ANALYSIS OF GENOMIC REARRANGEMENT AND PLASMID CONJUGATION OF AN INOCULANT STRAIN OF **RHIZOBIUM LEGUMINOSARUM** BV **TRIFOLII.**

A Thesis Presented in Partial Fulfillment of the Degree of Doctor of Philosophy.

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ABSTRACT

Variation of plasmid profile is the hallmark of *Rhizobium leguminosarum* bv *trifolii* strains isolated from the nodules of pasture plants. Previous attempts to demonstrate that these variant types were derived from the original inoculant were inconclusive. Subsequent laboratory based simulations revealed that the broad host range plasmid RP4 was capable of generating stable alterations in the plasmid and total genomic DNA profile. This variation involved an apparent loss of pSym, with concurrent loss of the *nod* and *nif* genes, but these strains produced nodules on white clover plants. The strains recovered from the nodules, while not identical, were clearly derived from the pSym$^-$ strain. A plasmid closely corresponding in size to pSym was detected in the nodule re-isolates and repeated trials of this experiment involving antibiotically marked (apparently) sym$^-$ strains confirmed this observation. This left the conclusion that the DNA was still there but in a form which was difficult to detect by conventional DNA hybridization procedures, a result which is not totally without precedent (Downs and Roth, 1987).

The second portion of this project involved an investigation of the transmissability of plasmids from the inoculant strain 2668. The strain was marked with Tn5 with the expectation that some of the movable DNA pieces would carry a Tn5 insert and could thus be selected for. A transmissable symbiotic plasmid was detected, as had been previously observed in other rhizobia by Johnston *et al* (1978). The plasmid was shown to be transferable, in an altered form, to soil microorganisms of unidentified genera, to a *sym*$^-$ strain of *R. leguminosarum* bv *trifolii*, to its original parent 2668 and to *E. coli*. In all strains, with the exception of *E. coli* the nodulation genes were functional, producing normal looking nodules (on the outside) and in many strains nitrogen was also fixed, though not generally as well as by the parent 2668. The significance of a self-transmissable broad host range symbiotic plasmid is discussed in the context of the microbial ecology of rhizobia.

INTRODUCTION

1.1 The Soil

In an investigation of interactions of members of the soil ecosystem, it is essential to consider the nature of the environment in which the microorganisms live. The forces which play a role in the dynamics of soil populations and the effect of the population upon its environment are governed to a large degree by the physical and chemical properties of the soil.

By definition soil refers to the loose, upper layer of material on the Earth's surface. It is the layer that supports plant life and hence terrestrial animal life. The basis of agriculture is in understanding how the soil will interact with factors ranging from continental climate to the presence of a particular bacterial species. This in turn will determine what life the soil might support and it is this vast array of influences that gives the soil its complexity and allows it to support a wide variety of life forms.

The environmental factors which shape the soil and its microbial population have been studied in some detail (Kononova et al, 1966; Paton, 1978; Chen and Avnimelech, 1986) and a degree of understanding has been reached. The degree to which each factor contributes has been quantified for a few soil systems and some conclusions about soil dynamics drawn. The soil components (in terms of particle size and composition) determine the physical and chemical nature of the soil (Haider et al, 1975; Butler, 1980) and are used to classify the soil into a number of defined types. Each soil type will have a different physical structure, chemical composition and biological content.

Soil structure is further described by its profile: a vertical section down through the thin outer mantle of the earth. The profile is sub-divided into horizons which are easily distinguished from one another on the basis of structure, texture and color (Hodgson, 1978). The uppermost layer or A horizon is the one of most interest in this analysis for it is here that the majority of biological activity occurs and here that any exchanges of genetic material are likely to

take place.

1.2 Life in the Soil

The microbiota of the soil is made up of five major groups: the bacteria, actinomycetes, fungi, algae and protozoa (Gray and Parkinson, 1968; Campbell, 1983). Bacteria are by far the most numerous, often in greater numbers than the sum of all other microfloral constituents, but many of the flaws in early research could be traced to ignoring the influence of these other members (Pugashetti et al, 1982; White, 1983; Wimpenny et al, 1983). Many soil microorganisms are found in close proximity to plant roots, in the region known as the rhizosphere, and rhizobia with their unique intracellular niche are no exception. Bacterial cells move downward with the growing plant roots, maintaining contact, presumably because nutrient concentration is higher near the root surface and because of the specific attraction exerted by plant exudates (Barber, 1982) and some may have passively adsorbed to the surface of the root.

Among the unsolved problems related to the study of soil microbial ecology is the determination of precisely what microorganisms are present. No one medium will isolate them all, nor will any one medium maintain them. In addition, some perfectly viable cells (in terms of their ability to respire) will not grow on media that have been shown to support laboratory adapted strains of these bacteria. It is suggested that they are adapted to the "normal" half-starved state of the natural environment and the rich laboratory media is therefore unsuitable (Roszak and Colwell, 1987). Many soil microorganisms have been only cursorily classified as interest has mostly centred on those microorganisms that impinge directly on human activities. Some studies relating what bacteria are present and what they do have been carried out (Stotzky and Krasovsky, 1981) but much more information is needed before a detailed picture of life in the soil can be drawn (Jensen et al, 1986; Roszak and Colwell, 1987). A great deal more study of the genetic variability within populations of soil microorganisms as well as on the extent to which the genetic material can be exchanged within and between populations is also

required. Further study may assist in resolving the ongoing debate regarding the usefulness of laboratory simulations of genetic variation and exchange in modelling actual field conditions (Wimpenny et al, 1983; Tempest et al, 1983; Saye et al, 1987; Trevors et al, 1987)

1.3 Conservation of Genomic Integrity and Adaptation to New Factors in the Soil

The maintenance of cellular identity and the propagation of the species is a salient feature of any organism, uni- or multicellular. All cells have homeostatic mechanisms which protect them from alterations in their internal environment (Tempest et al, 1983) and which maintain the integrity of the genome (Smith, 1988). The environment in which soil microorganisms exist also resists sudden fluctuations as the soil has substantial physical and chemical buffering capabilities. Therefore the microorganisms are protected from change both from within and without. Nevertheless, factors such as extremes of hydration, pH and nutrient level still act on a soil population and if we are to understand how a microorganism came to be the way it is, we must consider the factors that shaped it. Secondly, if we are to predict how a "new" stress may affect a microorganism, we must attempt to discover exactly how this stress affects it and what the range of possible responses of the microorganism are (Andrews, 1984).

Understanding the micro-evolution of an organism applies not only to whether or not a population can survive a challenge (environmental or otherwise) but what effect that stimulus will have on the integrity of the genome. In general, the genotype of a bacterial species is maintained primarily by the fidelity of DNA replication and populations that allow excessive error are eliminated (Pressing and Reanney, 1984). Secondary mechanisms exist to protect the genome from specific kinds of damage, the most well known being the SOS system of E. coli, which is induced in response to a variety of triggers including UV damage (Howard-Flanders, 1981; Gottesman, 1981; Little and Mount, 1982; Smith, 1988). However, these mechanisms

are not always appropriate to protect the cell against starvation, antibiotics or toxic xenobiotic compounds, such as pesticides. Despite all of these obstacles, microorganisms do survive and multiply in the soil and other, much harsher environments.

The topic of mutation, especially adaptive mutation, has attracted considerable interest over the years and many aspects and agents of DNA alteration have been investigated. The type of genomic alteration a bacterium may undergo is varied and at its most simple may be the alteration of one base for another. Certain DNA elements have been shown to be responsible for genomic alteration, particularly transposons and insertion sequences (Cohen, 1976; Broda, 1979; Kopecko, 1980; Chumley, 1981, Shapiro, 1983; Hall et al, 1983; Clerget, 1984; Syvanen, 1984; Smith, 1988). The genome shuffling identified in Halobacterium (Sapienza and Doolittle, 1982; Sapienza et al, 1982) and reported (but not yet confirmed by other groups) in the Rhizobiaceae (Heumann et al, 1983, 1984) involves genome reorganization on a staggering scale and could be responsible for enormous changes in a population. The degree of control that a bacterial species has over mutational events in its genome is a subject that is presently under investigation. Certainly, one response of a population to a "new" stress is to take advantage of an appropriate random mutation existing in one or more of its members or, if recent reports by Cairns et al (1988) and Hall (1988) prove to apply generally to bacteria, to direct an appropriate mutational event.

This leads directly to more general questions about the extent to which bacteria are endowed with the capacity to respond to environmental challenge by either random or directed genetic events.

1.4 Genetic Mechanisms of Adaptation

There are two broad classes of events which can lead to the alteration of the genome of organisms: those generated exogenously (such as conjugation and transduction) and those generated endogenously (such as mutation and recombination). Interest in this area has expanded recently and for further information readers are

directed to Trevors et al (1987) for a review of gene transfer in the soil and aquatic ecosystems and to Terzaghi and O'Hara (1989) for a review of the potential for genomic rearrangements among procaryotes. Some specific cases which may be relevant to the present study are covered in the following text.

Of the classic means of DNA transfer, transformation would appear to be the least likely to play any significant role in the natural environment. As a number of extra-cellular nuclease producing soil microorganisms exist, any "naked" DNA should be quickly broken down and ingested as a source of nutrient. However, some evidence has been produced that despite this hazard, DNA can adsorb to and be protected by clay minerals, particularly montmorillonite (Greaves and Wilson, 1973). It is possible that bacteria could take up this adsorbed DNA and incorporate it into their genome (Stotzky and Krasovsky, 1981). A laboratory model ecosystem showed that DNA adsorbed to sand could transform bacteria at frequencies significantly higher than in liquid culture and was substantially more resistant to DNase I (Lorenz et al, 1988).

It is more difficult to assess the role of transduction in the transfer of genetic material between bacteria in the soil. Bacteriophages are adsorbed to and concentrated by the presence of clay minerals in the soil (Stotzky and Krasovsky, 1981) and the frequency of transduction is increased by the presence of clays (Zeph et al, 1988). Reanney et al (1983) briefly review the information available on phage-mediated transfer of DNA in the soil and comment that transduction may be effective in disseminating genetic information where populations are large and concentrated. Concentration has been shown to significantly affect transduction frequency in both terrestrial (Zeph et al, 1988) and aquatic (Saye et al, 1987) environments. The importance of transduction remains to be determined, as at present there is only sufficient evidence to say that it can take place in the soil.

Conjugation is the transfer of DNA by cell-to-cell contact and is mediated by plasmids. It is also the way that any genes encoded by

the plasmid may be disseminated. The most well studied examples are the F (fertility) plasmid of E. coli which codes for its own transfer and maintenance (Hardy, 1981; Glass, 1982) and the various antibiotic resistance transfer factors. Plasmids act to extend a cell's phenotype, providing functions like antibiotic resistance and pathways for degrading unusual compounds. Most of the early work was of a clinical microbiological nature and concerned the transfer of R (antibiotic resistance) plasmids among and between a variety of bacteria of clinical importance in hospital environments (Falkow, 1975). It has since widened in scope considerably to include other habitats, such as the soil (Weinberg and Stotzky, 1972; Graham and Istock, 1979; Stotzky and Krasovsky, 1981; Polak and Novick, 1982; Trevors and Starodub, 1987). Most bacterial species examined have been shown to carry plasmids (Helinski et al, 1985) and a number harbor self-transmissible plasmids. Among the latter are Enterobacter cloacea (Kleeberger and Klingmuller, 1980), the pseudomonads (Manceau et al, 1986), Streptomyces lividans (Kendall and Cohen, 1987), Escherichia coli (Trevors and Starodub, 1987) and members of the genus Rhizobium (Johnston et al, 1978; Bedmar and Olivares, 1980; Pees et al, 1984). This list is by no means exhaustive and aims only to illustrate the ubiquity of plasmids. The combination of mobility and extension of phenotype can mean a significant advantage for a population over a non-plasmid carrying neighboring population. The presence of a plasmid in a strain can lead not only to the rapid dissemination of a function but it also has been shown to lead to a higher growth rate when compared to a non-plasmid carrying strain under the same conditions, without actual selection for any particular gene (Bouma and Lenski, 1988). However, this may well be a confession of our ignorance as to the function provided by the plasmid, rather than some non-specific enhancement.

The role of plasmids in the adaptive process has been studied by Reanney and colleagues over several years. The initial focus was on the ability of plasmids to act as agents of evolutionary change (Reanney, 1976). It was clear that the acquisition of a plasmid could

lead to the manifestation of new functions in bacterial strains. An extension and expansion of these ideas (Reanney, 1978; Reanney *et al*, 1983) suggested that while the potential of DNA transfer mechanisms to provide a selective advantage is great, the extent to which this potential is realized should not be overstated as there are many barriers to easy genetic exchange. The stability of the genotype is evidenced by the isolation of the same microorganism (with regard to standard identification procedures) over relatively long periods of time (e.g. with *Brucella ovis* this is about 30 years, O'Hara *et al*, 1985); if genes could be transferred quickly and easily, no chromosome could maintain its integrity.

Transduction, transformation and conjugation all involve transactions with exogenous DNA. However a cell can also generate heritable variation by internal genomic rearrangement. The potential of a species to undergo this type of DNA reorganization is difficult to assess as few organisms have been carefully studied from the genetic perspective and it has only been recently that the DNA of an organism could be examined directly. Some examples of internal genomic rearrangement are discussed below, but it is first useful to define two classes of alteration, programmed and unprogrammed (Borst and Greaves, 1987). Programmed rearrangements are events that occur at precisely specified end points, while unprogrammed events appear to lack the defined topology of the programmed rearrangements. Both seem, for the most part, to be randomly distributed in time, although representatives of either class may occur in response to specific signals.

Programmed genetic alterations often have repeated elements associated with their sites of rearrangement. These elements provide homologous DNA sequences which can be used by *recA* dependent (Smith, 1988) and *recA* independent (Gennaro *et al*, 1987) pathways of recombination.

Site specific recombination can lead to defined phenotypes in some organisms and this is particularly evident in the antigenic variation processes of *Neisseria*, *Borrelia* and *Salmonella*. While

there is no direct evidence for the involvement of repeat elements in these alterations, the site of action is highly conserved and the inverted block of DNA is bounded by repeated elements. The first and most well known example involves Salmonella flagellar phase variation which has been demonstrated to be the result of inversion of a particular 995 bp DNA sequence bounded by inverted repeats (Simon et al, 1980; Zieg and Simon, 1980) which causes the shift between H1 and H2 flagellar antigen synthesis. Secondly, Neisseria gonorrhoeae has the ability to alternate between several antigenic forms of pili and also a total phase shift involving the loss (or reacquisition) of pili. The former is due to the transfer of a gene "cassette" coding for that particular antigenic type to the pilus expression locus and the latter due to transfer of a non-functional cassette to this site (Swanson et al, 1986; Haas and Meyer, 1986). This process is analogous to mating type variation in Saccharomyces cerevisiae. Transformation also appears to have a major role in variability of the pilin genes; DNA from lysed Neisseria strains enters the cell at high frequency and any pilin genes present will preferentially recombine with the gene at the expression site, causing a switch of pilus type (Seifert and So, 1988). Thirdly, the agent of one form of relapsing fever, Borrelia hermsii, shows DNA rearrangements that are associated with the characteristic antigenic shifts. The mechanism of antigenic variation is suggested to be a duplicative transposition of the antigen-specifying gene from a storage site to an expression site and subsequent transcription of the gene (Meier et al, 1985). This is in many ways similar to antigenic variation in Neisseria.

Another example of a programmed rearrangement which leads to a defined phenotype is observed in the cyanobacterium Anabaena. Non-nitrogen fixing cells of Anabaena have the nifHD and nifK structural genes separated by 11 kb of DNA of unknown function. Formation of the heterocyst (a specialized cell for the nitrogen fixing process) triggers site-specific recombination between 11 bp direct repeats associated with the two gene sets and the intervening DNA, which interrupts nifD in the vegetative state, is looped out and excised

with the subsequent active transcription of the entire operon (Golden *et al*, 1985; 1987). The rearrangement involves the loss of the 3′ portion of *nifD* which is replaced by a sequence just upstream of *nifK* and results in a modified *nifD* gene and a fully functional *nifHDK* operon (Haselkorn *et al*, 1988).

