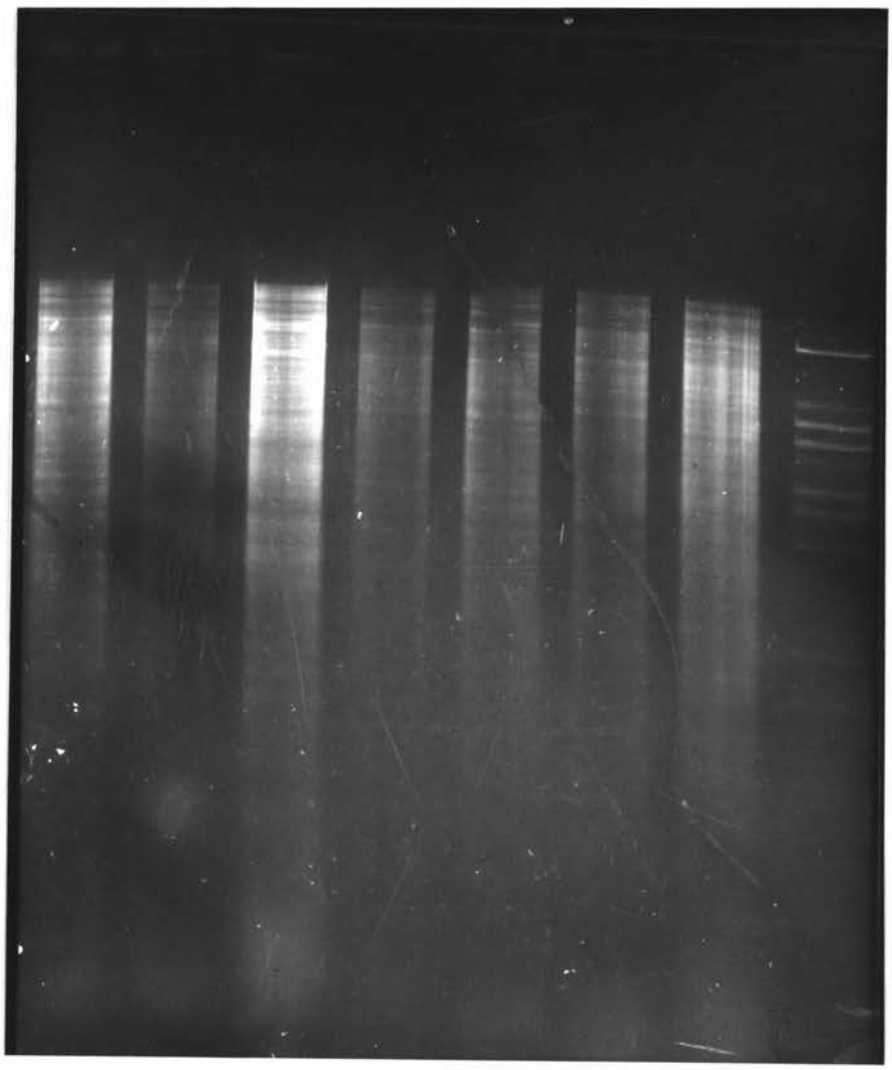


PLATE 8: Restriction endonuclease analysis of *R. trifolii* strains with the enzyme BstEII. Order (right to left): lambda, 2668, 0/19, 0/20, 0/22, 0/23, 0/24 and 0/25.



to that of the inoculant strain and amongst the field isolates. Comparison of the patterns was by eye and the ratings given were as follows (Table 5).

Patterns indistinguishable from 2668 were given a rating of "indistinguishable" (++++). Patterns that showed only one or a few bands different were given a rating of "very similar" (+++). Patterns that showed a number of band differences, but still displayed definite similarity were given a rating of "similar" (++). Patterns that showed major differences and showed notable similarity were given a rating of "related" (+). Patterns showing no similarity were given a rating of "unrelated" (-). Some care must be taken because of the subjective nature of these relationships. However, with care and practice, errors are few. The results of these experiments show that all the field isolates show a rating of "similar" or higher to the pattern shown by 2668. Differences are not totally unexpected; the plasmids are large enough to make significant contributions to the band pattern.

Fragment patterns were also compared between the field isolates and classes were assigned on the basis of similartiy. The results are summarised in Table 6. One of the patterns (K) is common. These strains were grouped on a basis of "+++" or better. The field isolates show a rating of "similar" or better to each other when compared.

Comparison of the isolates to the laboratory strains 2163 and TA1 shows that they are quite dissimilar and the rating would be "related" at best. The similarity between 2163 and TA1 is of a comparable level.

Restriction endonuclease analysis in other systems has shown that the degree of variability in fragment pattern is itself variable. The species *Brucella ovis*

TABLE 5: A comparison of the restriction endonuclease analysis patterns generated by *R. trifolii* strains.

TABLE 5:

Strain	Similarity to 2668	Class of Pattern
2668	++++	A
2163	+	NC
TA1	+	NC
0/0	++++	A
0/2	++	B
0/3	+++	C
0/4	+++	D
0/5	+++	D
0/7	+++	E
0/8	+++	F
0/9	+++	G
0/10	+++	H
0/11	+++	H
0/12	++	I
0/13	+++	J
0/14	++	K
0/15	++	K
0/18	++	L
0/19	++++	A
0/20	+++	K
0/22	+++	K
0/23	++	M
0/24	+++	N
0/25	+++	K
0/26	+++	K

++++, indistinguishable
+++, very similar
++, similar
+, related
-, unrelated
NC, not classed

TABLE 6: Common restriction endonuclease analysis patterns shown by *R. trifolii* strains.

TABLE 6:

Class	Members
A	2668, 0/0, 0/19
B	0/2
C	0/3
D	0/4, 0/5
E	0/7
F	0/8
G	0/9
H	0/10, 0/11
I	0/12
J	0/13
K	0/14, 0/15, 0/20, 0/22, 0/25, 0/26
L	0/18
M	0/23
N	0/24

is extremely homogeneous with no detectable differences in pattern (O'Hara *et al*, 1985). *Leptospira icterhaemorragiae* var. *harjo* can be divided into several types (corresponding to serovars) by this technique (Marshall *et al*, 1981). A higher degree of variability exists in the species *Mycoplasma ovipneumoniae*, with a large number of distinguishable restriction patterns in existence (Norman, personal communication). These *R. trifolii* field isolates fall into neither category. However, the contribution of the plasmids can not be underestimated. Some of these replicons are large. The degree of similarity shown by these isolates suggests they may be variations on a theme. The similarity to 2668 suggests they are likely to be derivatives of this strain.

Further investigation into the nature of the variation of the restriction profile would seem warranted. There are a number of approaches to this problem. Several strains (including 2668) could be totally cleared of plasmids and their DNA profiles compared by restriction endonuclease analysis. If the plasmid-free profiles were the same it would suggest that the different plasmids were the basis of the variation. Another comparison of profile would be to use random fragments of DNA from the 2668 profile as probes to the other strains. Alterations in the genome structure could be detected by shifts in band position. Closer examination of any altered bands may shed some light on the mechanism of genome variation.

3.5 Plasmid Profiles of *R. trifolii* Strains

Eckhardt plasmid gel electrophoresis was applied to the field isolates and laboratory strains of *R. trifolii* (Plates 9 and 10). The plasmid profiles obtained were compared and the information obtained is summarised in Table 7. The basis of comparison is electrophoretic mobility as measured by distance migrated in an agarose gel. Information obtained by this procedure is inconclusive but serves as a useful guideline for future investigation.

Of the twenty-two strains examined, seven had fewer plasmids than 2668, five had the same number and ten had more. A comparison of these profiles by distance migrated shows twelve different profiles (Table 8). These classes of profiles are shown diagrammatically in Figure 2. Plasmids common to several groups can be seen in this diagram. It appears that most of the plasmids in the field isolates are at least slightly different in size to those found in 2668.

The information obtained by measuring electrophoretic mobility in this way can only be regarded as an indicator. Confirmation must be obtained by mixing and lysing two strains in the same well. Differences in plasmid size are obvious using this procedure.

Analysis of field isolated plasmids has shown some regional conservation of structure (Huguet *et al*, 1980). Hybridization studies have revealed sequence homologies between *R. leguminosarum* and *R. trifolii* plasmids and plasmids from *A. tumefaciens* (Denarie *et al*, 1981). Plasmid number is variable and large plasmids are common. There is very little information on alteration of plasmid profile in the soil. Further investigation on the source of the extra-chromosomal bands is required. If the re-organization of the genome can occur under stress (Heumann *et al*, 1983), then it is conceivable that these plasmids are chromosomally derived. To distinguish between internal and external derivation of the plasmids a gene library could be constructed.

PLATE 9: Eckhardt gel electrophoresis of *R. trifolii* strains. Left to right: 2668, 0/0, 0/2, 0/4, 0/5, 0/7, 0/8, 0/10, 0/11, 0/12, 0/13 and 0/14.

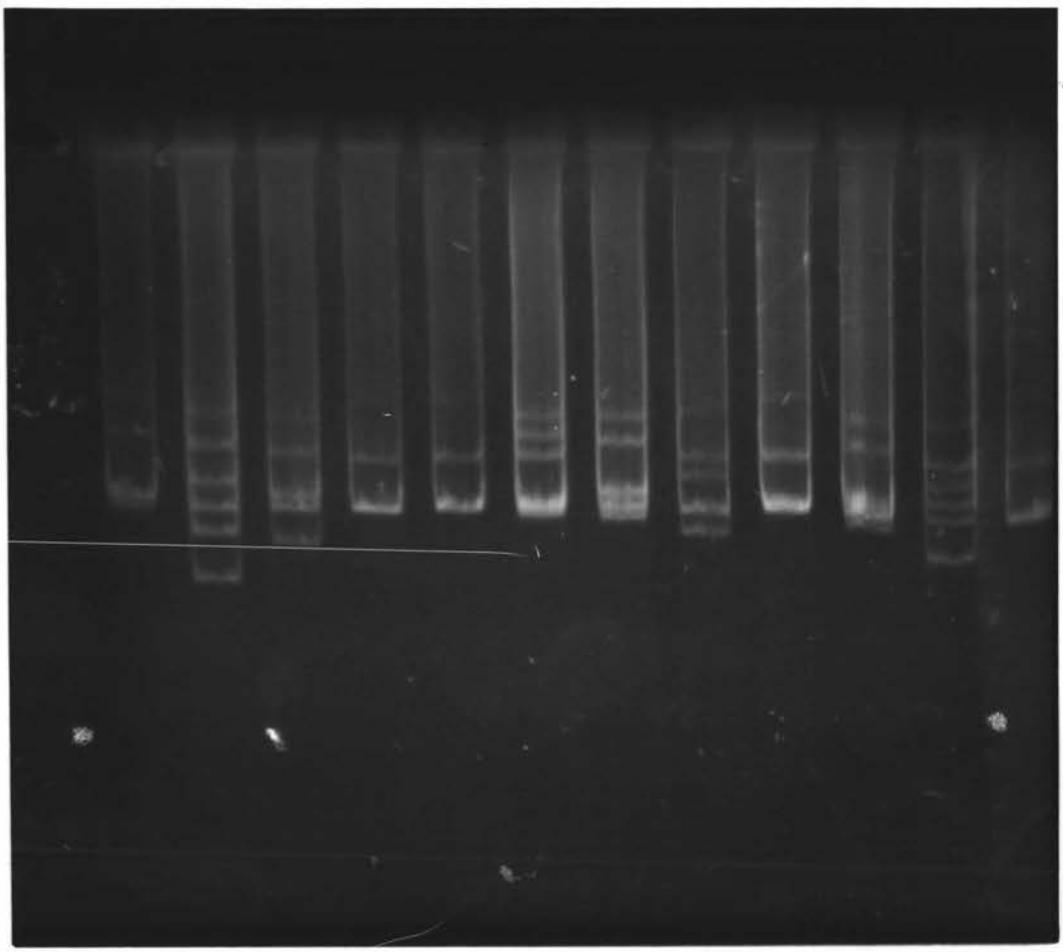


PLATE 10: Eckhardt gel electrophoresis of R. *trifolii* strains. Right to left: 0/15, 0/18, 0/19, 0/20, 0/22, 0/23, 0/24, 0/25 and 0/26.