Repeated elements are involved in what appears to be a programmed reorganization relating to the life-cycle change of *Caulobacter crescentus*. The organism alternates between a mobile (swarmer) and sessile (stalked) form. Rearrangement of the not inconsiderable quantity of repeated DNA of this organism is invariably associated with cell cycle changes (Nisen and Shapiro, 1980) but it is not known whether the rearrangements are an intrinsic part of the cycle change mechanisms, or are merely incidental consequences of the change.

It appears that plasmids can also undergo programmed rearrangements, an example being the IncIα plasmid R64. The plasmid has four overlapping invertible regions spaced over a 2kb region which may invert together or independently in a controlled fashion, regulated by a *trans*-acting gene (Komano *et al*, 1986). These sequences have been designated A,B,C and D and the authors have suggested the term **shufflon** as a descriptive name for the entire region. A consideration of the structures these inversions produce suggests that they could act as a biological switch with the ability to select any one of seven open reading frames. The result would be translation products with a constant N-terminus and a variable C-terminus (Komano *et al*, 1987). Investigation is proceeding to determine the function of this region. A simpler type of plasmid rearrangement, involving plasmid-to-plasmid DNA exchange, has been observed in *Haemophilus influenzae* (Balganesh and Setlow, 1986).

The most obvious example of the unprogrammed classes of events are the localized DNA alterations of which nucleotide substitutions and small deletions/insertions are representative. The effect of these changes in sequence can vary from no obvious phenotypic difference to radical alteration of the gene in an either positive or

negative way, as determined from the result of the change for the cell. These small DNA alterations are non-directed random events and can affect regulatory or structural sites within a gene.

Genomic rearrangements have been identified as being associated with the expression of genes not normally considered part of the cells repertoire ("cryptic" genes). Several functions have been detected in *E. coli* which fall into the category of cryptic genes (Hall, 1983; Hall *et al*, 1983). The first is *ilv* which is involved in isoleucine and valine synthesis and about which little is known. The second is *bgl*, the third *cel* and the fourth *sac*, all three of which are involved in β-glucoside metabolism, *cel* specifically with cellobiose hydrolysis. The expression of *bgl* required the introduction of a promoter which was supplied by the insertion of an IS element at the appropiate site providing a transcriptional start signal. Expression of the *cel* glucosidase requires another, as yet unidentified, event (Kricker and Hall, 1984) and the *sac* gene expression requires three separate events (Parker and Hall, 1988). The frequency of mutation causing the activation of the *bgl* operon, far from being random seemed rather to be linked to the presence of the compound to be utilized by the activated gene. The presence of salicin in the culture medium enabled a pair of mutations, whose combined frequencies should have been on the order of 10^{-17}, to occur much more often. This increase in frequency (on the order of 10^{10}-fold) was specific, as no change was observed in the frequency of an unselected mutation (Hall, 1988). No mechanism has been advanced for this astounding result and it seems to be another case of what Cairns *et al* (1988) described as "directed mutation". The *galK* operon of pRF100 is non-functional but under certain circumstances rearrangement of the element IS 30 can lead to reactivation of this gene (Dalrymple, 1987). *Neisseria gonorrhoea* is often found as an auxotroph but members of the population can revert to full prototrophy under appropriate selective conditions. The mechanism of reversion is not known, but may involve DNA repair functions (Juni and Heym, 1980).

The results of random insertions of Mud*lac* (a Mu phage construct containing *lacZ* used for detecting promoters) in *E. coli* and *Pseudomonas putida* have been examined by Shapiro (1985; 1986) in order to assess genotypic and phenotypic alterations that may routinely occur during colony growth. None of the promoters which became manifest, by virtue of the appearance of any one of a variety of *lac*$^+$ sectors, have been identified but they evidently represent "decryptification" of functions not expressed in the bulk of the colony and are most commonly seen on old plates. Whether or not these expressed functions have adaptive significance is not known at present. Evidence exists for regular decryptification in other systems. For example, the marine microorganism *Vibrio harveyii* when grown on solid media, will produce colonies of which some sectors synthesize a bioluminescent pigment (Simon and Silverman, 1983). Restreaking of a non-producing sector will again result in segmented colonies. Other pigment variation is seen in *Streptomyces reticuli* and *Serratia marcescens* (Simon and Silverman, 1983). Dimorphic colony formation has been identified in the bradyrhizobia (Sylvester-Bradley *et al*, 1988). The implications of the demonstrated colonial polymorphism are fascinating: how homogeneous is a colony of cells? Conventional dogma states that a colony which arises from a single original cell is a homogeneous population identical to the founder. Yet if certain members of a population exhibit traits not generally shown it may be more correct to consider a colony as a cooperative venture of cells, members of which are all of the same genetic makeup but are not necessarily manifesting the same phenotypic capabilities. The behaviour of a colony may, under certain circumstances, be analogous to that of a multicellular organism (Shapiro, 1988).

The archaebacteria are an unusual group of microorganisms which have some of the characteristics of procaryotes and others more commonly associated with eucaryotes (Woese, 1981; 1987). Their habitats are generally the more marginal environments (Belay *et al*, 1984), examples being high salt concentrations and extreme temperatures. It has been observed that some members of this group

undergo large scale genomic rearrangements (Sapienza et al, 1982; Sapienza and Doolittle, 1982) apparently facilitated by short highly conserved repeated sequences. No phenotypic alterations have yet been associated with these rearrangements. High degrees of genomic variation are also seen among Mycoplasma ovipneumoniae isolates (Mew et al, 1985), with no obvious corresponding phenotypic differences. These isolates were collected in a small area over a short time period and may or may not be representative of the general population of M. ovipneumoniae.

Evidence presented in recent papers, some of which has been reviewed here, suggests that the genome of a microorganism has more "plasticity" than has been generally recognized (Terzaghi and O'Hara, in press) and under the appropriate conditions quite radical changes can be induced in the organization of a cell's genome. This brief but wide-ranging discussion of cellular plasticity was designed to indicate to the reader the potential variabilty which exists among procaryotes. Some of these cases may be discussed further later, where they are more directly relevant to the genus Rhizobium or this project.

1.5 Plasmids: Their Role in Bacteria and Induction of Genomic Rearrangement.

Plasmids are widely dispersed in nature and nearly all bacterial species carry them (Helinski et al, 1985). Plasmids are extra-chromosomal DNA elements encoding information for their own replication and maintenance. Novick (1980) presents them as intracellular entities on the verge of independent existence and certainly it is possible to so regard the self-transmissible plasmids such as F. Plasmid structure and function has been well studied and a number of reviews exist covering aspects of structure (Clowes, 1972; Stanisich, 1984), replication (Scott, 1984), conjugation (Willets and Wilkins, 1984), incompatability (Couturier et al, 1988) and function (Timmis and Puhler, 1979). The interest of this study is to focus on the potential of plasmids to extend the phenotype (e.g. transfer of symbiotically important genes) and to undergo rearrangement as well

as cause other DNA structures to undergo rearrangement.

One class of genes that are often plasmid-borne, especially in Pseudomonas species, are those encoding the enzymes required for the degradation of exotic substances such as 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) and other herbicides. Halogenated aromatic compounds are of special interest because of their potential environmental toxicity. Experimental analysis in a chemostat showed that Pseudomonas putida strains able to use 3-chlorobenzoic acid (3-Cba) via a plasmid-encoded pathway were able to extend their utilization range to 4-Cba and 3,5-diCba. The mechanism was found to be a combination of genetic exchange with a resident TOL (toluene utilization) plasmid and a gene duplication-deletion event in the original 3-Cba$^+$ plasmid (Chatterjee and Chakrabarty, 1982). This can be turned to the advantage of the agriculturalist, as it has been shown that the addition of a 2,4,5-T degrading microorganism can lower herbicide level in the soil sufficiently so that even sensitive plants can then be grown (Kilbane et al, 1983). Strains of bacteria able to degrade unusual compounds can be isolated from nature and there is considerable interest in producing laboratory strains with specific abilities (Ghosal et al, 1985a; 1985b). The types of functions attracting interest include the utilization of xylene, toluene and their derivatives in Pseudomonas species; many of the characteristics that the genetic engineer will attempt to produce may well have been already selected by the natural environment.

A variety of mechanisms of sequence rearrangement which can lead to new functions have been described and commonly involve repeated elements of one kind or another. The Sym plasmid (carries symbiotically important genes) of Rhizobium phaseoli was found to undergo changes which affected its nodulation and nitrogen fixation functions (Hombrecher et al, 1981). Repeated sequences are well distributed in R. phaseoli strains and it was suggested that they are involved in these alterations (Soberon-Chavez et al, 1986). The loss of nodulation function of a Rhizobium leguminosarum biovar phaseoli symbiotic plasmid without concurrent decrease in molecular size was

suggested to be due to homologous recombination between reiterated sequences (Soberon-Chavez and Najera, 1989b). Plasmid instability has resulted in change in the symbiotic properties of Rhizobium trifolii (Djordjevic et al, 1982) and in this species there are also a number of known repeated sequences on pSym (Watson and Schofield, 1985). However, experiments which would identify the repeated elements that could be involved in the rearrangements described above have yet to be reported.

A further family of repeated sequences, which may or may not be IS elements, has been identified in several rhizobial species and the related genus Agrobacterium (Flores et al, 1987). The element ISRm1, first identified in Rhizobium meliloti, has also been found in other rhizobia and an unidentified gram negative bacterium (Wheatcroft and Watson, 1988). The ability of IS elements to catalyse rearrangements is well known (Shapiro, 1983), but whether all of that potential is realized and provides the sole mechanism for the rearrangements described by Soberon-Chavez and Najera (1989b) and Djordjevic et al (1982) remains to be determined. Variation in plasmid structure in Bacillus subtilis has been shown to be due to interactions between inverted and direct repeats on the replicon (Peeters et al, 1988).

The broad host range plasmids such as RK2 (Thomas, 1981) have been of interest to clinical and molecular biologists since their discovery (Lowbury et al, 1969; Datta and Hedges, 1972). This incompatability grouping (IncP) encompasses the plasmids RK2, RP4, RP1, R68 and R18, which were all shown to be indistinguishable from one another (Burkardt et al, 1979; Stokes et al, 1981). Subsequent analysis of the IncP group has led to division into two sub-classes; IncPα (RP4) and IncPβ (R772 of Proteus mirabilis, R906 of Bordetella bronchoseptica and others). The cluster of essential functions related to transfer and maintenance, and the locations of restriction sites are similar between the two groups (Smith and Thomas, 1987).

Initial restriction mapping of this plasmid was made difficult by the lack of suitable enzyme sites. On a statistical basis a hexanucleotide recognition sequence should occur about fifteen times

on the 60 kb plasmid. It was found that most common enzymes generated fewer fragments than predicted, with EcoR1, HindIII and BamHI having but a single site. Jacob and Grinter (1975) suggest that the plasmid may have evolved this relative immmunity to these enzymes as part of its broad host range lifestyle. The availability of commercial restriction enzymes from the less common bacterial species led to the generation of an excellent physical map and paved the way to identifying the gene loci associated with transfer and replication (Lanka et al, 1983). The origin of replication was identified and shown to contain a number of direct repeats which are important for binding of the replication proteins and copy number regulation (Filutowicz et al, 1987). A second set of repeat elements are associated with the origin of transfer and their secondary structure is believed to be important in the transfer process (Guiney and Yakobsen, 1983).

A number of genes have been identified which also have an effect on the transfer process, either to abolish it or to modify the host range. The transfer related functions trfA and trfB were identified when mutations in this region abolished transfer (Kornacki et al, 1984). One very deleterious host range mutant was mapped to 40 kb (with the single EcoR1 site at 0/60 kb) and identified as the gene encoding a DNA primase (Krishnapillai et al, 1984). The DNA primase is central to the transfer of a copy of RP4 from one host to another and appears to be required for second strand synthesis on the single strand of DNA transferred during conjugation. It is unclear whether the primase is transferred also during conjugation or expressed from the transferred single strand (Lanka and Barth, 1981; Lanka and Furste, 1984). It is hoped that some of these questions will be answered once high-expression vector translation products are available (Furste et al, 1986). A regulatory gene mutant labelled incC was identified and was found to act to modulate the **TrfA** replication protein (Thomas, 1986). A further set of host-range mutants were identified and named the kil genes, and have been shown to have a host-lethal effect if expressed in an uncontrolled

manner. These genes are regulated by another group of loci, the kor genes, which suppress their effect (Figurski et al, 1982). The exact role of the kil genes is unclear but appears to relate to the stable maintenance of the plasmid (Thomas et al, 1988). The kil and kor genes interact to regulate the important transfer-related functions trfA (Schreiner et al, 1985) and trfB (Theophilus and Thomas, 1987) and regulation of three recently identified genes of unknown function (Thomas et al, 1988). It was suggested by Thomas et al (1988) that the interaction of the various gene loci was what allowed the stable maintenance of RP4 in its many hosts. Despite the many RP4 derivatives constructed in the course of examining host range and plasmid maintenance, only the entire RP4 plasmid has the impressive host range and stability for which this group of plasmids is reknowned (Barth et al, 1984: Pinkney and Thomas, 1987).

Conjugative plasmids of various incompatability groups are known to mobilize plasmid and chromosomal markers and the IncP replicons are no exception. RP4 and a deletion derivative R68.45 mobilize other plasmids at high frequency (Haas and Holloway, 1976) and cases of chromosomal marker transfer are also documented (McLaughlin and Ahmad, 1986). The plasmid can mediate the transpositions of certain pieces of the chromosome to other plasmids (Berry and Atherly, 1984) and inserts of great size (circa 285 kb) could be maintained in and transferred by RP4 (Julliot et al, 1984). RP4 appears to be able to generate a variety of DNA alterations in its recipients by marker mobilization and DNA rearrangement (Jaoua et al, 1987) and its broad host range means that there is potential for genes to be transmitted over large taxonomic distances.

Of more direct relevance to this dissertation is the effect of RP4 on members of the genus Rhizobium. There is no doubt that members of this genus are among the potential recipients of this plasmid (Pinkney and Thomas, 1987) and some of the work mentioned above (Berry and Atherly, 1984; Julliot et al, 1984) suggest that rhizobia are not immune to the DNA rearranging ability of RP4. If, as is suggested by Reanney et al (1983), the soil micro-population (of

which the rhizobia are transient members) is potentially linked into one gene pool, then surely broad host range plasmids would be an important factor in maintaining that link.

1.6 Identification and Characterization of **Rhizobium** Species and their Plasmids.

Isolation of members of the genus Rhizobium is straightforward because they form specialized structures (called nodules) on the roots of host legume plants. A host plant can serve as a specific "trap" for any of the appropriate bacteria that may be present. Differentiation into species and strains is more complex; originally the specific plant-bacteria interaction was used as a taxonomic criterion (Vincent, 1970) and often it still is. However, as many of the fast-growing rhizobia had the majority of the genes encoding symbiotic functions on plasmids, questions were raised as to the suitability of a plasmid based classification system (Schmidt et al, 1984; Downie et al, 1985). The discovery that some of these plasmids were transferrable (Brewin et al, 1980) to other genera, including Agrobacterium (Hooykaas et al, 1981; 1982; Hirsch et al, 1984) and Pseudomonas (Plazinski and Rolfe, 1985) has only increased the concern of the taxonomists; if the factor that is being used to classify a bacterial species is able to transfer to other bacterial species then it is scarcely an ideal identification procedure.

A number of standard methods exist for differentiating bacteria, including serology, biochemical testing and DNA homology, and most of these have been applied to the rhizobia. Serological analysis by the use of strain-specific antisera (Holland, 1966) and fluorescent labelling of nodule homogenates (van der Merwe and Strijdom, 1973) were useful but, as with most antigenic analyses, similar determinants on otherwise unrelated bacteria can lead to difficulties in interpretation. Some cellular antigens are less prone to this cross-reactivity (Sadowsky et al, 1987) and the addition of numerical taxonomic analyses to serological studies increases the resolving power of serology (Dudman and Belbin, 1988) and hence its usefulness, but difficulties remain with this procedure. The analysis of

intrinsic (low level) antibiotic resistance patterns has been shown to be useful for R. leguminosarum but less so for R. phaseoli (Josey et al, 1979). R. trifolii appears to stably maintain intrinsic antibiotic resistance levels (Ronson, personal communication). Examination of strains isolated from the soil environment reveals considerable diversity in the patterns of resistance, indicating large-scale strain variation, in both R. phaseoli (Beynon and Josey, 1980) and R. trifolii (O'Hara, 1985) which would limit the taxonomic usefulness of this character. Serology and intrinsic antibiotic resistance have been previously used in this laboratory to discriminate between rhizobial isolates, but closely related isolates proved to be difficult to differentiate by these criteria (O'Hara, 1985).

Direct examination of genotype is more readily, reliably and precisely interpreted. The simplest analysis of a genome is by direct comparison of restriction endonuclease patterns generated from total cellular DNA, a procedure which has been applied to Leptospira (Marshall et al, 1981), Mycoplasma (Darai et al, 1981; Mew et al, 1985), Brucella (O'Hara et al, 1985) and Rhizobium (Mielenz et al, 1979). The technique is most useful in determining whether an isolate is the same as a type strain but is difficult to quantify without densitometric analysis of the band pattern.

Genome analysis by total DNA hybridization was applied to the genus Rhizobium initially in an attempt to order the various members of the legume-nodulating family of bacteria. Early results showed R. leguminosarum and R. trifolii to be closely related (Gibbins and Gregory, 1972) and later work has served to include R. phaseoli in that cluster (Crow et al, 1981). The refinement of using the conserved rRNA sequences as probes has allowed the definition of four homology groups: group 1 contains R. meliloti, R. fredii and R. leguminosarum (with trifolii and phaseoli reduced to the status of biovars of this species), group 2 contains R. loti, group 3 is comprised of the Galega rhizobia and Bradyrhizobium is a separate and distinct genus (Jarvis et al, 1986). More work is needed to

determine more detailed relationships and to allow more accurate and direct identification, straight from isolated nodules (Hodgson and Roberts, 1983). Future analysis and taxonomic identification must, especially in fast-growing rhizobial strains, be based on chromosomal DNA as plasmid based identities are not necessarily fixed. It may prove helpful to utilize probes that identify symbiosis-specific, plasmid-specific and chromosome-specific sequences independently, as has been done by Schofield et al (1987). It is extremely important that a taxonomy be coherent and reflect the evolutionary events that shaped a bacterial group (Woese, 1987), although some taxonomists do not believe that an identification system need be evolutionarily consistent to be useful.

The use of r-RNA and DNA hybridization has allowed a more accurate grouping of rhizobial species and has given some insight into the evolutionary relationships between the species. However, far less is known about the plasmids carried by the rhizobia. Structural analysis with restriction endonucleases of geographically related R. meliloti plasmids has revealed a regional conservation of electrophoretic banding patterns (Huguet et al, 1980) but generally identification is on the basis of size of the intact plasmid as measured by electrophoretic mobility (Hirsch et al, 1980) or electron microscopy (Tichy and Lotz, 1981). Sequence analysis and DNA homology studies will require a much larger data base than currently exists, although some success with whole plasmid hybridization has been reported (Adachi et al, 1983). Identification of the symbiotic plasmid is most easily accomplished by hybridization with one of the important symbiotic operons, commonly nifKDH (Christensen and Schubert, 1983), although heat curing of the plasmid followed by a nodulation assay on plants has been used for presumptive identification (Morrison et al, 1983). Currently, several groups are trying to use specific probes and other means to accurately identify symbiotic plasmids (Schofield et al, 1987; Harrison et al, 1988; Young and Wexler, 1988) with a view to more easily identifying different strains of a particular rhizobial species isolated from the

soil.

The number of plasmids maintained by a single Rhizobium cell is highly variable, with anywhere from one (Pankhurst et al, 1983) to ten (Thurman et al, 1985) or more. This can include multiple copies of the symbiotic plasmid (Harrison et al, 1988). Plasmid size is similarly varied and ranges from the enormous (1000 kb) symbiotic "mega-plasmid" of R. meliloti (Burkardt et al, 1987) to a 60 kb self-transmissable plasmid, also in R. meliloti (Bedmar and Olivares, 1980) with many Sym plasmids in the 180-350 kb range (Watson and Schofield, 1985; Soberon-Chavez et al, 1986; Espuny et al, 1987; Schofield et al, 1987; Mozo et al, 1988; Harrison et al, 1988). Summation of the molecular weights of the plasmids indicates that they can represent an appreciable portion of the genomic coding capacity (Casse et al, 1979). It has always been assumed that there must be a reason for the cell to maintain this amount of extra-chromosomal material, but if so, many genes of importance remain to be recognized. Some attempts have been made to identify genes on other plasmids in the rhizobial strains (Bedmar and Olivares, 1980; Bromfield et al, 1985).

Some plasmid-encoded functions, other than nodulation and nitrogen fixation genes, have been identified and include the production of bacteriocins (proteins which inhibit other strains of that species and related organisms) by several R. leguminosarum strains (Hirsch et al, 1980). Several less well defined properties have been associated with the symbiotic plasmid, examples of which are the production of an abundant cryptic protein by R. leguminosarum (Dibb et al, 1984) and an increased growth rate in R. trifolii (Thurman et al, 1985). Even less clear is the function of the repeat elements found on the symbiotic plasmid of R. trifolii (Schofield and Watson, 1985; Watson and Schofield, 1985) and R. fredii (Barbour et al, 1985; Masterson and Atherly, 1986); it is possible they may act similarly to the repeat elements discussed in section 1.4, namely as sites for rearrangement and regulation of function.

The phenomenon of bacterial conjugation has been referred to

The phenomenon of bacterial conjugation has been referred to previously and although it would seem superfluous for a symbiotic plasmid to exhibit self-transmissibility, at least with regard to the bacteria-plant symbiosis, many of them do. Self-transmissible plasmids, including pSym, have been demonstrated in R. leguminosarum bv viceae (Johnston et al, 1978; Hooykaas et al, 1982; Pees et al, 1984), R. leguminosarum bv. trifolii (Hooykaas et al, 1981; Christensen and Schubert, 1983; Plazinski and Rolfe, 1985; Espuny et al, 1987; Schofield et al, 1987), bv. phaseoli (Martinez et al, 1987) and R. loti (Pankhurst et al, 1983). Bacterial species which have received and can express at least some of the symbiotic material include A. tumefaciens (Hooykaas et al, 1981; 1982; Pankhurst et al, 1983; Wong et al, 1983; Martinez et al, 1987), Pseudomonas and Lignobacter (Plazinski and Rolfe, 1985) and unidentified gram negative soil microorganisms (Jarvis et al, 1989). Subcloned pieces of the R. meliloti pSym megaplasmid have allowed the formation of pseudonodules on alfalfa by Agrobacterium and E. coli (Hirsch et al, 1984). The existence of transmissible plasmids has important implications with regard to the ability of the bacterium to genetically interact with soil microorganisms. There is some evidence for recombinant Sym plasmids (Schofield et al, 1987) and organisms have been isolated from the soil that did not previously have the ability to nodulate legumes, yet gained the abilty to do so once symbiotic genes were transferred into them (Soberon-Chavez and Najera, 1989a; Jarvis et al, 1989). A theoretical study of plasmid transfer (Knudsen et al, 1988) predicts that most of the conjugation will occur very soon after the rhizobia enter the rhizosphere of the plant and with coated seed, this is probably as the seed germinates.

Plasmid incompatibility is one of the factors limiting a free exchange of genetic material (Reanney et al, 1983). The classic definition of incompatibility refers to the inability of two plasmids to be stably maintained, replicated or segregated in a bacterial host cell. There is evidence that some type of incompatibility operates between the symbiotic plasmids of R. leguminosarum biovars trifolii

Espuny et al, 1989), biovar trifolii and a Rhizobium sp. (Espuny et al, 1987) and biovar trifolii and pRi of A. tumefaciens (O'Connell et al, 1987). The mechanism of incompatibility has not yet been elucidated. Non-classical forms of incompatibility may exist and there may be other factors as yet unrecognized restricting the host range of symbiotic plasmids. Analysis of the transfer of plasmids among a number of R. leguminosarum strains showed that there appeared to be a correlation between chromosomal background and the symbiotic plasmid present in a strain (Young and Wexler, 1988). Interactions between symbiotic plasmids of biovars trifolii and viceae resulted in deletions and hybrid forms of pSym (Christensen and Schubert, 1983) and there is evidence indicating there is an incompatibility of function in Rhizobium species such that even if the plasmids were compatible in the classical sense, some of the genes were not and deleted forms of the plasmids were the result (Wang et al, 1986).

Rearrangements need not necessarily be the result of plasmid incompatibility and R. leguminosarum bv phaseoli has been shown to undergo substantial genomic rearrangements (Palacios et al, 1987) which can also affect pSym (Soberon-Chavez et al, 1986). A recent isolation of a biovar phaseoli "hyper-mutant" (higher frequency of mutational events) suggests that in this case a mutation in the DNA metabolic pathways was responsible for the high degree of recombination (Soberon-Chavez and Najera, 1989b). This may be an isolated example and not indicative of mutation in biovar phaseoli generally.

Isolations of rhizobia from the soil show the existence of multiple symbiotic plasmids in one strain (Harrison et al, 1988) and a higher degree of restriction fragment polymorphism associated with pSym than with the chromosome (Harrison et al, 1988; Young and Wexler, 1988). The evidence presented, briefly in this synthesis and in more detail in the cited papers, questions the stability of the symbiotic genotype. This instability refers not only to the evidence that it rearranges itself and recombines with other plasmids and the chromosome, but also to its ability to move to new hosts, possibly

making them "rhizobia" also. It is not anticipated that rearrangements and transfer occur wholesale in the natural environment, but the fact that rearrangements can occur and their importance in the general scheme of microbial ecology and genetic interaction should be carefully considered.

1.7 The Molecular Anatomy of Symbiosis

There are two gene clusters involved in the establishment of the plant-bacteria symbiosis and subsequent conversion of molecular nitrogen to a form useful to the plant. The first group comprise the nodulation (*nod*) and host specific nodulation (*hsn*) genes whose functions are to initiate responses in the plant which will lead to the formation of the specialized nodule structures from cortical tissue. The second group include the nitrogen fixation (*nif*) and related functions (*fix*) which code for the nitrogenase structural proteins and ancillary functions, respectively. A thorough treatment of the process of nodulation and nitrogen fixation will not be attempted because it is not directly relevant to this study, however it is important to the process of understanding how rhizobia fit into the soil ecosystem to understand how they establish the symbiosis. The genes involved in the process are depicted in diagram 1 and for further detail the reader is directed to Rossen *et al* (1987) for a review of nodulation and to Ausubel (1984) and David *et al* (1988) for a review of nitrogen fixation.

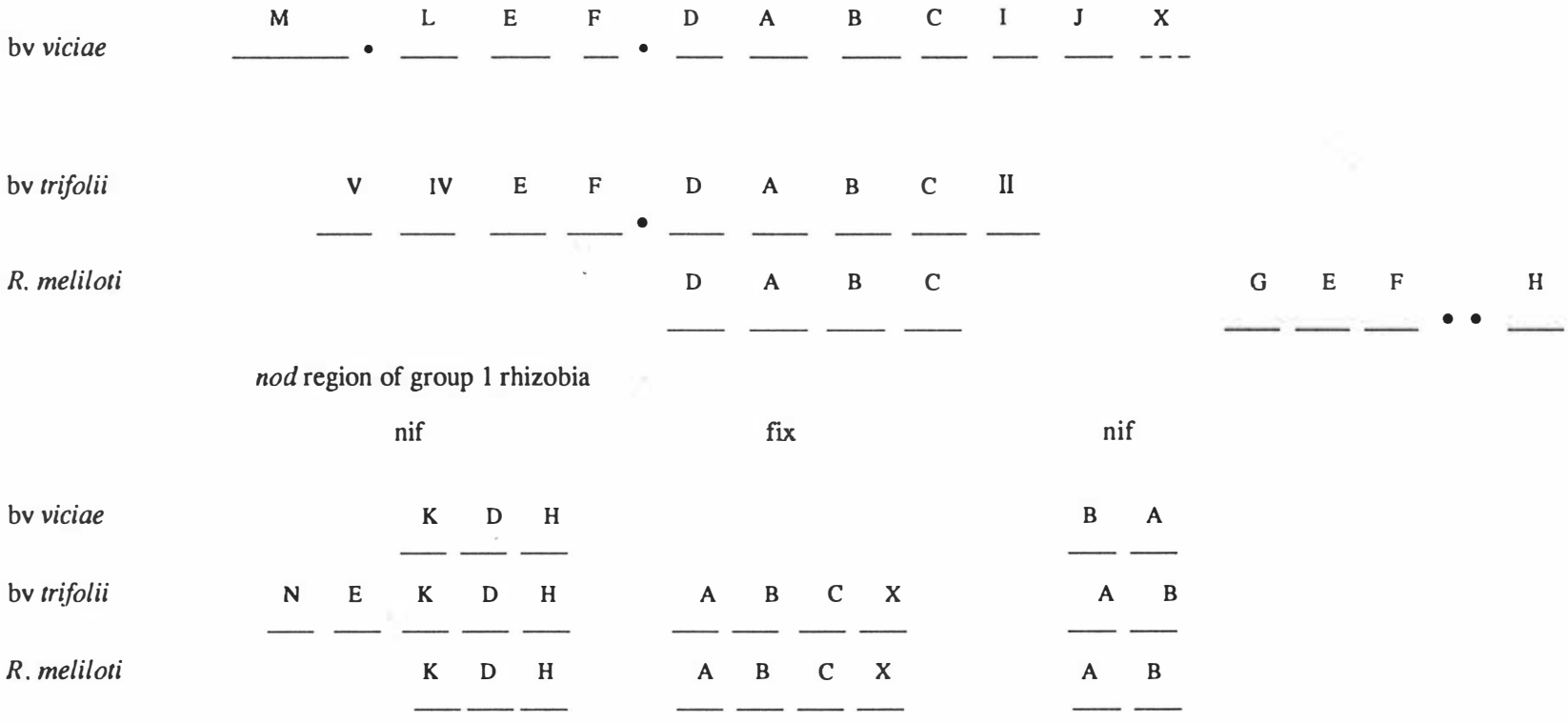


Diagram 1: Organisation of the nodulation and nitrogen fixation genes of some homology group 1 rhizobia. (after Scott *et al.*, 1984; Earl *et al.*, 1987; Rossen *et al.*, 1987; Innes *et al.*, 1988).

Initiation of symbiosis appears to be triggered by the plant in the form of complex ring compounds called flavones. These flavones were shown to interact with nodD (Peters et al, 1986) which in turn acts on nodABC and nodEF. Flavones act to stimulate nodulation in biovar trifolii (Innes et al, 1985; Redmond et al, 1986) and can act to retard nodulation in biovar viceae (Firmin et al, 1986). Flavone activation/suppression is nonspecific, as compounds from clover will function with biovar viceae (Firmin et al, 1986). Control of other nodulation genes by nodD is exerted by a sequence upstream of the operons called the nod box, a conserved sequence located near each operon (Fisher et al, 1988). The cascade runs:

flavone → nodD → nodABC,EF.

Presumably the other nod genes are also part of this cascade. The nodABCD genes appear to be involved in the early stages of plant nodule formation and are often referred to as the "common" nod genes because the nodABCD genes from R. meliloti can complement mutants in these genes in the R. leguminosarum biovars and vice versa. It has also been shown that the nodABCD genes alone are sufficient to allow A. tumefaciens to carry out the initial steps of the nodulation process (Schofield et al, 1984). The second set of nodulation genes, the host-specific nodulation (hsn) genes, appear to code for the specificity of the microsymbiont for the macrosymbiont (Horvath et al, 1986; Weinman et al, 1988).

The nif and fix genes are involved in the actual process of fixing atmospheric nitrogen and were first identified as homologs of the equivalent genes in Klebsiella pneumoniae (Ausubel, 1984). A cascade of activation appears to operate here also:

low O_2 → fixLJ → nifA → nifKDH, other fix

Low partial pressure of oxygen acts on the products of fixLJ (Ditta et al, 1987; David et al, 1988) which can activate nifA. Activation of other genes by the nifA protein appears to require the presence of various upstream elements (Alvarez-Morales et al, 1986) but less is known about nif regulation than about nod regulation. The enzyme responsible for the fixation of the nitrogen is nitrogenase

and is encoded by nifHDK (Scott et al, 1984). This gene cluster is often chosen as a probe for pSym. The function of the fix genes is not clear but the fixABCX operon may code for a nitrogenase specific electron transport system (Earl et al, 1987). The nod and nif genes are closely linked in biovar trifolii (Schofield et al, 1983) and the nif and fix genes are closely linked in biovars viceae and phaseoli (Hombrecher et al, 1981) but understanding the functional and regulatory link between these two vital sets of genes will require further study.