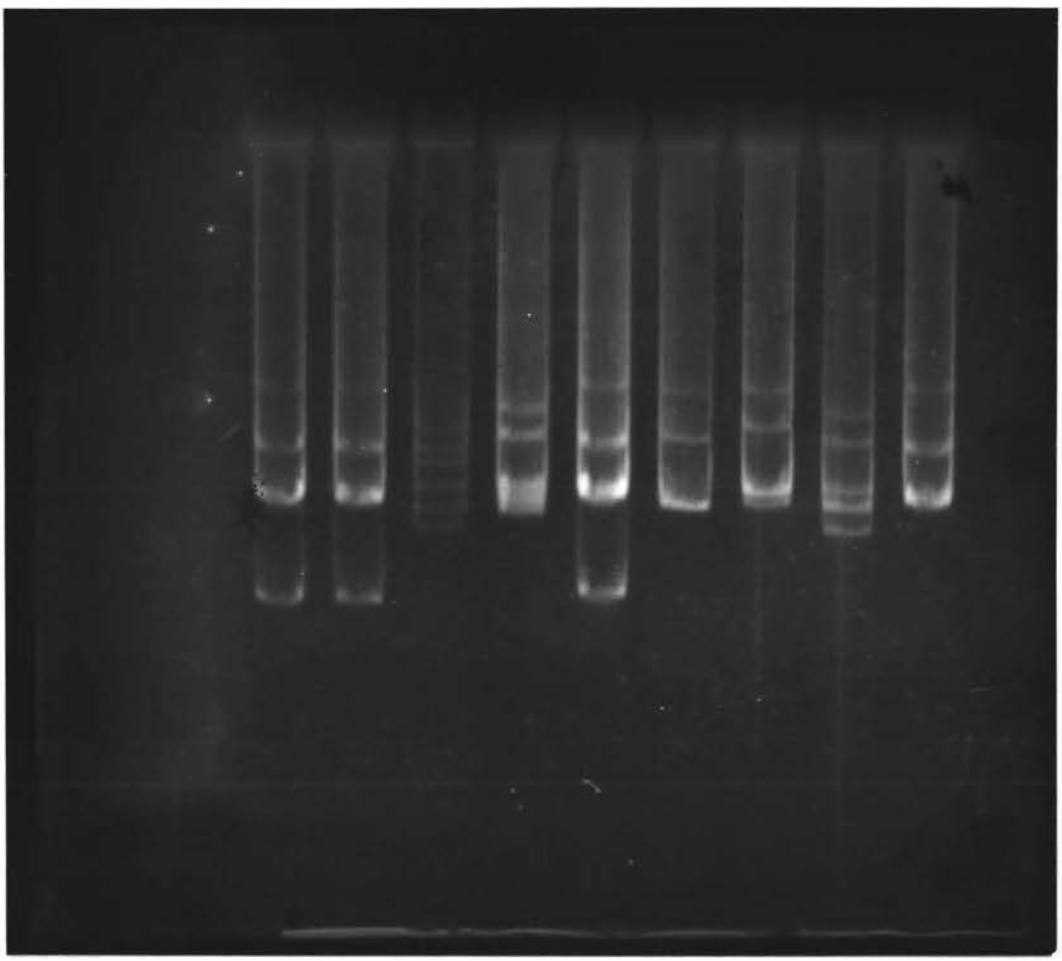


TABLE 7: Plasmid profiles obtained on agarose gels after Eckhardt treatment of *R. trifolii* field isolates.

TABLE 7:

Strain	Number of Plasmids	No. in common with 2668	Pattern
2668	4	4	A
2163	3	ND	ND
TA1	3	0	TA1
0/0	6	1	B
0/2	5	2	C
0/3	3	1	D
0/4	3	1	D
0/5	3	1	D
0/7	5	1	E
0/8	5	3	F
0/9	5	2	G
0/10	5	1	H
0/11	3	1	D
0/12	4	1	I
0/13	6	1	J
0/14	3	1	D
0/15	3	1	D
0/18	5	2	C
0/19	4	4	A
0/20	3	1	D
0/22	4	1	K
0/23	5	3	F
0/24	6	1	L
0/25	4	1	K
0/26	4	1	K

ND, not done.

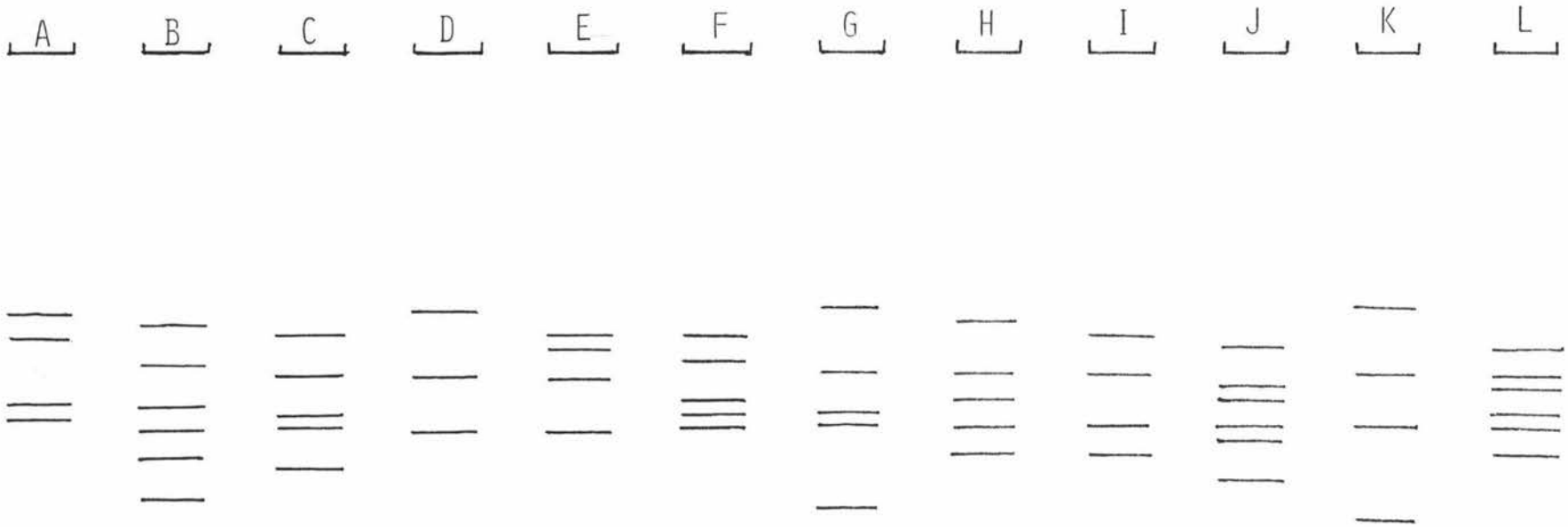
TABLE 8: Groups of plasmid profiles obtained by comparison of electrophoretic mobility.

TABLE 8:

Group	Strains in the Group	No. of Plasmids
A	2668, 0/19	4
B	0/0	6
C	0/2, 0/18	5
D	0/3, 0/4, 0/5, 0/11, 0/14, 0/15, 0/20	3
E	0/7	4
F	0/8, 0/23	5
G	0/9	5
H	0/10	5
I	0/12	4
J	0/13	6
K	0/22, 0/25, 0/26	4
L	0/24	6

FIGURE 2: Plasmid profiles obtained after Eckhardt gel electrophoresis shown diagrammatically in classes.

FIGURE 2:



The library would contain the total plasmid DNA of a strain, obtained from a cesium chloride gradient. Such a library could be used as a source of probes to compare plasmid structures and to examine the genome. The library itself can also be investigated for specific sequences, such as the *nif* genes.