Of more direct relevance, perhaps, to the theme of microbial plasticity that underlies the present study is the existence of a number of repeat elements associated with the nodulation and nitrogen fixation genes. A functional fix gene repeat has been identified on the R. meliloti megaplasmid. Both genes are apparently fully operational as inactivation of one copy does not produce the Fix^- phenotype (Renalier et al, 1987). A repeat of the nifH gene has been identified in biovar phaseoli (Quinto et al, 1982) but not all isolates have this repeat (Martinez et al, 1985). Repeat elements identified in biovar trifolii are promoter elements associated with the nifHDK operon and in a few cases the N-terminus of nifH also (Watson and Schofield, 1985). They are conserved and appear to be specific for pSym of biovar trifolii (Schofield and Watson, 1985). No purpose or role has been identified for these repeated elements and their presence may be accidental. However, it is possible that as well as being potential regulatory sites for the Nod and Nif genes they may act as loci to promote some of the recombinational events described in section 1.6

It is interesting to note that a rising proportion of the studies published in the literature are taking a more holistic approach and are attempting to assess rhizobia in terms of their environment, rather than just as convenient bags of nodulation and nitrogen fixation enzymes and proteins (Harrison et al, 1988; Young and Wexler, 1988; Soberon-Chavez and Najera, 1989a; Jarvis et al, 1989). The importance of this approach is addressed in the next

section.

1.8 *Rhizobium* in the Soil

In order to set the aim of this study in perspective, it is necessary to examine the rhizobia as soil microorganisms with a specialized lifestyle and to see what the various types of genetic changes discussed previously may mean to this genus in its natural habitat.

An analysis of genetic diversity in strains of *Pseudomonas* across a "landscape contour" by McArthur *et al* (1988) showed that the genetic diversity of the bacterium was positively correlated with the diversity of the habitat (as measured by percent organic matter, concentration of metal ions and other factors). A similar study with *R. leguminosarum* biovar *viceae* found variation in the genetic composition of the population despite the close proximity of the sample sites. This field had been continuously re-inoculated and cultivated and the authors commented that the intensive pea plant cultivation may have provided continued selection for adapted genotypes resulting in the strong correlation between particular serotypes, intrinsic antibiotic resistances and plasmid profiles (Brockman and Bezdicek, 1989). There seemed to be clear preferences for certain plasmid profile groups within a serogroup at different topographic locations, suggesting a microniche component in the selection of the dominant rhizobial strain (Brockman and Bezdicek, 1989). In an examination of a small number of clover nodule isolates from a limited area, Schofield *et al* (1987) found good evidence for genetic exchange amongst the isolates. Interestingly, three of their isolates would not grow on the standard undefined rhizobial growth medium (TY). This could have been due to a severe auxotrophic mutation or some other disadvantageous change that had occurred. Alternatively the strain carrying the symbiotic plasmid in this case wasn't able to grow on TY because it wasn't a *Rhizobium*. It is impossible to do more than speculate until further studies are carried out on these three strains (Schofield *et al*, 1987).

A comparison of the degree of polymorphism (as measured by the number/position of hybridization patterns generated by specific DNA probes) of the chromosome and pSym of a number of Rhizobium leguminosarum biovar viceae isolates showed the plasmid to be substantially more polymorphic than the chromosome (Young and Wexler, 1988) as mentioned in section 1.6. However, the DNA probes in question were different (lac for the chromosome and five overlapping probes of the symbiotic region) and the choice of probes may have introduced a certain bias. Young and Wexler (1988) found a clear correlation between particular symbiotic plasmids and chromosomal backgrounds and suggested three hypotheses to explain this correlation. Firstly, that opportunities for plasmid transfer are rare in the soil, so that the association between plasmid and chromosome that arises as a clone expands are not readily dissipated. Secondly, that the plasmid is restricted to certain host backgrounds and although conjugation occurs, only certain plasmid-chromosome combinations are stable. Thirdly, that although many plasmid-chromosome combinations exist in the soil only some are favorable enough to compete successfully for nodulation sites. The three hypotheses espoused by Young and Wexler (1988) could be designated, respectively, the linkage hypothesis, the genomic compatibility hypothesis and the selection hypothesis. Evaluating each hypothesis in turn, and in light of other studies, some contradiction between these proposals and the work of other researchers in the field can be found.

The linkage hypothesis stands up least well in regards to other studies. The study of Schofield et al (1987) involved only sixteen samples from a very small area and still identified a recombinant Sym plasmid. Similarly, Harrison et al (1988) compared plasmids from laboratory and field-isolated strains of Rhizobium leguminosarum biovar trifolii and commented that on the basis of their data regarding size, number and host specificity of Sym plasmids, the potential for recombination and plasmid transfer appeared high. Secondly, a large number of groups have studied the transmissibility

of pSym (Johnston et al, 1978; Hooykaas et al, 1981; 1982; Martinez et al, 1987; Espuny et al, 1987) and have found it to transfer readily, and not just to Rhizobium. Although these studies are laboratory-based there is evidence to support the theory of plasmid transfer, even over great taxonomic distances, in the soil (Richaume et al, 1989).

The genomic compatibility hypothesis has more to commend itself in terms of observed data, as incompatibility between Sym plasmids (Espuny et al, 1987; Ollero et al, 1989) and between genes on these plasmids (Wang et al, 1986) is known. However, bacteria from genera other than Rhizobium have been identified which will accept and at least partially express pSym (Plazinski and Rolfe, 1985; Jarvis et al, 1989) and unless the genomic compatibility is more universal than proposed by Young and Wexler (1988) this hypothesis must be regarded as flawed.

The third hypothesis is selection and here the plant is as important as the bacteria. There is certainly evidence that genes expressed by the rhizobia, such as antibiotic resistance, can affect nodulation effectiveness (Schwinghammer, 1967; Schwinghammer and Dudman, 1973) and that the plant cultivar can select among bacterial strains (Demezas and Bottomley, 1987). Furthermore, rhizobial strains carry host specific nodulation (hsn) genes which act to link a rhizobial microsymbiont to a plant macrosymbiont. At present, there is insufficient evidence to decide how important "selection" might be.

Other soil-based studies, notably those of Soberon-Chavez and Najera (1989a) and Jarvis et al (1989), have identified other factors that have bearing on rhizobial ecology. In the first case, Soberon-Chavez and Najera (1989a) were able to isolate an organism from the soil that responded to antibiotic selection and behaved in culture in the same way as R. leguminosarum biovars but contained no symbiotic plasmid. Introduction of pSym into this strain was quite sufficient to confer full $Nod^{+}Fix^{+}$ phenotype. The authors also suggest that in the sites they examined, the number of "non-symbiotic" rhizobia in

the soil may be larger than the number containing pSym (Soberon-Chavez and Najera, 1989a). The second case was a study conducted by Jarvis and co-workers (1989) in which soil-isolated organisms, which had demonstrated some degree of homology on a non-quantified dot blot probed with a strain of biovar trifolii lacking pSym, had a symbiotic plasmid mobilized into them by the broad host range plasmid R68.45. These soil bacteria were uncharacterized gram negative rods isolated from a white clover-ryegrass pasture and the introduction of symbiotic genes enabled some of the soil organisms to form nodules (Jarvis et al, 1989). The identity of these bacteria which are able to accept pSym and nodulate clover is not yet clear.

The studies of Young and colleagues (Young, 1985; Young and Wexler, 1988) suggest that there is a non-random distribution of symbiotic plasmids across chromosomal backgrounds. They suggest that plasmid transfer does occur, but not indiscriminately among rhizobial strains. However another study in which Young was involved (Harrison et al, 1988) suggseted that the potential for recombination and transference of pSym was high. Schofield et al (1987) also identified significant levels of plasmid transfer and Broughton et al (1987) suggested that rather than certain dominant genetic lines of bacteria there was a "continuum of symbiotically proficient strains under conditions of maximum diversity". Furthermore, the studies of Soberon-Chavez and Najera (1989a) and Jarvis et al (1989) suggset that there are large numbers of bacteria in the rhizosphere which can accept and express pSym.

There is an obvious contradiction implied in the above studies which is likely to be resolved once further information is available. The implication from the studies of Broughton et al (1987), Soberon-Chavez and Najera (1989a) and Jarvis et al (1989) is that not only are there rhizobia present, but also bacteria that may become rhizobia with the addition of pSym, and it is this pool of symbiotically competent bacteria that can nodulate legumes. The idea of a linked pool of soil bacteria has been proposed by Reanney (1976; 1978) and colleagues (1983) and the pool of "rhizobia" (defined as

bacteria which can nodulate legumes in association with pSym) could be a subset of and genetically linked to this larger pool of soil-dwelling microorganisms.

A recent review paper by Eberhard (1989) suggests that the optional traits carried on plasmids are the result of a bacterial strain trying to adapt to local conditions. The use of the plasmid as the DNA structure coding for the adaptive trait means that the information can be spread throughout the population. The author regards the symbiotic plasmid as an extreme form of adaptation to local conditions in the form of a symbiotic lifestyle (Eberhard , 1989). We have already seen that pSym is self-transmissable (Johnston et al, 1978) and to other genera (Hooykaas et al, 1981; 1982; Plazinski and Rolfe, 1985; Jarvis et al, 1989), thus it could be said that a Rhizobium is any bacterium that can accept and express pSym. If the various symbiotic plasmids and rhizobial strains are examined in this light, the degree of genetic dissimilarity is understandable. The presently defined species of Rhizobium could be described as microorganisms that have had pSym in their populations for a sufficient length of time to adapt to the symbiotic lifestyle (Eberhard ,1989). Those species in which the symbiotic information is encoded by the chromosome (Bradyrhizobium) may initially have had carried a symbiotic plasmid which became integrated.

There is already a substantial body of evidence regarding the ability of pSym to undergo rearrangement (Zurkowski, 1982; Soberon-Chavez et al, 1986; Wang et al, 1986; Palacios et al, 1987; Schofield et al, 1987; Espuny et al, 1989; Ollero et al, 1989; Soberon-Chavez and Najera, 1989a and b). There is some evidence for rearrangements involving plasmid and chromosome in an apparently specific manner (Berry and Atherly, 1984), which is a possible mechanism to explain the chromosomal location of symbiotic genes in Bradyrhizobium and several authors have commented on the highly polymorphic nature of pSym, especially in regard to the region around the symbiotic genes (Harrison et al, 1988; Young and Wexler, 1988). The final statement of a paper by Soberon-Chavez and Najera (1989a) is that:

"The study of this type of bacteria [natural pSym⁻ *R. leguminosarum*] will help elucidate the *Rhizobium* life cycle and will shed light on the importance of rearrangements of plasmids and chromosomes in the establishment and perpetuation of the symbiosis between *Rhizobium* and legumes".

Coupled to the importance of the rearrangement of the symbiotic plasmids is the importance of the ability of these plasmids to transfer to other bacteria, whether rhizobia or not. The combination of rearrangement and plasmid transfer gives the rhizobia a powerful means of adapting to change and experimenting with various gene combinations. Although most of the genetic recombination and plasmid transfers have been generated or identified in the laboratory, the fact that they can occur can assist in generating and testing realistic hypotheses which can account for what is observed in nature.

1.9 Competition and Genetic Interaction: the Fate of Inoculant **Rhizobium**.

The question of the fate of inoculant rhizobia arose in the context of a previous study undertaken in this laboratory (O'Hara, 1985). Access was gained to a freshly sown pasture which had been recently cleared from virgin bush. The area had theoretically had no previous exposure to rhizobia, enabling studies of the short-term modification and adaptation of the inoculant rhizobia, *R. leguminosarum* bv *trifolii* 2668. Samples of plant material in the form of soil cores were taken at known time intervals following the initial sowing and rhizobia were isolated from the clover nodules.

The original inoculant strain *R. leguminosarum* bv *trifolii* 2668 (NZP 560) was compared to a number of isolates made from the field by the criteria of gel diffusion immunoprecipitation (Vincent, 1970) intrinsic antibotic resistance (Josey *et al*, 1979; Beynon and Josey, 1980), plasmid profile (Casse *et al*, 1979; Hirsch *et al*, 1980; Tichy and Lotz, 1981; Scott and Ronson, 1982; Christensen and Schubert, 1983) and genomic restriction endonuclease digest pattern (Mielenz *et al*, 1979; Marshall *et al*, 1981; O'Hara *et al*, 1985). Initial isolates

showed a high degree of similarity to the inoculant but as time passed the level of similarity found in the isolates declined as did the frequency of isolates indistinguishable from 2668. Taken at face value this observation would suggest that either the inoculant was being displaced by a number of similar strains which are already present in the soil or is the result of plasmid transfer (Jarvis et al, 1989). Alternatively, the inoculant could be adapting to the soil by utilizing genes it already has in its genome in a cryptic state (Hall, 1988) or by acquiring genes as a result of plasmid transfer (Richaume et al, 1989) and thereby changing its response to the various test parameters. However, in analysing the field environment the first possibility must be considered, namely that a pre-adapted strain or strains present in the soil are out-competing the inoculant. The site in question was adjacent to a field that had been inoculated with a different strain two years before and which consequently was likely to contain rhizobia adapted to local conditions. In defence of the conclusion that the inoculant was still present, but altered, it should be stated that it was unlikely that large numbers of rhizobia would cross from the old site to the new (for topographical reasons), that the new inoculant was introduced in enormous numbers and that on the basis of the various identification tests the initial isolates were more like the inoculant 2668 than the inoculant of the nearby field. It was therefore felt that some type of genetic alteration, either endogenous and/or exogenous, had resulted in the alteration of the inoculant but it was impossible to rule out other explanations including the presence of "contaminant" strains. Experiments analysing the response of soil microorganisms in the soil are difficult to interpret because of the large number of uncontrolled and uncontrollable factors. It was for this reason that a second series of experiments was planned, using a laboratory simulation to attempt to mimic possible natural influences and thus attempt to confirm the results obtained with the soil-based studies.

It was decided to continue experimentation with 2668 in order to maintain as much similarity with the field study as was possible.

The initial focus of the project was to attempt to explain the variation of the field isolated strains by generating a stress-altered version of the inoculant. It was hypothesized that the bacteria might encode a system analogous to the E. coli **SOS** genes which would mediate genetic rearrangements and decryptification of the genome in an attempt to survive. The broad host range plasmid RP4 was introduced into 2668 as a form of genetic "sponge" that could accept any DNA sequences that were being translocated. In retrospect it was overly ambitious to detect this type of effect without any readily selectable marker and not surprisingly, no variants were detected after stress treatment.

One event that was detected at low frequency was a rearrangement of the rhizobial plasmids following the introduction of RP4 into the cells. This rearrangement was stable on sub-culture, once formed, and involved what could have been a deletion in one plasmid (or replacement with a smaller plasmid) and an apparent loss of another. Examination of the electrophoretic plasmid and total genomic DNA restiction endonuclease profiles of these strains with radioactive probes of the nod and nif gene fragments suggested that it was pSym, or a portion thereof involving the symbiotic genes, that had been lost. However, nodules were produced when this strain was inoculated onto clover plants (Trifolium repens cv **Huia**) and the bacteria isolated from the nodules were shown to be derived from apparently nod$^-$ rhizobial strains and had recovered the capacity to hybridize with the nodABC and nifKDH probes. Repeated trials with genetically marked derivatives of the apparently pSym-deleted strains merely served to confirm this result. Failure to satisfactorily provide a mechanism whereby a seemingly nod$^-$ strain of rhizobia forms nodules resulted in termination of this line of inquiry until such time as a reasonable hypothesis could be generated and investigated.