3.6 Hybridization of Plasmid Profiles With a Radioactively Labelled Probe

Plasmid profiles of *R. trifolii* field isolates were transferred to nitrocellulose filters using the Southern blotting procedure. Radioactively labelled *R. trifolii nif HDK* probe was added to the filters. Radioactive labelling was detected by autoradiography. The results are summarised in Table 9.

The plasmid which hybridized to this probe was variable in size (Plate 11). Fifteen of the twenty-two field isolates showed hybridization to the same band. This band was also common with strain 2668. This band appeared to be one of the 2668 "doublet" - the pair of smallest plasmids.

Of the four strains which did not hybridize to the same band, two showed one different band of hybridization and two strains had two different. The two strains not showing hybridization to the same band as 2668 did not show hybridization to a common band either. The two strains which showed two different bands of hybridization probably have neither of the hybridized plasmids bands in common. Three strains show an extra band of hybridization. This extra band corresponds to a small plasmid seen in all three strains. These strains have the same plasmid profile (Figure 2, profile K).

There has been a large number of changes in the position of the *nif* genes in the field isolated *R. trifolii* strains. Despite the lower intensity of the secondary bands, it is unlikely these bands are non-specific hybridization.

PLATE 11: DNA hybridization of Eckhardt plasmid profiles of *R. trifolii* strains using *R. trifolii nif KDH* probe. Left to right: 2668, 0/2, 0/3, 0/4, 0/5, 0/7, 0/8, 0/9, 0/10, 0/11, 0/12, 0/13, 0/14, 0/15, 0/18, 0/19, 0/20, 0/22, 0/23, 0/24, 0/25, 0/26, and 2668.

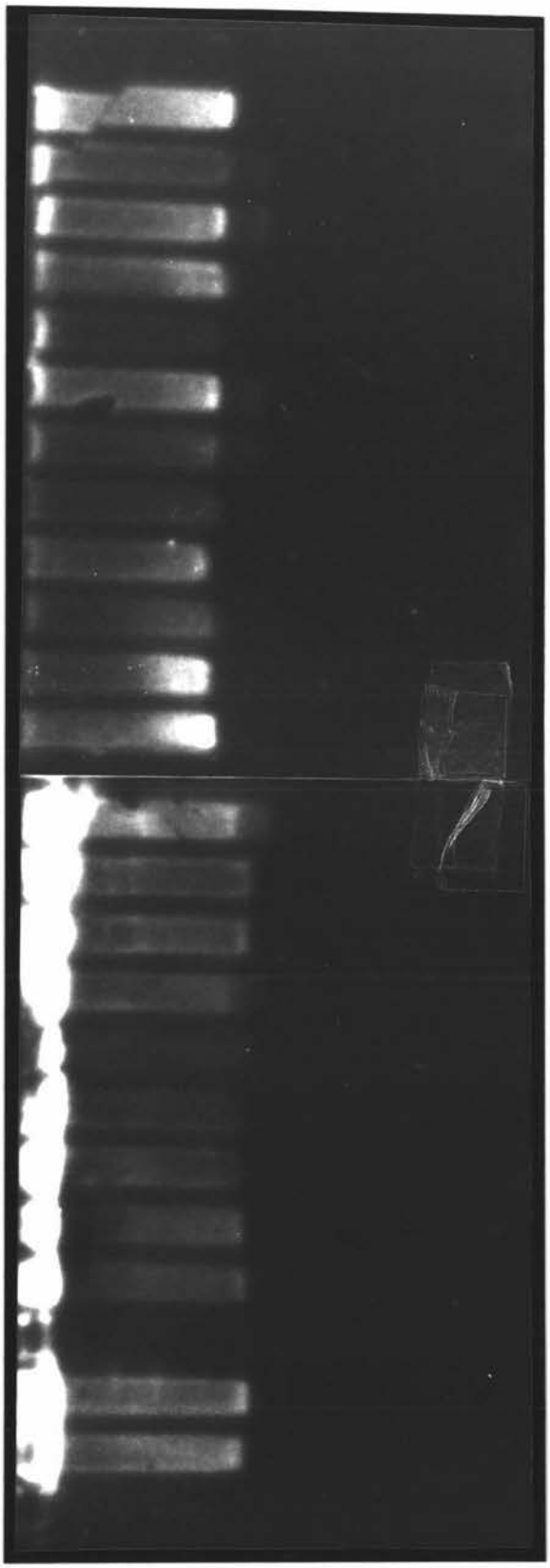


TABLE 9: Hybridization of *R. trifolii nif HDK* radioactively labelled probe to plasmid profiles of *R. trifolii* strains.

TABLE 9:

Strain	No. of Plasmids	Hybridized Plasmid Profile	
		No. of Bands	No. same as 2668
2668	4	1	1
0/0	6	1	0
0/2	5	1	0
0/3	3	ND	ND
0/4	3	1	1
0/5	3	1	1
0/7	5	1	1
0/8	5	1	1
0/9	5	1	1
0/10	5	2	0
0/11	3	1	1
0/12	4	1	1
0/13	6	2	0
0/14	3	1	1
0/15	3	1	1
0/18	5	1	1
0/19	4	1	1
0/20	3	1	1
0/22	4	2	1
0/23	5	1	1
0/24	6	1	1
0/25	4	2	1
0/26	4	2	1
TA1	4	ND	ND
2163	4	ND	ND

ND, not done.

The position of the secondary hybridization corresponds to the position of a plasmid in the profile in all cases. The conditions of hybridization are also relatively stringent. There are other possible explanations for the secondary band. A "module" of DNA may have been deleted from the main band (*sym* plasmid) in some cases. Yet the plasmids seen in the gel are not significantly lower in intensity. A more likely explanation is the hybridization to a homologous sequence, but not necessarily the structural genes themselves. For example, contained within the *nif HDK* probe is a short "promoter" sequence (as discussed previously). It is possible that this sequence exists on plasmids other than p*sym*.

Some of the symbiotic plasmids are showing a large difference in size (notably 0/10 and 0/13) to that seen in 2668. Both appear to be larger than the 2668 p*sym*. It is possible that a "module" of DNA, such as a transposon, has inserted and increased the size of these plasmids.

A degree of variation among symbiotic plasmids exists. Two strains of *R. leguminosarum* have been isolated. One carries the *sym* plasmid pRL1JI, which is a small (195kb), transmissable plasmid which produces a bacteriocin. The *sym* plasmid pRL6JI (285kb), is non-transmissable and carries no bacteriocin. The strain will uptake hydrogen (hup^+), however. Therefore, the range of sizes of symbiotic plasmids which occurs in the field isolated *R. trifolii* strains is not remarkable. What is surprising is the rapidity at which changes to the basic 2668 pattern are occurring. Further investigation must be undertaken to confirm and extend the results obtained so far. A probe of the *DK* structural genes only would not contain the repeat sequence and might prove useful for detecting the *nif* structural genes only. Similarly, a probe consisting of the "promoter" alone may determine whether or not this sequence is present on other plasmids. The greater homology of the probe should balance the hybridization intensity, assuming the number of repetitions is equal. Other analysis must involve the isolation of the

the plasmids showing hybridization, examination of these replicons by restriction endonuclease analysis and comparison to the 2668 *sym* plasmid.