In the second portion of the project the ability of the inoculant strain to transmit genetic information, in the form of plasmids, to other organisms was examined. To this end four gram negative rods of uncertain genera which had previously demonstrated

the ability to accept, express and maintain parts of pSym introduced as a cointegrate with a broad host range vector (Jarvis *et al*, 1989) were chosen as recipients. The donor strain (2668) was marked randomly with Tn*5* and exconjugants from crosses between this strain and the soil microorganisms were co-selected for the presence of Tn*5*, giving a selectable marker on any mobile piece of DNA detected. In all soil bacteria crosses a plasmid of approximately the same size was visualized by electrophoresis in each of the exconjugants. This new plasmid was shown to hybridize with probes containing Tn*5*, *nod* and *nif*. Conjugation between the soil strains containing these small plasmids and a *sym*$^-$ bv *trifolii* strain, demonstrated that the plasmids would transfer back into rhizobia from this background. Similar crosses with *E. coli* and the original parent rhizobium 2668 demonstrated the ability of these Tn*5*-marked plasmids to mediate conjugation with HB101 or 2668 and be stably maintained in these recipients. Plant tests with all of the exconjugant strains previously mentioned showed that these Sym-derived plasmids would provide sufficient information for nodulation of and nitrogen fixation in clover, with the exception of *E. coli*. The ecological consequences of the broad host range transmissibility of the symbiotic plasmid is discussed.

2.0 MATERIALS AND METHODS

2.1 Bacterial Strains: Maintenence, Selection and Identification

The *Rhizobium leguminosarum* biovar *trifolii* strain chosen for this project was the microsymbiont used to coat the clover seed in the standard ryegrass-clover seed pasture mixes employed in New Zealand. The strain is referred to as 2668 and is held in the DSIR Culture Collection, Palmerston North as NZP 582. It is naturally sensitive to most antibiotics (table 5) in comparison to the other bacterial strains used in this study.

MO 103 was a mutant detected in a cross between 2668 and HB101(RP4)-see section 2.5 for procedure. The aberrant plasmid profile (fig. 15A, lane b) was observed only once among 52 isolates screened by Eckhardt electrophoresis (section 2.9) in four separate crosses of 2668 and HB101(RP4). There are no obvious differences in colony morphology or growth rate in liquid culture between 2668 and MO 103 (estimated from Absorbance at 600nm in a spectronic 20 spectrophotometer). Most exconjugants of the 2668 X HB101(RP4) cross appear identical to 2668 strains aside from the extra RP4 band. MO 104 was isolated from a tube of stress medium initially inoculated with MO 103 that had been incubated at 28°C for four weeks with shaking (see section 3.1 and 3.5). It initially had a slower growth rate than MO 103 (as estimated from A_{600}), but continued subculture on TY (section 2.2) removed this characteristic. In all other ways, MO 103 and 104 are identical (see section 3 and figs. 15A, lanes b and c; 19, lanes c and d) and are therefore considered serial isolates of the same strain.

MO 110 was a nodule re-isolate from a plant inoculated with MO 103 and MO 111 from a plant inoculated with MO 104. Because the inoculants MO 103 and 104 are serial isolates of the same strain, MO 110 and 111 are also two isolates of the same strain. In four trials a total of sixteen nodule re-isolates have been compared by Eckhardt electrophoresis and all are the same (figure 1) as MO 110/111, suggesting that MO 110/111 is a stable variant. MO 110 and 111 are indistinguishable on the basis of the tests used here (figs. 15A,

lanes d and e; 19, lanes e and f). Inoculation of plants with the spontaneous antibiotic resistant mutants of MO 104 (MO 105-Cloramphenicol^R, MO 107-Rifampicin^R, MO 109-streptomycin^R) yielded MO 110/111-type strains with these antibiotic resistances.

The spontaneous antibiotic resistant mutants listed in table 1 were selected by plating 0.1ml of cultures containing 3±2 X10^8 cells/ml (as measured by plating onto the nonselective medium TY) onto plates of TY containing the antibiotic. Concentrations of antibiotics (abbreviations in brackets) used to select mutants were:

Chloramphenicol (Cm), 100 ug/ml; Rifampicin (Rif), 100 ug/ml; Streptomycin (Sm), 200 ug/ml; Spectinomycin (Sp), 100 ug/ml.

Resistant colonies appeared after 6 days at 30°C and were single colony purified two times on antibiotic plates. Frequency of the antibiotic resistant mutants was 10^{-6} to 10^{-7} (see table 5B).

The soil organisms received from Dr. B.D.W. Jarvis (Jarvis et al, 1989) were isolated from a white clover-ryegrass paddock. The four strains used in this study were ones shown by Jarvis et al (1989) to be able to accept symbiotic information and manifest a Nod$^+$ phenotype. They were taxonomically uncharacterized when received, beyond being shown to be gram negative rods. Before receipt, spectinomycin resistant mutants of all four were derived as described above by Dr. Jarvis.

Gram staining showed all four to be small gram negative ovoid rods. Description of the bacterial colonies and the results of a variety of tests are reported in the results. The tests included motility, growth on a variety of solid and liquid media, growth, acid and gas production in several sugar and sugar-alcohol media, litmus milk and the API 20NE test strip. Test results were read and compared to the standard key provided and also compared to published information on the suggested species (Skerman, 1967: Bergey, 1974; 1984).

Antibiotic resistant cultures were maintained on antibiotic containing plates and sub-cultured every two months (every month for E. coli). The TY plates were stored at 4°C. Growth temperature for

the rhizobia, the soil bacteria and the exconjugants was 30°C. The soil bacteria received from Dr. Jarvis had adapted to this temperature by the time we obtained them. Liquid cultures were grown at 28°C with shaking. E. coli cultures were grown at 37°C in Luria broth or on Luria agar, but otherwise were as described for the rhizobia.

Strains PN 104, PN 291, PN 435 and PN 302 were kindly supplied by Dr. D. B. Scott. The non-rhizobial strains used in this study are listed in Table 2.

TABLE 1.: Strains of rhizobia used.

References listed in the table are as follows:

(1). Scott and Ronson, 1982.

(2). Jarvis *et al*, 1989.

TABLE 1:

Laboratory designation	Antibiotic markers carried	Culture Collection Number or Derivation	Reference
R. trifolii			
2668		NZP 582	
MO 800	S		this study
MO 103	A/T/K	2668 x HB101(RP4)	this study
MO 104	A/T/K	slow growing mutant of MO 103	this study
MO 105	C/A/T/K	spont. mutant MO 104	this study
MO 107	R/A/T/K	spont. mutant MO 104	this study
MO 109	S/A/T/K	spont. mutant MO 104	this study
MO 110/111		nodule re-isolates	this study
PN 104	nod^-/S/R	NZP 514 *sym*$^-$	(1)
NR 41	Sp	Unknown sp	(2)
NR 42	Sp	Unknown sp	(2)
NR 64	Sp	Unknown sp	(2)
OR 168	Sp	Unknown sp	(2)
MO 120	N	2668 x *E.coli* PN302	this study
MO 121	Sp/N	MO 120 x NR 41	this study
MO 122	Sp/N	MO 120 x NR 42	this study
MO 123	Sp/N	MO 120 x NR 64	this study
MO 124	Sp/N	MO 120 x OR 168	this study
MO 201	S/R/N	MO 121 x PN 104	this study
MO 203	S/R/N	MO 122 x PN 104	this study
MO 204	S/R/N	MO 123 x PN 104	this study
MO 205	S/R/N	MO 124 x PN 104	this study
MO 810-813	Sp/N/R	MO 121 x 2668	this study

NZP: Palmerston North Culture Collection, DSIR. A=ampicillin
C=chloramphenicol. K=kanamycin. N=neomycin.
R=rifampicin. S=streptomycin. Sp=spectinomycin.
T=tetracycline.
All antibiotic concentrations are 50 ug/ml.

TABLE 2.: Non-rhizobial strains used.
References listed in the table are as follows:

(1). Scott *et al*, 1985.

(2). Simon *et al*, 1983.

(3). Schofield *et al*, 1983.

TABLE 2:

Laboratory designation	Characteristics and Markers	Culture Collection Number or Derivation	Reference
Escherichia coli			
HB 101	F⁻ *pro leu thi lacY* S r⁻m⁻ EndoI⁻ *recA*⁻	MU 672	(1)
HB 101(RP4)	S/A/T/K	MU 668	
PN 302 or HB 101(pSUP 1011)	S/N/K	Tn5 donating suicide vector	(2)
PN 291	T/C	*nod ABC* EcoRI 7.2 kb fragment from bv *trifolii* ANU843 in pBR328	(3)
PN 435	A	*nif KDH* EcoRI 5.2 kb fragment from bv *trifolii* in pBR328	
MO 300	S/R	Spont. mutant of *E. coli* HB 101	this study
MO 301	S/R/N+K	MO 121 x MO 300	this study
MO 302	S/R/N+K	MO 122 x MO 300	this study
MO 303	S/R/N+K	MO 123 x MO 300	this study
MO 304	S/R/N+K	MO 124 x MO 300	this study

MU: Massey University Culture Collection, Palmerston North.
A=ampicillin. K=kanamycin. N=neomycin. R=rifampicin.
S=streptomycin. T=tetracycline.
All antibiotic concentrations are 100 ug/ml, except tetracycline which is 50 ug/ml.

2.2 Media Used for Strain Maintenance

2.2.1 Tryptone Yeast Extract Broth (TY) (Beringer, 1974) contains (g/l): Tryptone (Difco), 5.0; Yeast Extract (Difco), 3.0. The broth was sterilized by autoclaving at 121°C for 15 minutes. For solid media; 12g of agar (Davis) was added for base, per litre of broth. The mixture was then autoclaved. If the medium was to be used immediately, 5 ml sterile 1M $CaCl_2.6H_2O$ was added per litre, otherwise it was stored without any additions. This was the standard undefined growth medium.

2.2.2 Luria Broth contained (g/l): Tryptone (Difco), 10.0; Yeast Extract (Difco), 5.0; NaCl, 5.0. For solid medium: 12g of agar (Davis) was added for base. Autoclaving conditions as above. This was the standard E. coli growth media.

2.2.3 Nutrient agar was made by dissolving 8.0g of nutrient agar base (Difco) and 15g of agar (Davis) in one litre of water and autoclaving at 121°C for 15 minutes

2.2.4 Yeast Extract Mannitol Agar (YEM) (Vincent, 1970) contained (g/l): mannitol (BDH), 10.0; Yeast extract (Difco), 0.4; K_2HPO_4, 0.5; $MgSO_4.6H_2O$, 0.2; NaCl, 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 S10 Defined Medium (Chua et al, 1985)

Stock Solutions

Salts (g/l): $MgSO_4.7H_20$, 25.0; $CaCl_2.2H_2O$, 2.0; $FeCl_3$, 0.6; Na_2EDTA, 1.5; NaCl, 20.0.

Ammonium Chloride (g/l): NH_4Cl, 18.

Vitamins (g/l): Thiamine HCl, 1.0; Biotin (1 mg/ml), 1.0 ml; Calcium Pantothenate, 2.0. Dissolve with gentle heating.

Trace Elements (mg/l): $ZnSO_4.7H_2O$, 15.0; H_3BO_3, 250.0; $NaMbO_3$, 200.0; $MnSO_4$, 200.0; $CuSO_4.5H_2O$, 20.0.

Phosphates (g/l): K_2HPO_4, 100.0; KH_2PO_4, 100.0.

Carbon Source (g/l): Sodium Succinate, 81.0.

Bromothymol Blue (g/100ml): 0.2.

Medium (ml/1): Salts, 10 ; trace elements, 1 ; vitamins, 1 ; NH_4Cl, 6 ; 2-(N-Morpholino)ethanesulphonic acid (MES), 10.0g; Histidine (BDH), 100.0 mg; bromothymol blue, 10. Adjust pH to 6.2 with conc. NaOH. Add 12g agar (Davis). Autoclave for 15 minutes at 121°C. Cool to 50°C and aseptically add 5 ml sterile phosphates and 10 ml sterile carbon sources. S10 is the standard defined media for rhizobia used in this lab.

2.2.6 Jensen's Agar Medium (Vincent, 1970) (g/1): $CaHPO_4$, 1.0; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.2; NaCl, 0.2; $FeCl_3$, 0.1. Adjust pH to 6.5-7.0, add 15g agar and dissolve by heating. Dispense into required containers and autoclave. This medium is used for germinating seeds and growing plants.

2.2.7 Stress Medium, adapted from Heumann et al (1983, 1984). Stock solutions (g/100ml): NH_4Cl, 0.1; KNO_3, 0.1; glucose, 0.5; dextrin, 0.5.

Stress Medium (ml/50 ml): S10 salts, 0.5 ; S10 trace elements, 0.05 , S10 vitamins, 0.05 ; MES, 0.5g; Histidine, 0.005g; NH_4Cl or KNO_3, 0.3. Adjust to pH 6.5. For agar add 0.6g agar (Davis). Autoclave, cool to 50°C. Add: S10 phosphates, 0.25 ml; glucose or dextrin, 0.5 ml. Two combinations were used: glucose+nitrate or dextrin+ammonium.

This is basically a defined medium in which either the carbon or nitrogen source is replaced with a more difficult to utilize version, e.g. NO_3^{2-} instead of NH_4^-. This medium was used for nitrogen or carbon source stress of strains.

2.2.8 Selection media (Heumann et al, 1983). These are the four solid media onto which culture was plated after incubation in Stress Media. All 4 media contained 15g agar per litre of media.

(a). MM. Minimal medium consisted of (g/1): KH_2PO_4, 2; K_2HPO_4, 7; $(NH_4)_2SO_4$, 1; trisodium citrate.$2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.1; glucose, 5; S10 vitamins, 1 ml; nutrient broth (Difco), 1 %.

(b). YEM. As specified above.

(c). LL. Lab Lemco contained (g/1): lab lemco powder (Oxoid), 8.

(d). Azo. Burk's nitrogen-free agar contained (g/1): KH_2PO_4, 0.1; K_2HPO_4, 0.9; $CaCl_2.2H_2O$, 0.1; $MgSO_4.7H_2O$, 0.1; $Na_2MoO_4.2H_2O$, 0.005; $FeSO_4.7H_2O$, 0.25; glucose, 10; 0.05N H_2SO_4, 20 ml.

2.3 Antibiotic Containing Media

Antibiotic stock solutions were prepared as follows:

2.3.1 Ampicillin was prepared by dissolving 200mg of ampicillin (Sigma) in 20 ml distilled water for a final concentration of 10 mg/ml.

2.3.2 Chloramphenicol was prepared by dissolving 400mg of chloramphenicol (Sigma) in 20 ml of absolute ethanol for a final concentration of 20 mg/ml.

2.3.3 Kanamycin was prepared by dissolving 400 mg kanamycin sulfate (Sigma) in 20 ml water for a final concentration of 20 mg/ml.

2.3.4 Neomycin was prepared by dissolving 2g of neomycin sulfate (Sigma) in 20 ml of distilled water for a final concentration of 100 mg/ml

2.3.5 Rifampicin was prepared by dissolving 400mg rifampicin (Sigma) in 20 ml methanol for a final concentration of 20 mg/ml.

2.3.6 Spectinomycin was prepared by dissolving 500mg spectinomycin dihydrochloride (Sigma) in 20 ml water for a final concentration of 25 mg/ml.

2.3.7 Streptomycin was prepared by dissolving 2g of streptomycin (Sigma) in 20 ml of distilled water for a final concentration of 100 mg/ml.

2.3.8 Tetracycline was prepared by dissolving 200mg of tetracycline HCl in 20 ml of methanol for a final concentration of 10 mg/ml.

All antibiotic solutions were sterilized by passage through a 0-22 micron filter (Millipore). Sterile antibiotics were added aseptically to the media after it had been autoclaved and cooled to 50°C. Antibiotic solutions were stored at -20°C.

Final concentrations used are listed (where necessary) in the text.

2.4 General Purpose Solutions

All solutions were autoclaved at 121°C for 30 minutes after preparation.

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

(a). 1M Tris contained (g/l): Trizma base (Serva), 121.1. This was dissolved in 850 ml of distilled water, the pH adjusted and the volume made up to 1 litre. Two pHs were in general use, 7.5 and 8.0. pH was adjusted with concentrated HCl.

(b). 0-1M Tris pH8 was prepared by diluting 1M Tris pH8 1/10 with distilled water and checking pH prior to autoclaving.

(c). 0.1M Tris pH8 + 2-mercaptoethanol was prepared as above except the 2-mercaptoethanol was added aseptically after autoclaving. Allowance was made for its volume in the dilution process.

2.4.2 Ethylene diamine tetraacetic acid solution. 0.25M Na_2EDTA contained (g/l): EDTA (BDH), 83 dissolved in 850 ml. The pH was adjusted to 8 with conc. HCl and the volume adjusted to 1 l. This solution was also used in conjunction with the Tris buffers.

2.4.3 Tris-EDTA buffer (TE) was prepared at several concentrations. Buffers were made from stock solutions listed above.

(a). 50/20 TE was final concentration 50 mM Tris and 20 mM EDTA. 50 ml of 1M Tris pH 8 and 80 ml 0.25M EDTA were added to 800 ml of water, the pH adjusted to 8.0 (if needed) and the volume to 1 l.

(b). 10/1 TE was final concentration 10 mM Tris and 1 mM EDTA. 10 ml of 1M Tris pH 8 and 4 ml of 0.25M EDTA were added to 950 ml of water, the pH adjusted to 8.0 (if needed) and the volume to 1 l.

(c). 10/1 TE + sarkosyl was the same buffer containing N-lauryl-sarcosine to 0.1 % added before final volume adjustment.

2.4.4 5M NaCl was prepared by dissolving 292g NaCl in 700ml distilled water and adjusting the volume to 1l.

2.4.5 SDS. 10% sodium dodecyl sulfate was prepared by dissolving 10g of the solid in 100 ml of water. **Note**: care should be taken with the powder, inhalation can cause lung irritation.

2.4.6 Borate electrophoresis buffer (TBE) contained 89 mM Tris, 89 mM boric acid and 2.5 mM EDTA, pH 8.2. A 10X stock was prepared by

dissolving in 800 ml distilled water: 108g Tris, 55g Boric acid, 9.2g EDTA. pH was checked, as this buffer is at pH 8.2 by virtue of the relative concentrations of the ingredients, and no adjustments were required. The volume was adjusted to 1 l.

2.4.7 Standard Saline Citrate (SSC) contained 150 mM sodium chloride and 15 mM sodium citrate, pH 7. A 20X stock was prepared by dissolving 175.3g of NaCl and 77.4g $(CH_2)_4(COONa)_2.3H_2O$ in 800 ml distilled water. The pH was adjusted to 7 and the volume to 1 l. This solution was used at a variety of concentrations, most commonly 20X, 2X and 0.5X.

2.4.8 Salt-Tris-EDTA (STE) contained 100 mM sodium chloride, 10 mM Tris pH 8 and 1 mM EDTA. It was prepared by adding 20 ml 5M NaCl, 10 ml 1M Tris pH 8 and 4 ml 0.25M Na_2EDTA to 950 ml water, adjusting the pH to 8 and the volume to 1 l.

2.4.9 Dilution buffer was prepared by adding 2.5g $MgSO_4.7H_2O$ to 90 ml water, dissolving, adjusting the volume to 100 ml and autoclaving. 10 ul of Tween-20 was added aseptically afterward. Final concentrations of ingredients were 10 mM $MgSO_4$ and 0.01 % Tween-20.

2.5 Conjugation of Bacterial Strains

A single colony of each strain to be crossed was inoculated into the appropriate growth medium and grown two days at 28°C with shaking (or 24 hrs at 37°C with shaking for *E. coli*). The A_{600} of the culture at this point was 0.8±0.2. From this culture, a 50-fold dilution was made and grown overnight at 28 °C (37°C) with shaking. The A_{600} of this culture was 0.3±0.1, which corresponds to 4±2 $X10^8$ cells/ml.

TY agar was used as a basal medium for conjugation. A sterile 25 mm diameter 0.45 um pore size filter was placed on the plate and 100 ul of each parent pipetted onto the filter. Each parent was spotted separately on the plate away from the filter to ensure that both parent cultures were viable and the plate incubated with the lid off for one hour at room temperature (≈20°C) in a laminar flow cabinet (Airpure) to dry the cross. The plate was inverted and incubated overnight at 30 °C. This temperature was standard in this lab and all

strains used grew well at this temperature.

The filter was removed and resuspended in 1 ml of dilution buffer (see 2.4.7). The mix was diluted to 10^{-5} and 0.1 ml of each dilution plated on the selective media. For the crosses between HB101(RP4) and 2668, the selective medium was S10.Nm (50 ug/ml), for the crosses of 2668 and the soil microorganisms it was TY.Sp (50 ug/ml).Nm (50 ug/ml), for the crosses of the 120 series and PN 104 it was TY.Rif (50 ug/ml).Nm (50 ug/ml), for the crosses between the 120 series and HB 101 it was Luria.Sm (100 ug/ml).Km (100 ug/ml) and for the cross between MO 121 and 2668 it was S10.Sm (50 ug/ml).Nm (50 ug/ml). In each cross 0.1 ml of the 10^{-5} dilution was plated on media appropriate to each of the parents, e.g. HB101(RP4) X 2668, the cross mix at 10^{-5} was plated on Luria.Km (100 ug/ml) to enumerate the donors and at the same dilution on S10 to enumerate the recipients. Frequency of transfer of the antibiotic marker was calculated as follows:

Frequency of transfer = no. exconjugants expressing both antibiotic markers/no. recipents expressing the appropriate marker

Note 1: HB 101 is an auxotroph and S10 a defined medium lacking proline and leucine and with a Carbon source not usable by HB101. Pure cultures of 2668(RP4) and 2668::Tn5 could be obtained from S10 plates at lower dilutions where colony density was insufficient for crossfeeding. Dilution in the dilution buffer, which contains a detergent, should maximize the probability that single colonies arise from single cells.

Note 2: In this study cultures found to be resistant to kanamycin were also resistant to neomycin and vice versa. Cultures listed as resistant to one were assumed to be resistant to both. Rhizobia are inherently kanamycin resistant, by virtue of their inability to uptake this antibiotic, and respond poorly to ampicillin, with high concentrations required for inhibition.

2.6 Isolation of **Rhizobium** from Nodules

Clover plants were removed from growth tubes in a laminar flow cabinet (Airpure) and any agar carefully cleaned away. The plants were examined for overall appearance and the roots for nodule size and color. The roots were carefully washed in sterile water to remove stray material. A section of the root carrying the nodule was cut away and each nodule was surface sterilized independently 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 to select rhizobia and on to nutrient agar to check for non-rhizobial contamination. Contaminated cultures were discarded.

Nodules chosen were the largest and healthiest present on that root. Normally only one nodule per plant was examined, except in part I, where two nodules per plant were tested. A portion of the streak from the YEM+actidione plate was restreaked for single colonies. The plasmid profile of one or more colonies were subsequently determined by the Eckhardt gel electrophoresis procedure and the culture tested for antibiotic resistances (where appropriate).

2.7 Germination of Seeds, Inoculation of Clover Plants and Re-isolation from Nodules

In all experiments the seeds were Trifolium repens bv **Huia** which had been obtained as certified stock from Hodder and Tolley (NZ Ltd).

The seeds were rinsed in a petri dish with sterile distilled water to which 100 ul 20 % sarkosyl had been added to break the surface tension. This was followed by the addition of a saturated solution of $CaOCl_3$ to a final concentration of 10 %. The bleach was mixed in and the seeds allowed to stand for 10 minutes. The seeds were washed five times with sterile distilled water, and a sample from the final wash was inoculated into a TY broth culture to test the effectiveness of the procedure. Finally, 10 ml of sterile water was added to the dish and the seeds allowed to germinate overnight in the dark. After this time, the water was decanted and the seeds placed aseptically onto Jensens agar plates, sealed and grown for 48 hours in a light and temperature controlled room (14 hour day at 22°C).

The seedlings were aseptically transferred to 25 ml volume tubes containing 5 ml of Jensens agar set on a slope and capped with loose plastic lids, with two seedlings per tube. To determine levels of nitrogenase activity, 125 ml bottles with cotton plugs containing 25

ml of media were used for growth of the seedlings instead of tubes.

48-hour cultures were made from single colonies and overnight dilutions prepared from them as has been described previously (section 2.5). 1 ml of these cultures was spun down and resuspended in 300 ul sterile distilled water. 250 ul of the washed culture were added aseptically to the tube and the plants transferred to the growth room used to grow the seedlings described above. Negative controls consisted of seedlings to which only distilled water was added and 2668 was the positive control. Nodulation by 2668 could be scored after 14 days, but the soil organisms took as long as five weeks. Nitrogen fixation was scored at 3 1/2 weeks (where possible).

It was occasionally necessary to demonstrate that the inoculant strain and the nodulating strain were the same. Nodules were isolated as described in section 2.6. Antibiotic resistance markers, plasmid profile and total genomic DNA profiles were obtained for the isolated strain and compared to the inoculant.

2.8 Microscopic Analysis of Nodule Structure and Chromatographic Analysis of Nitrogenase Activity

It is difficult to estimate the ability of a plant to fix atmospheric nitrogen by direct examination. It is known that acetylene can be reduced to ethylene (ethyne to ethene) by the nitrogenase enzyme and this reaction has been used to quantify the nitrogen fixing ability of the plant-microbe symbiosis.

Plants were placed in bottles and inoculated as described in section 2.7. The plants were grown for three to four weeks before assay. The cotton wool plugs in the bottles were replaced by greased caps. 1 ml of air was removed from each bottle and 1 ml acetylene injected in its place. Samples were taken at 4, 6, 25 and 28 hours. The percent reduction of acetylene to ethylene was measured by analysis of a 100 ul sample through a Poropak T column fitted to a Varian Aerograph series 2700 gas chromatograph (Moduline). A measure of nitrogenase activity was gained by calculating from the calibrated chart printout of the percent conversion of acetylene to ethylene.

In conjunction with the chromatographic analysis of the nodules,

sections of three and a half week old nodules were made and examined by light and electron microscopy. The sectioning and photography (EM) were carried out by Mr. D. Hopcroft and R. Bennett of DSIR (P.N.) and the LM demonstrated by Dr. C. O'Kelly, Dept. of Botany and Zoology, Massey U.

The following is a brief description of the procedure employed to fix, section and examine the nodules (D. Hopcroft, personal communication). Solutions used in this procedure were:

(a). Primary fixative. This solution consisted of 3% glutaraldehyde, 2% formaldehyde in 0.1M Na_2HPO_4 buffer pH7.2.

(b). Phosphate buffer. This solution consisted of 0.1M Na_2HPO_4 buffer pH7.2.

(c). Osmium tetroxide buffer. This solution consisted of 1% OsO_4 in Na_2HPO_4 buffer pH7.2.

(d). Acetone/resin. This solution consisted of a 50/50 mix of acetone (AnalR) and Polarbed S12 resin.

Nodules were excised from the root and fresh tissue was sliced in the primary fixative in a plastic petri dish. The samples were transferred to glass vials and fresh fixative was added. The samples were left to fix for two hours at room temperature. The samples were washed 3 times in phosphate buffer at room temperature and then stained in osmium oxide buffer for 30 minutes at room temperature. The samples were washed 3 further times in phosphate buffer and then dehydrated by a series of acetone/water washes, culminating in 2 washes in 100% acetone. The samples were infiltrated by stirring in an acetone/resin mix on a magnetic stirrer overnight and then transferred to a 100% resin solution for a further 8 hours stirring. Specimens were embedded in fresh resin using silicone rubber moulds and cured at 60°C for 48 hours.

Thin sections were cut using a diamond knife and a Reichert Ultracut E microtome. Sections for light microscopy were placed on slide, covered with coverslips which were sealed onto the slides with clear nail polish. Sections for electron microscopy were picked up on copper grids, double stained with ethanolic Uranyl Acetate followed

by Lead Citrate.

Slides for light microscopy were examined on a Zeiss Standard microscope equipped with apochromatic objective lenses and a Zeiss MP 63 35mm automatic camera system. Photographs were taken on Kodak Technical Pan film (C. O'Kelly, personal communication). Grids for electron microscopy were examined on a Phillips ZOIC transmission electron microscope and photographed with Kodak Fine Grain Positive film.

2.9 Plasmid Characterization by Electrophoresis

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

2.9.1 The solutions for this method were as follows:

Solution 1 contained (g/10 ml 1xTBE): Ficoll 400 (Sigma), 1.0; Bromophenol blue (Sigma), 0.005; RNase, 0.01 ml. The RNase (Amersham) solution was at a concentration of 10 mg/ml in 50/20 TE pH 8 and was treated at 100°C for 2 minutes to remove DNase activity. Immediately prior to use 0.1 ml of a 2 mg/ml solution of egg white lysozyme (Sigma) was added per 1 ml of solution 1.

Solution 2 contained (g/10 ml of 1xTBE): Ficoll 400 (Sigma), 1.0; sodium dodecyl sulfate (Sigma), 0.2.

Solution 3 contained (g/10 ml of 1xTBE): Ficoll 400 (Sigma), 0.5; sodium dodecyl sulfate (Sigma), 0.2.

2.9.2 Single colonies were initially removed from plates and inoculated into 5 ml of TY. This culture was incubated at 28°C with shaking for 48 hours to an A_{600} of 0.8±0.2. A subculture at a concentration of 1/100 in TY was prepared and incubated overnight (18 hours) with shaking at 28°C and grew to an A_{600} of 0.3±0.1. In the case of strains carrying antibiotic resistance markers, the appropriate antibiotic was added to the liquid medium to maintain selection for the strain/plasmid.

0.3 ml of culture was centrifuged at 12,000g for 3 minutes at room temperature in a microfuge (Eppendorf). The cells were washed

once with 1 ml 10/1 TE + sarkosyl and once with 1 ml 10/1 TE. 20 ul of solution 1 were added to each pellet. The pellet was briefly resuspended with the autopipette tip and transferred to the well of an 0.7 % agarose (Sigma) horizontal gel poured at least four hours previously. The transferred cells were incubated **in situ** for 10 minutes. 20 ul of solution 2 were added and the cells stirred gently twice. The well was filled with solution 3. The gel was submerged by filling the tank (12 cm width, 27 cm length, 600 ml capacity) to within 1 cm of the top with 1xTBE and the plasmids electrophoresed at 0.6 V/cm for one hour and then overnight at 3 V/cm at 4 °C.

2.9.3 The gels were removed from the tanks and placed in trays of fresh ethidium bromide (**Care**: carcinogen) at 0.5 ug per ml for 30 minutes and destained in distilled water for 10 minutes. The gel was then examined by ultraviolet light on a transilluminator (UV Products) and photographed at f11 for 1 minute on Kodak Tri-X film through a Wratten 23A (red) filter.

2.10 Restriction Endonuclease Analysis

The present technique used for obtaining sufficient quantities of total genomic DNA for analysis is a modification of that of Scott et al (1984). Restriction enzyme analysis is a very sensitive approach to identification of bacterial strains and clearly shows similarities and differences between test strains.

2.10.1 The solutions used for this procedure were as follows:

(a). Phenol. Phenol (AnalR, BDH) was melted at 70°C and 8-hydroxyquinoline added to 0.1%. The phenol was washed twice with 1M Tris pH8 and twice with 0.1M Tris pH8 + 2-mercaptoethanol. The phenol was tested to ensure pH was 8 and stored a 4°C in a brown glass bottle under 0.1M Tris pH8 + 2-mercaptoethanol. It was stable for about 2 months.

(b). Chloroform. The chloroform solution was actually chloroform (AnalR, BDH) and iso amyl alcohol (AnalR, BDH) in a 24:1 mixture

(c). Lysozyme. Lysozyme was made fresh every time and dissolved in sterile distilled water at 10 mg/ml.

(d). Proteinase K. The proteinase K solution was made fresh

every time and dissolved in sterile distilled water at 12 mg/ml.

(e). Sarkosyl. A solution of N-lauryl sarcosine was made to 60 mg/ml in sterile distilled water and stored at room temperature until required. It was aliquoted into 0.5 ml amounts.

(f). Sodium acetate. 24.6 g of CH_3COONa was dissolved in 70 ml of water and the volume adjusted to 100 ml. Final pH of the solution was 4.5 and concentration 3M.

(g). Loading Buffer (TEGS). This solution contained (ml/10 ml): 1M Tris pH7.5, 0.1; 0.25M EDTA, 0.4; Glycerol (AnalR, BDH), 2; 10% SDS, 0.05. The volume was made to 10 ml and 30mg of bromophenol blue was added. The buffer was stored at room temperature.

2.10.2 A single colony was picked from a plate and inoculated into 5 ml of TY broth. The culture was grown for two days at 28°C with shaking to an A_{600} of 0.8±0.2. A 1/50 subculture was made from this into 10 ml of TY and this was grown overnight at 28°C with shaking to an A_{600} of 0.4±0.1.