3.7 Transfer of a Pattern of Intrinsic Antibiotic Resistance

R. trifolii strain 0/18 was crossed with *E. coli* HB101 and incubated overnight. The crossed bacteria were resuspended, diluted and plated onto selective media. *E. coli* HB101 did not grow on $10ug/cm^3$ of naladixic acid. The *R. trifolii* strain was doubly selected against; the level of antibiotic and the media used for selection, as *R. trifolii* does not grow on Luria. The selective plates were incubated for three days at 30C.

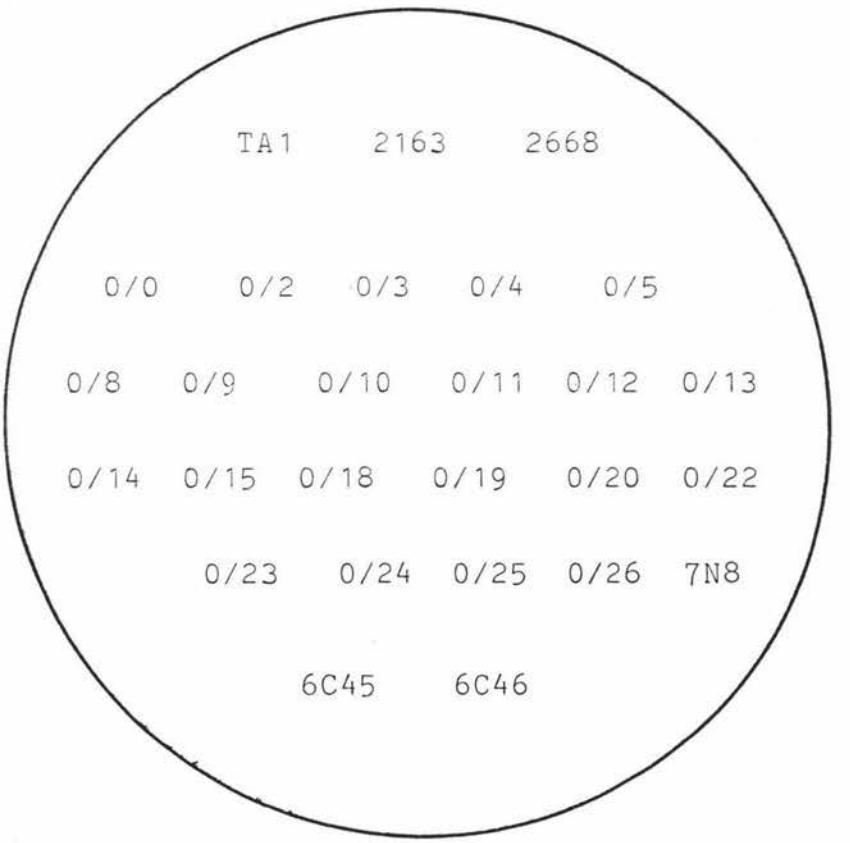
Several trials of this cross were carried out. None of the selective plates showed any signs of colonies. It is possible that attempting to transfer the pattern of intrinsic antibiotic resistance across a genera barrier is too difficult. A test which could be performed would be to substitute a streptomycin resistant 2668 culture for the *E. coli*. Transfer of these traits into another *R. trifolii* strain may prove less difficult.

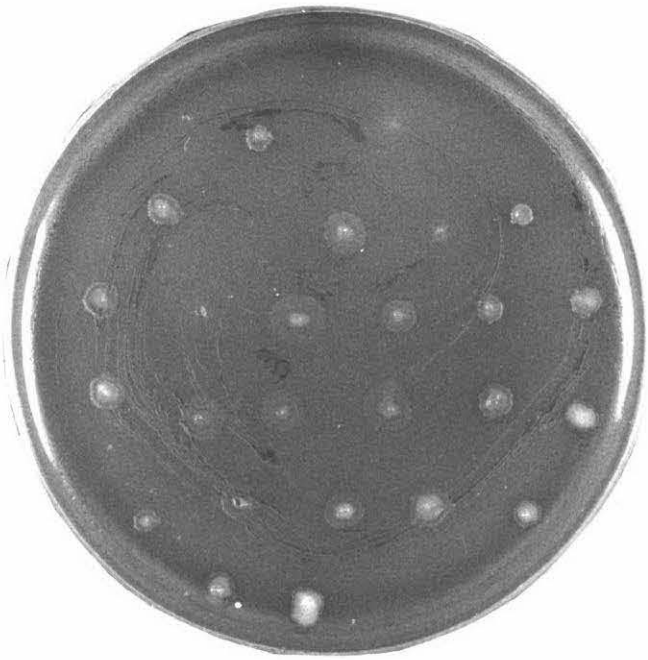
3.8 Production of Bacteriocins by *R. trifolii* Strains

Strains of bacteria to be used as indicators were overlaid onto plates and test cultures stabbed into the overlay. The plates were examined for zones of inhibition after two days. Two classes of bacteriocin producers were seen. The first inhibited only non-rhizobial species and the second inhibited rhizobia and non-rhizobia (Plates 12 and 13). Of all the tested rhizobia strains, only 2668 and 2163 did not produce bacteriocins. The first class of producers included the strains TA1, 0/0-0/4, 0/7-0/10 and 0/13. The second classes comprised strains 0/5, 0/11, 0/12, 0/14 and 0/18-0/26. The results are summarised in Table 10. The sizes of the zones varied according to the indicator. Zones of stimulation were seen at times outside the zone of inhibition. No inhibition of the laboratory strains, nor of the field isolates 0/9, 0/10, 0/11, and 0/18 was exhibited by any tested strain. The inhibiting agent, strictly speaking, should not be called a bacteriocin. The range of inhibition shown by the "bacteriocin" is wide, both Gram positive and Gram negative micro-organisms. Because not all rhizobia are showing exactly the same range of inhibition, it seems likely there is more than one "bacteriocin". Strains did not always produce the bacteriocin - occassionally no zone of inhibition could be seen. This lack of inhibition could be due to other factors, including low amounts of bacteria on the stab.

Bacteriocin production is exhibited by many species of micro-organisms. *R. leguminosarum* produced a *medium* bacteriocin, the determinant of which was shown to be transferrable (Hirsch, 1979). Chromosomal genes were shown to be mobilized and there is indirect evidence as regards the mobile nature of this function. Another *R. leguminosarum* strain carries a 195kb plasmid (pRL1) which has the functions Nod, Nif, Tra (transfer), production and immunity to *medium* bacteriocin, repression of and sensitivity to *small* bacteriocin (Priem and Wijffelman, 1984). A bacteriocin associated with *Streptococcus cremoris* was shown to be plasmid borne and transferrable (Neve, Geis and Teuber, 1984). Some of the

PLATES 12 and 13: *R. trifolii* strains stabbed into indicator bacteria. The indicator on Plate 12 is *Serratia marcescens* and on Plate 13 is *Bacillus cereus*. The order of strains in both plates is shown by the following representation:





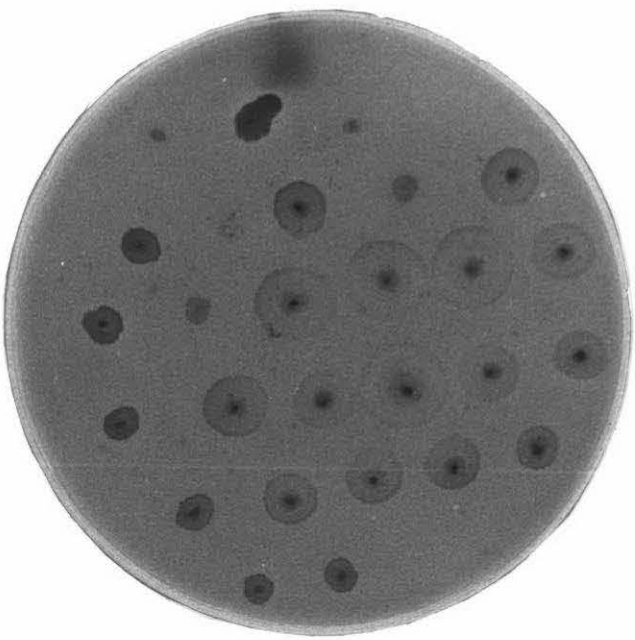


TABLE 10: Patterns of inhibition produced by *R. trifolii* strains on various indicator bacteria.

TABLE 10:

Stab	Indicator											
	TA1	2163	2668	0/0	0/10	0/23	Bc	Bs	Pa	Pd	Pf	Sm
TA1	-	-	-	-	-	-	0	0	-	-	-	-
2163	-	-	-	-	-	-	-	-	-	-	-	-
2668	-	-	-	-	-	-	-	-	-	-	-	-
0/0	-	-	-	-	-	-	0	0	-	-	-	+
0/2	-	-	-	-	-	-	-	-	-	0	-	0
0/3	-	-	-	-	-	-	-	-	-	-	-	-
0/4	-	-	-	-	-	-	0	0	-	0	0	0
0/5	-	-	-	-	0	0	0	-	-	-	-	0
0/7	-	-	-	-	-	-	0	0	-	-	-	0
0/8	-	-	-	-	-	-	0	0	-	-	-	-
0/9	-	-	-	-	-	-	-	-	0	0	-	+
0/10	-	-	-	-	-	-	0	0	-	-	-	0
0/11	-	-	-	0	0	0	0	-	-	0	-	+
0/12	-	-	-	0	-	-	0	0	-	0	-	+
0/13	-	-	-	+	-	-	-	0	-	0	0	+
0/14	-	-	-	0	-	-	0	0	-	-	0	+
0/15	-	-	-	0	0	0	0	0	-	0	-	+
0/18	-	-	-	0	0	0	0	0	0	-	0	+
0/19	-	-	-	0	-	-	0	0	-	-	-	+
0/20	-	-	-	0	-	+	0	-	-	-	-	+
0/22	-	-	-	0	-	+	0	0	-	-	-	+
0/23	-	-	-	0	-	+	0	-	-	-	-	+
0/24	-	-	-	0	-	-	0	-	-	-	-	+
0/25	-	-	-	0	-	-	0	-	-	-	-	+
0/26	-	-	-	0	-	-	0	0	-	-	-	+

Bc, *B. cereus* Bs, *B. subtilis* Pa, *P. aeruginosa*
Pd, *Ps. denitrificans* Pf, *Ps. fluorescens*
Sm, *S. marcescens*
0, inhibition of indicator +, stimulation of indicator
-, no effect on indicator

"bacteriocins" produced have an extremely wide range of activity. A bacteriocin-like substance produced by a *Staphylococcus aureus* strain inhibited Gram positive and negative micro-organisms (Kader, Sahl and Brandis, 1984). It was shown to inhibit the biosynthesis of DNA, RNA, protein and polysaccharide and there is some evidence the function is plasmid borne.

Many of the bacteriocins found for other rhizobia have been shown to be transmissable. An attempt to establish whether this was the case for the field isolates was made. *R. trifolii* strain 0/18 was chosen as the "donor" of the bacteriocinogenic function because of the range of inhibition it generated. The dilution series of the mating mixture was added to Luria overlay containing one of the four indicators. Low numbers of presumptive exconjugants exhibiting inhibition were recovered, the highest number being six on the 10^{-1} *B. cereus* indicator. Seven *E. coli* cultures were further examined (cultures x1 through x7). An Eckhardt plasmid analysis showed two of the exconjugants (x2 and x5) contained a plasmid. These two cultures also exhibited the "bacteriocin" producing phenotype on the indicators discussed previously. After subculturing, Eckhardt plasmid analysis could no longer detect a plasmid in any of the exconjugants. However, x5 still produced a "bacteriocinogenic" substance as shown by zones of inhibition on indicator bacteria. Control cultures which were included at all stages behaved as would be predicted, namely bacteriocin production by *R. trifolii* 0/18 and none by *E. coli* HB101.

The transfer of the bacteriocinogenic factor between genera suggests that the property either resides on or can be easily mobilized by an existing broad host range replicon. The soil micro-organisms are known to carry a number of such replicons (Reanney, 1978). The ability of the field isolates but not the inoculant to produce bacteriocins suggests that the production may be a plasmid-borne trait acquired from another soil micro-organism. The inhibition of some rhizobial strains by others would indicate that there was

more than one "bacteriocin"; immunity to a particular bacteriocin is always associated with its production.

Experimental evidence indicates that "bacteriocin" production is plasmid borne in these *R. trifolii* field isolates. This must be confirmed, either by stable transfer to a non-producing recipient or by curing plasmids from a cell and subsequent loss of bacteriocin production. Some characterization of the inhibitory compound may prove valuable. It may be possible to identify the protein band by comparing the supernatant of media from producing and non-producing cells. If it can be shown to be plasmid borne, the replicon in question can be transferred to a different host and examined in more detail.

CONCLUSIONS

Two basic problems presented themselves in the course of this study. The first problem involves the identification of the field isolated strains; are the "alterations" in perceived genotypes and phenotypes because of changes in the genome or the immigration of adapted strains from other pastures. Differentiation between these two possibilities is necessary. The second problem, which arises from the first, was the question of how the genomic changes occurred. The possible agents of the alterations include the acquisition of genetic information from other soil micro-organisms and the internal rearrangement of the rhizobial genome.