The cells were centrifuged at 3,000g for 15 minutes at room temperature in a fixed head centrifuge (Sorvall). The pellets were resupended in 1 ml of 10/1 TE + sarkosyl and centrifuged at 12,000g for 2 minutes at room temperature in a microfuge (Eppendorf). The cells were washed once with 10/1 TE and spun as above.

The cells were resuspended in 0.25 ml of 10/1 TE and 10 ul of fresh lysozyme was added to the suspension. It was then incubated at 37°C for 45 minutes. At this time 10 ul of fresh proteinase K and 10 ul of fresh sarkosyl were added and incubation continued at 50°C overnight.

The cell lysate was vortexed for ten seconds at maximum speed. 0.5 ml of a 50/50 mix of phenol and chloroform was added to each tube of cell lysate and the mix vortexed thoroughly. It was then centrifuged at 12,000g for 15 minutes at RT in a microfuge (Eppendorf). The lysate was transferred to a fresh tube. This process was repeated until the lysate was clear and the phenol removed with a final extraction with chloroform. The final clear supernatant was precipitated with $1/10^{th}$ volume of 3M sodium acetate (pH 4.5) and 2

volumes 95 % ethanol. This was incubated at -20°C for 1 hour and then centrifuged at 12,000g for 20 minutes at 4°C in a microfuge (Eppendorf).

The final pellet was resuspended in 50 µl of distilled water. A 5 ul sample was mixed with 2.5 ul of 1 ug/ml ethidium bromide and spotted onto a sheet of gladwrap placed on a transilluminator. DNA standards at 100, 75, 50, 35, 20, and 10 ug/ml were mixed with ethidium bromide and spotted next to the samples. The entire field was photographed at f4-5 for 1 second on Polaroid 667 Instant film and concentrations estimated by comparison of the standards and the unknown (Ausubel et al, 1988).

2.10.3 DNA digests were carried out in a total volume of 25 µl. 2 ug samples of DNA were digested by restriction enzymes (all BRL) in buffers provided by the manufacturer. Digests were from 2-3 hours, although information received casts doubts on the activity of some enzymes (Crouse and Amorese, 1986) at the end of this time. Table 3 lists the enzymes used. After digestion, 8 µl of loading buffer was added to each sample and it was transferred to the well of a 0.85 % agarose gel (Bio-Rad). The gel was submerged and electrophoresed overnight at 1.5 V/cm in a BioRad DNA Sub Gel™ tank.

The gel was stained and photographed as described in section 2.9.3.

TABLE 3: Restriction endonucleases used. The buffers listed were supplied by the manufacturer of the enzyme.

TABLE 3.

Restriction enzyme	Buffer	Incubation Temp (°C)	Source
Bcl I	2	50	BRL
Eco RI	3	37	BRL
Hin dIII	2	37	BRL

BRL: Bethesda Research Labs. React 2 consists of :50 mM Tris (pH 8), 10 mM $MgCl_2$, 50 mM NaCl. React 3 consists of :50 mM Tris (pH 8) 10 mM $MgCl_2$, 100 mM NaCl.

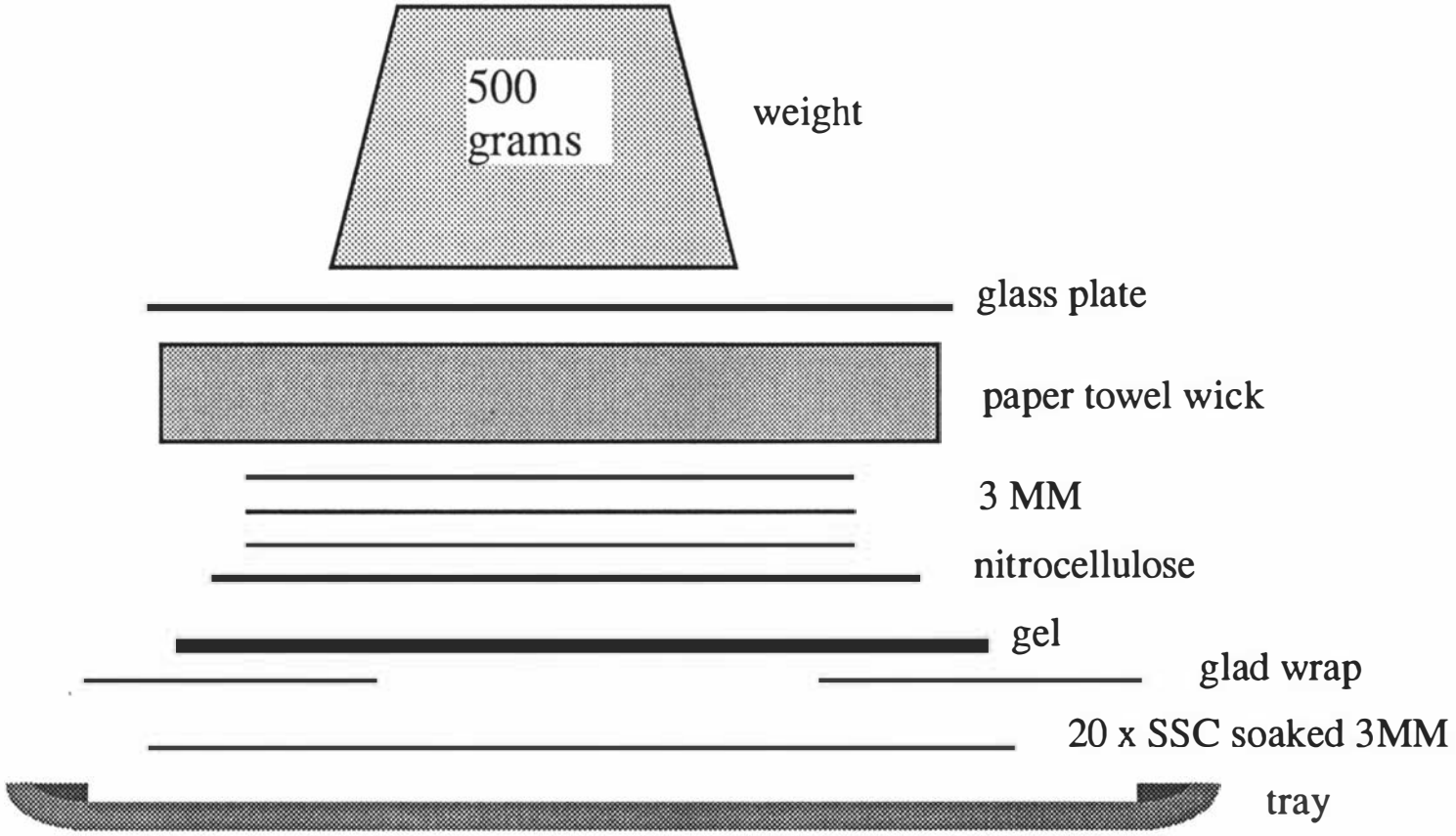


Diagram 2. Assembly of apparatus for Southern blotting (Maniatis et al., 1982).

2.11 Preparation of Gels for Hybridization

Gels were prepared by the method of Southern (1975) with slight modifications to the concentrations of the solutions and times of immersion in the solutions.

2.11.1 The solutions used in this procedure were as follows:

(a). Depurination. The DNA within the gels was depurinated by immersion in 0.25M HCl. This was prepared by mixing 125 ml conc. HCl and 4875 ml water.

(b). Denaturation. The DNA within the gels was denatured by immersion in 0.5 M NaOH 0.5M NaCl. This was prepared by dissolving 100g NaOH and 146.1g NaCl in 4l water. The volume was then made up to 5l.

(c). Neutralizer. The denaturing solution was neutralized by immersing the gel in 0.5M Tris pH7.4 2.0M NaCl. This was prepared by dissolving 302.45g Tris and 584.4g NaCl in 4l water and then adjusting the pH to 7.4 with conc. HCl. The volume was then adjusted to 5l.

2.11.2 Before the gels were removed from the tanks and the run finished, 10 ul of dye marker was added to one track and electrophoresis continued a further 15 minutes. The dye was added to act as a control to ensure the various solutions had totally penetrated the gel. After the gel had been photographed, excess agarose was trimmed away with a scalpel. The gel was then agitated gently in depurinator until the dye changed color and then a further 10 minutes to ensure that adequate depurination had occurred (usually 30 minutes total for Eckhardt gels). The gel was rinsed with distilled water and then the DNA denatured by washing the gel in denaturing solution. The gel was agitated until the dye in the gel returned to its original color and then 10 minutes more to ensure complete denaturation of the DNA (usually 30 minutes total for Eckhardt gels). The gel was again rinsed with distilled water and agitated gently for 1 hour in 0.5 M Tris pH7.4 2 M NaCl. The gel was placed into a tank lined with four pieces of Whatman 3 MM paper which had been wetted to capacity with 20X SSC. The paper was covered with

gladwrap to reduce evaporation, except for a hole slightly smaller than the gel. The gel was placed in the tank and covered with a piece of nitrocellulose cut slightly larger than the gel which had been wetted in 2X SSC. A piece of Whatman 3 MM cut to the same size as the gel was soaked in 2X SSC and placed over the nitrocellulose. This was followed by two dry pieces of Whatman 3 MM and a stack of paper towels to act as a wick. The towels were covered with a glass plate and a 500 g weight placed on top (see diagram 2). The apparatus was left to stand overnight, with the addition of small amounts of 20X SSC to keep the base paper wet.

The following day, the nitrocellulose was removed, rinsed with 2X SSC, placed in an envelope of blotting paper and vacuum dried at 80 °C for two hours. It was then stored until required. The gel was restained in ethidium bromide to ensure that the DNA had in fact been transferred to the nitrocellulose.

2.12 Recovery of DNA Fragments from Agarose Gels

This method was used to directly recover DNA which had been electrophoresed into agarose gels. The method is an adaptation of the technique of Thuring et al (1975).

2.12.1 Solutions used in this procedure were as follows:

(a). TE-equilibrated phenol. Preparation is described in 2.10.1, except 10/1 TE pH8 is substituted for 0.1M Tris + 2-mercaptoethanol.

(b). Chloroform. Preparation is described in 2.10.1.

2.12.2 The DNA to be recovered is electrophoresed normally. A high purity agarose was used (e.g. SeaPlaque, Marine Colloids) to minimize DNA-gel interactions. The fragment was visualized briefly on a normal transilluminator (UV Products) but excised under long wave length ultraviolet. The excised fragment was placed in a 1.5 ml eppendorf centrifuge tube to which an equal volume of TE-equilibrated phenol was added and placed overnight at -20°C.

The sample was centrifuged at 12,000g for 10 minutes at 4°C. A clear layer of electrophoresis buffer formed on the phenol and this was recovered for further purification. The aqueous sample was washed with phenol/chloroform and then chloroform. The DNA was precipitated

as described in section 2.10. Pure fragments of DNA can be quickly obtained this way and are suitable for further use such as DNA probes.

2.13 Rapid Isolation of Plasmid DNA

The rapid boiling method for recovering plasmids from bacteria is well known and most procedures are based on that of Holmes and Quigley (1981). It is suitable for plasmids of up to 60-70 kb, but the efficiency of recovery declines with the increase in size.

2.13.1 The solution used in this procedure was as follows:

(a). HQ-STET contained (ml/100 ml): 1M Tris pH8, 5; 0.25M EDTA, 20; sucrose, 8g. The mix was made up to 95 ml and autoclaved, after which 5 ml Triton-X100 was added aseptically. Final concentrations were 50 mM Tris pH8 50 mM EDTA 8% sucrose 5% triton-X100.

2.13.2 A colony of cells containing the plasmid of interest was inoculated into 3 ml of LB and grown 18 hours with shaking at 37°C (cell number is not critical). 1-4 ml of this culture were spun down in a bench microfuge and resupended in 350 µl of HQ-STET buffer. To the resuspended cells 25 µl of freshly made 10 mg/ml lysozyme were added. The cells were placed in a boiling water bath for 40 seconds and then spun for 10 minutes in a microfuge. The pellet of chromosomal and cellular debris was removed and the plasmids remaining in the supernatant precipitated with 500 ul isopropanol at -20°C for 10 minutes and then spun 10 minutes in a microfuge (Eppendorf). The DNA pellets were washed once with 70% ethanol, dried under vacuum for 30 minutes and resuspended in 50 ul of distilled water.

A more pure product could be obtained by phenol purification before precipitation, as was described for total DNA isolation (section 2.10).

2.14 Preparation of Radioactively Labelled DNA Probes

Radiolabelled DNA was produced by random priming of the probe sequence. The method used was modified from that of Whitfeld *et al* (1982). The probes used in part I were a 5.2 kb *Eco*RI fragment of PN 435 carrying *nifKDH* and a 7.2 kb *Eco*RI fragment of PN 291 carrying

nodABC. Whole plasmid was digested as described in 2.10 except all volumes are doubled. High-purity agarose gels (Seakem analytical grade) were loaded with digested probe, electrophoresed and stained. The bands were dissected from the gel as discussed in 2.12. The probes used in part II were not isolated fragment probes, except where indicated otherwise.

The probe DNA was incubated at 37°C for 45 minutes with the enzyme Hae III. 4 µl of random primers at 50 mg/ml (obtained from Prof. D.B. Scott) was added and the mix incubated in a boiling water bath for 2 minutes and then cooled on ice for 30 seconds. The ingredients for the radiolabelling reaction were added as follows: distilled water, 2.5 µl; react 2(DNA digest buffer, BRL), 1.5 µl; 20 mM dATP, dTTP and dGTP (BRL), 1 µl; ^{32}P dCTP (10 mCi/ml, New England Nuclear), 3 µl; Klenow fragment of DNA polymerase (Boehringer-Mannheim), 2 units. The reaction mix was incubated at 37°C for one hour and stopped by the addition of 2 µl of 250 mM EDTA.

The crude probe was extracted with phenol/chloroform (described in section 2.10) and the phenol/chloroform back extracted with water until the radioactivity was gone. The aqueous phases were all combined (usually to a volume of 250 ul) and ethanol precipitated as described in section 2.10. The dried pellet of radioactively labelled DNA was resuspended in 100 µl of water, incubated for 2 minutes in a boiling water bath, cooled on ice and added to the bag containing the blot as described in the following section (2.15).

2.15 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).

2.15.1 The solution used in this procedure was as follows:

(a). 10X Denhardts' solution was used to prepare the nitrocellulose for hybridization and contained (amount/500 ml): 1M

Hepes (Sigma) pH 7.0, 25 ml; 20 x SSC, 75 ml; 3 mg/ml herring sperm DNA Sigma, (phenol purified), 3 ml; 10 mg/ml E.coli tRNA (Sigma), 1 ml; 20 % sodium dodecyl sulfate (Sigma), 2.5 ml; Ficoll 70 (Sigma), 1g; bovine serum albumin (Sigma), 1g; polyvinylpyrrolidine-10 (Sigma), 1g. The solutions and solids were added to 300 ml sterile distilled water and the volume adjusted to 500 ml.

2.15.2 Nitrocellulose filters were prehybridized for two hours in 25 ml of 10X Denhardts at 60 °C in a sealed plastic bag. At the end of this time all except 2 ml of the Denhardts was poured out and 100 ul of freshly boiled probe was added and the bag re-sealed. The bag was placed in a shaking water-bath and incubated overnight at 60 °C.

The probe mix was decanted and the filter removed and washed three times in 200ml of 2X SSC at 60 °C and then once in 0.5X SSC at room temperature (about 25°C). The filter was air dried and placed on a piece of Whatman 3 MM paper and wrapped in gladwrap. The blot was placed in a cassette with a sheet of Kodak X-ray film and an intensifying screen on both top and bottom. The cassette was stored overnight at -20 °C. The film was developed in an automatic X-ray developing machine (Kodak). If this length of exposure was insufficient to adequately visualize the level of hybridization of the radiolabelled DNA, a fresh piece of film was added and the cassette re-exposed for a longer period of time.

2.15.3 It was of interest to reprobe some of the filters and for this purpose it was necessary to remove the hybridized radioactivity. The filters were washed for 20 minutes in 20 mM sodium hydroxide, followed by 15 minutes in neutralizer (section 2.11) and finally 15 minutes in 2 x SSC. The washed membrane was tested to ensure the previous probe had been completely removed by overnight exposure with a fresh film. The filter was then treated for DNA hybridization as previously described in 2.15.2.