Samples were obtained from plants chosen randomly in a transect across the field. One nodule was sampled from each plant. Because of the huge quantity of inoculant rhizobia applied to the fields, it is very unlikely that a nodule chosen randomly would be the result of an immigrant strain. The topography of the field is such that water run-off is unlikely to carry any bacteria from the old pasture into the new.

The question of alteration as against immigration was addressed by using three identification techniques. The results from the gel diffusion tests were inconclusive. All the field isolates (except 0/3) showed at least one line of precipitation with the antiserum. The strains showed common antigens with strain 2668 and could be grouped by antigens common amongst themselves. The technique did not distinguish the isolates from the other laboratory strains; both 2163 and TA1 showed lines of precipitation with this antiserum. Further, more selective testing is required, either with absorbed antisera or with a more discriminating immunological test.

The patterns of intrinsic antibiotic resistance exhibited by the strains vary in a non-random way. The earlier isolates exhibited the same antibiotic resistances as the inoculant, 2668. Later isolates showed an increasing number of antibiotic resistances, always the same resistances as the earlier

members and one further. The resistance profiles appear to be a basic pattern that has gained low level immunity. This basic pattern, as shown by the common resistances and sensitivities, is that of 2668. The field isolates appear to be 2668 derivatives which have gained low level antibiotic resistances.

Restriction endonuclease analysis (REA) is the most sensitive of these three techniques, because it is most sensitive to alterations. Only two field isolates have REA patterns indistinguishable from that of 2668. However, most of the other strains were very similar (+++). Differences between strains are to be expected considering the number of plasmids each contains; these could contribute significantly.

While no one technique is sufficient to generate firm identification, especially as genomic change is one of the proposed reasons for divergence, an amalgamation of results from all three should be. All of the field isolated strains exhibited a positive response to anti-2668 serum. The basic intrinsic antibiotic resistance pattern was the same as that of 2668 and the restriction endonuclease fragment patterns generated by total genomic DNA from the strains was obviously related to that shown by 2668. Therefore it is most probable that these field isolated strains are 2668 strains which have undergone changes in genotype and phenotype. There are no obvious correlations between the groups of intrinsic antibiotic resistance patterns, antigenic groups and classes of restriction endonuclease profile.

The alterations in plasmid profile and *nif* gene location are indicative of large scale genomic change. Consideration of the mechanism of this change leads to the second question, namely whether the change is internally or externally derived, or whether both sources may be significant. The alteration of the restriction, plasmid and hybridization pattern could be explained by re-organization of the existing genome. The genus *Rhizobium* is capable of major alteration of its genome under stress. This can include asymbiotic fixation of nitrogen, the disappearance of plasmids and the production of

unusual pigments (Heumann *et al*, 1983). A proposed mechanism for these alterations involves stepwise amplification of parts of the genome. The existance of insertion elements is also responsible, at least in part, for the dynamic nature of the genome. Theoretically such large scale changes could lead to the expression of previously silent genes. This in turn could lead to the alteration of antigens and production of bacteriocins.

The corollary is that the changes are due to the acquisition of genetic information from other soil micro-organisms. This could also explain the change in restriction pattern, the number of new plasmids and the apparent "shifting" of the *nif/nif* homologous genes. The acquisition of more genetic information would account for the alteration in intrinsic antibiotic resistance pattern and antigen profile. The ability of a "bacteriocin" to transfer between genera provides a clue to the possible mechanism of acquisition. The potential for interactions in the soil is high. Micro-organisms can arise quickly to utilize novel substrates, for example 2-4-5-T (2,4,5, trichlorophenoxyacetic acid). There are two steps in this process, the accumulation of strains which can carry out the degradation and the assimilation of the entire pathway into a single strain. Thsi does not always occur or may only proceed to partial completion (Reanney *et al*, 1983). The existence of broad host range replicons and the amount of naked DNA in the soil would allow rapid alteration of genotypes. As far as the *R. trifolii* field isolates are concerned, no obvious correlation between the plasmid carried and bacteriocin production exists. Strains 0/22, 0/25 and 0/26 have the same intrinsic antibiotic resistance pattern, the same REA pattern, the same plasmid profile and hybridization response. This is the highest degree of similarity amongst the strains.

The bacteriocin produced by *R. trifolii* #0/18 has been shown to be transferrable. The host range of the transfer is wide and although the plasmid appears unstable in *E. coli*, some strains still produce bacteriocin. This suggests that

either the entire plasmid or some portion thereof has integrated into the chromosome. It is possible that this "portion" is a transmissable element in its own right. Further studies on the nature of the bacteriocin, and the structure with which it is associated, are necessary.

Bacterial strains are able to adapt quickly to new challenges in the soil. Examples of this are the development of "new" pathways for unusual substrates (Reanney *et al*, 1983). This suggests that the changes in the *R. trifolii* field isolates may be due to adaptive acquisitions of genetic information. Supportive evidence includes the method of obtaining samples; unless the field is absolutely saturated with extremely competitive immigrant strains, it is unlikely that many of the field isolates have arrived from the one adjacent pasture. The inoculant should vastly outnumber any immigrants; the field is sown with nearly 20 tons of rye grass - clover seed. Other evidence includes the degree of similarity found for the REA profiles, the pattern of 2668 is quite distinct from that of 2163 and TA1. The field isolates resemble 2668 most closely. However, the amount of change which has occurred in the sixth month isolates makes it very difficult to show relationships to 2668 using these tests.

Despite the adaptation that occurs, the bacterial genome remains stable. The adaptive process is one of expansion, rather than mutation. These processes act, at least in part, to preserve pre-evolved genes from the hazards of a variable environment and assure continuation of a species (Reanney *et al*, 1983). It should be noted that the method of isolation probably only samples "successfully adapted" strains, the "unsuccessful" strains may not nodulate plants. These suggestions of alteration have important consequences for the production of seed inoculant strains of *Rhizobium trifolii*.

TABLE 11: Summary of responses of *R. trifolii* strains to the tests applied.

TABLE 11:

Strain	Intrinsic Resistance	Antigen Reaction	REA	Number of Plasmids	Hybridization No. of Bands	Bacteriocin Production
TA1	10	TA1	TA1	3	ND	Yes, 1
2163	10	2163	2163	3	ND	No
2668	11	1,2,4	A	4	1	No
0/0	11	1	A	6	1	Yes, I
0/2	11	1	B	5	1	Yes, I
0/3	11	NR	C	3	ND	Yes, 1
0/4	11	1	D	3	1	Yes, I
0/5	11	1	D	3	1	Yes, II
0/7	9	2	E	5	1	Yes, I

TABLE 11 (continued):

Strain	Intrinsic Resistance	Antigen Reaction	REA	Number of Plasmids	Hybridization No. of Bands	Bacteriocin Production
0/8	10	2	F	5	1	Yes, I
0/9	10	2	G	5	1	Yes, I
0/10	9	2	H	5	2	Yes, I
0/11	9	2	H	3	1	Yes, II
0/12	9	3	I	4	1	Yes, II
0/13	8	3	J	6	2	Yes, I
0/14	7	2	K	3	1	Yes, II
0/15	8	2	K	3	1	Yes, II
0/18	8	2	L	5	1	Yes, II

TABLE 11 (continued):

Strain	Intrinsic Resistance	Antigen Reaction	REA	Number of Plasmids	Hybridization No. of Bands	Bacteriocin Production
0/19	8	2	A	4	1	Yes, II
0/20	8	2	K	3	1	Yes, II
0/22	8	2	K	4	2	Yes, II
0/23	8	2	M	5	1	Yes, II
0/24	8	4	N	6	1	Yes, II
0/25	8	4	K	4	2	Yes, II
0/26	8	4	K	4	2	Yes, II

NR, no reaction ND, not done
For a description of scoring symbols see Results.

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APPENDICES

APPENDIX 1

DISTILLATION OF PHENOL

All materials used for DNA extractions should be of as pure a grade as possible and redistillation of the phenol removes minor impurities. The procedure involved was as follows:

The solid phenol was melted and approximately 750cm^3 poured into the 1l round bottomed flask. Anti-bumping chips were added and the distillation equipment assembled. The phenol was heated to 180C and maintained at this temperature until approximately 100cm^3 of the original volume remained. The heat was turned off and the equipment allowed to cool. All glassware was washed in hot water, then 95% ethanol and then hot water again. The redistilled phenol was stored at -20C until required.

Prior to use the solid phenol was thawed (care was taken to ensure the container did not crack) and a sufficient amount for the extraction removed. The melted phenol was transferred to a separating funnel and 8-hydroxyquinoline (Sigma) to 0.1% was added and dissolved. The phenol was then washed twice with 1M Tris pH 8.0 and twice with 0.1M Tris pH 8.0 containing 2-mercaptoethanol to 0.2%. This was usually sufficient to raise the pH of the phenol to 8.0. The phenol was transferred to a brown glass bottle and an equivalent volume of 0.1M Tris pH 8.0 containing 2-mercapto-ethanol to 0.2% added. The phenol was stored (up to one week) at 4C until required.

APPENDIX 2

PREPARATION OF DIALYSIS TUBING

Before dialysis tubing can be used for DNA extraction it must be prepared. The best size for this purpose is the 12mm diameter tubing.

The procedure was as follows:

1. Boil one packet of tubing in 1l of 5% sodium carbonate for 15 minutes. Change for a further litre and repeat this process until there is no detectable colour or odour in the solution.

2. Pour off the final amount and boil for fifteen minutes in 1l of distilled water.

3. Boil for 15 minutes in 1l of 1mM EDTA.

4. Wash the tubing for five minutes in distilled water.

5. Boil for fifteen minutes in 1l of 1mM EDTA. Allow this solution and the tubing to cool. Cover the beaker with foil and store at 4C.

APPENDIX 3

RESTRICTION ENDONUCLEASE BUFFERS

The formulations for these buffers were taken from the New England Biolabs Catalogue and make up at 5 x concentration. The formulations were as follows:

1. 5 x AluI contained 250mM NaCl, 30mM Tris-HCl pH 7.6, 30mM $MgCl_2$, 30mM 2-mercaptoethanol and 500$\mu g/cm^3$ of bovine serum albumin.

2. 5 x BamHI contained 250mM NaCl, 30mM Tris-HCl pH 7.4, 30mM $MgCl_2$, 50mM 2-mercaptoethanol and 500$\mu g/cm^3$ of bovine serum albumin.

3. 5 x BglII contained 500mM NaCl, 50mM Tris-HCl pH 7.4, 50mM $MgCl_2$, 50mM 2-mercaptoethanol and 500$\mu g/cm^3$ of bovine serum albumin.

4. 5 x BstEII contained 750mM NaCl, 30mM Tris-HCl pH 7.9, 30mM $MgCl_2$, 30mM 2-mercaptoethanol and 500$\mu g/cm^3$ of bovine serum albumin.

5. 5 x EcoRI contained 250mM NaCl, 500mM Tris-HCl pH 7.5, 25mM $MgCl_2$, and 500$\mu g/cm^3$ bovine serum albumin.

6. 5 x HindIII contained 250mM NaCl, 250mM Tris-HCl pH 8.0, 50mM $MgCl_2$ and 500$\mu g/cm^3$ bovine serum albumin.

7. 5 x KpnI contained 30mM NaCl, 30mM Tris-HCl pH 7.5, 30mM $MgCl_2$, 30mM 2-mercaptoethanol and 500$\mu g/cm^3$ of bovine serum albumin.

8. 5 x PstI contained 500mM NaCl, 50mM Tris-HCl pH 7.5, 50mM $MgCl_2$ and 500$\mu g/cm^3$ bovine serum albumin.

9. 5 x SalI was the same as 5 x BstEIII.

APPENDIX 4

PREPARATION OF RANDOM PRIMERS

1. Dissolve the calf thymus DNA in DNase buffer (20mM Tris-HCl, pH 7.4, 10mM $MgCl_2$) at a concentration of 30-40 mg/cm^3.

2. Add DNase I to a final concentration of 1mg enzyme per 500mg of DNA. Incubate at 37C for 45 minutes.

3. Add pronase to $1mg/cm^3$ and SDS to 1%. Incubate at 37C for 45 minutes.

4. Wash the DNA twice in phenol : chloroform : isoamyl alcohol (25:24:1) as described in section 2.5.31.

5. Denature the DNA at 100C for 10 minutes and then plunge into an ice bath to cool.

6. Add NaCl to a final concentration of 0.1M and load into a DEAE cellulose (Pharmacia) column equilibrated with 5mM Tris pH 7.4, 1mM EDTA and 100mM NaCl. Use $1cm^3$ column volume per 50-60mg of DNA.

7. Wash the columns with the Tris-EDTA-salt until all the O.D. (260) has been washed free.

8. Elute the calf thymus primer with Tris-EDTA, 300mM NaCl until all the O.D. (260) has been removed.

9. Ethanol precipitate the DNA at -20C overnight with 1/10 volume of 2M sodium acetate and 2 volumes of ethanol. Centrifuge at 17,000g for 15 minutes at -10C in an SS34 rotor (Sorvall RC-2B).

10. Dissolve the pellet at $50\mu g/cm^3$ in water and store at -20C.