DISCUSSION OF RESULTS

3.0 Perspective

This section is a presentation of the results obtained from an examination of the genomic rearrangement of Rhizobium leguminosarum biovar trifolii 2668 and an interpretation of what these results might mean in the context of genomic stability. A more general discussion of this work in relation to analyses reported in the literature will be presented in the Conclusions.

The initial aim of part I was to use starvation (in the form of recalcitrant carbon and nitrogen sources) of inoculant rhizobia in an attempt to induce a stress response like that described by Heumann et al (1983; 1984) as a possible explanation for previously observed variability among field isolates (O'Hara, 1985). The use of recalcitrant carbon and nitrogen sources was chosen from several stress procedures listed by Heumann et al (1983; 1984) as it was simple, and of the treatments used in Heumann 's studies, most similar to what might occur in the soil. No genomic rearrangements were detected as a result of the treatment but a rearrangement event detected after the introduction of the RP4 was chosen for further characterization. In terms of explaining variation in soil isolates, the rearrangement was felt to have some relevance as RP4 has been identified in soil bacteria (Datta and Hedges, 1972) and is known to induce rearrangements (Berry and Atherly, 1984). Diagram 3 summarizes the experiments of part I.

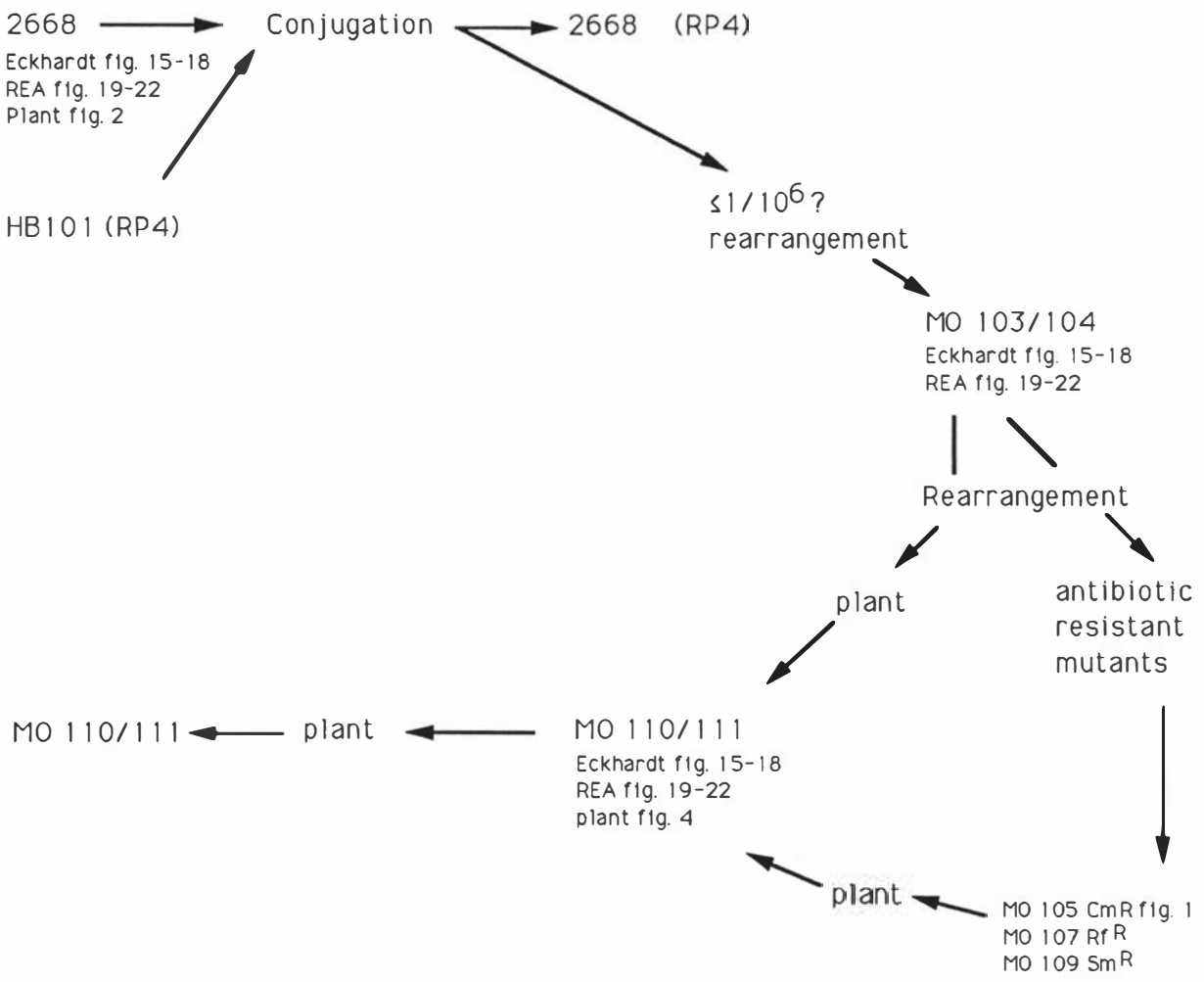


Diagram 3: Summary of the experiments performed in part 1.

The aim of part II was to survey the ability of pSym to transfer and be expressed in a variety of hosts. A number of previous studies have examined the effect of a particular pSym in different rhizobial species as well as A. tumefaciens (Johnston et al, 1978; Hooykaas et al, 1981; 1982; Martinez et al, 1987; Espuny et al, 1987) but only a few studies have looked further afield in terms of variety of potential recipients (Plazinski and Rolfe, 1985: Jarvis et al, 1989) and these have used mobilizing vectors to facilitate the transfer of pSym. Soil isolates obtained from Dr. B.D.W. Jarvis were used as the initial recipients, both because of the success of his study (Jarvis et al, 1989) and to maintain some relevance to the soil situation. Secondary transfers from these strains were performed as attempts to further characterize the pSym host range and to provide further evidence for the plausibility of the soil gene pool hypothesis advanced by Reanney et al (1983), at least under laboratory conditions. Diagram 4 summarizes the experiments of part II.

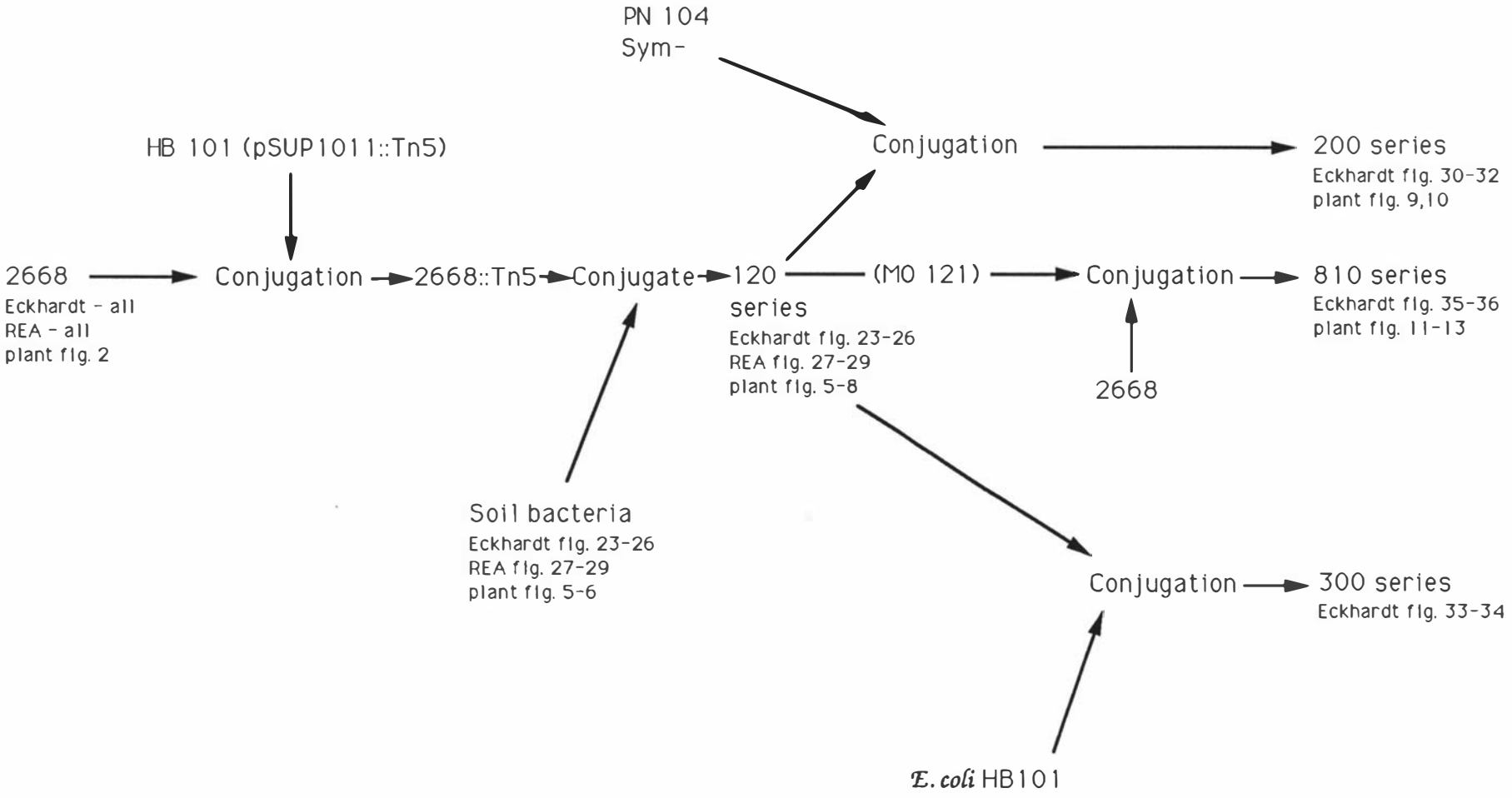


Diagram 4: Summary of the experiments performed in part II.

3.1 Selection of Strains

R. leguminosarum biovar trifolii 2668 was chosen as the strain to be subjected to stress and as donor of pSym as it was the inoculant strain in the field sampled in the earlier study (O'Hara, 1985) and is currently the standard inoculant strain for pasture production in New Zealand.

The cross between 2668 and HB101(RP4) was performed on TY and exconjugants selected on S10.Nm. The minimal medium S10 will not support the growth of HB101 and neomycin selects for the RP4 replicon. The frequency of transfer of RP4 to 2668 was $2X10^{-3}$ which is comparable to that observed for similar crosses by other investigators (Beringer et al, 1978; McLaughlin and Ahmad, 1986). Of the exconjugants examined, one of the 8 colonies examined in trial 1 showed signs of rearrangement of the plasmids of 2668. No further rearrangements were observed in the 16 colonies examined from trial 2, nor the 24 colonies of trial 3, nor the 4 of trial 4. It is impossible to set a frequency on the rearrangement observed from this data but loss of DNA from pSym was observed by Soberon-Chavez and Najera (1989b) to occur at a frequency between 1% from growth at the normal 30°C and 65% at the elevated temperature of 37°C. Spontaneous rearrangement of Staphylococcus aureus was observed at a frequency of 5×10^{-6} by Gennaro et al (1987). Higher frequencies of rearrangement under certain conditions have been reported (Soberon-Chavez et al, 1986; Palacios et al, 1987). The variation in frequency of rearrangement reported in the literature makes it difficult to even guess at a figure. We would suggest that frequency is 10^{-6} or lower because of the rarity of observed spontaneous variation in the cultures from this part of the study over long time periods.

The soil bacteria NR 41, NR 42, NR 64 and OR 168 were obtained as spectinomycin resistant mutants from Dr. B.D.W. Jarvis (Jarvis et al, 1989). These soil-isolated bacteria were adapted to laboratory growth media (TY, YEM) and temperature (30°C) when received.

The exconjugants from the cross between 2668 and PN 302 (HB101[pSUP1011::Tn5]) arose at a frequency of $5X10^{-3}$, which is 20-

100 times as higher than other investigators observed between species (Simon et al, 1983; Christensen and Schubert, 1983). It is unclear why such a high frequency of transfer of Tn5 was observed, as the conditions of the cross are very similar to those used by Christensen and Schubert (1983). It is possible that R. leguminosarum biovar trifolii 2668 is a good recipient.

The symbiotic plasmid of 2668 is approximately 190kb and the other plasmids sum to about 1160kb (Harrison et al, 1988) while the chromosome is about 5200kb (Crow et al, 1981), thus pSym is about 1/32nd of the genome and 2668::Tn5 plasmid inserts should occur in about 1 of every 32 colonies, assuming a random distribution of insertion sites. Rather than isolating a Tn5-marked pSym, a plate with sufficient colonies to contain at least one pSym::Tn5 (50-100 colonies) was resuspended in S10.Neo liquid medium and grown overnight at 28°C with shaking. 0.1 ml of this stock was plated on L.Kan to ensure there were no residual PN 302 bacteria present. This stock was used as described in section 2.5 as the pSym::Tn5 donating parent in further crosses to the selected soil bacteria.

The frequencies of the exconjugant strains from the various crosses are compared to frequencies observed in similar studies by other workers. The frequency of Neo^R soil bacteria was $3X10^{-4}$ for 2668::Tn5 X NR 41 and NR 42, $1X10^{-4}$ for 2668::Tn5 X NR 64 and $7X10^{-5}$ for 2668::Tn5 X OR 168. The symbiotic plasmid has been shown to transfer at a frequency of between 10^{-2} (Johnston et al, 1978; Soberon-Chavez and Najera, 1989b) and 10^{-4} (Brewin et al, 1980; Wang et al, 1986) to Rhizobium and Agrobacterium respectively. Frequency of transfer of the symbiotic plasmid to the "non-symbiotic" R. leguminosarum isolated from the soil was not reported (Soberon-Chavez and Najera, 1989a) and both Plazinski and Rolfe (1985) and Jarvis et al (1989) used broad host range mobilizing plasmids to transfer pSym. Crossing of the genus barrier by broad host range plasmids such as RP4 occurs at between 10^{-1} (Schilf and Krishnapillai, 1986) and 10^{-6} (Datta and Hedges, 1972) depending on the actual donor and recipient, so these figures (if the soil bacteria are of a different genus and

pSym a broad host range plasmid) are plausible. The exconjugant bacteria, one colony of which was designated the 120 series isolate for that cross, where NR 41 X 2668::Tn*5* produced MO 121 (fig.23, lane d), NR 42 X 2668::Tn*5* produced MO 122 (fig. 23, lane f), NR 64 X 2668::Tn*5* produced MO 123 (fig. 23, lane h) and OR 168 X 2668::Tn*5* produced MO 124 (fig. 23, lane j), were selected for and maintained on TY.Sp.Nm. The stability of these strains varied, with differences in plasmid profile appearing during subculture (discussed in section 3.6).

The cross between the 120 series isolates (MO 121-124) and the $pSym^-$ rhizobia PN 104 was an attempt to return the symbiotic plasmid to a rhizobial background and observe the effects of passage through an alternative host. The frequency of transfer was $4X10^{-2}$ for PN 104 X MO 121, $3X10^{-2}$ for PN 104 X MO 122 and MO 123 and $3X10^{-3}$ for PN 104 X MO 124, which is comparable to pSym transfer frequencies among rhizobia (Johnston *et al*, 1978; Christensen and Schubert, 1983) including *Rhizobium* (*Hedysarum coronarium*) to *R. leguminosarum* biovar *trifolii* transfers (Espuny *et al*, 1987). The 200 series strains, where one exconjugant colony of each cross was designated the 200 series isolate for that cross, where MO 121 X PN 104 produced MO 201 (fig. 30, lane c), MO 122 X PN 104 produced MO 202 (fig. 30, lane e), MO 123 X PN 104 produced MO 203 (fig. 30, lane g), MO 124 X PN 104 produced MO 204 (fig. 30, lane i), were selected for and maintained on S10.Rif.Nm and were relatively stable on subculture.

The cross between the 120 series and *E. coli* HB101 was an attempt to determine how far taxonomically pSym could be transferred and was also designed to get the reduced derivative into a host from which it could be readily extracted and characterized. The frequency of the Km^R *E. coli* HB101 was $5X10^{-3}$ for HB101 X MO 121, $3X10^{-3}$ for HB101 X MO 122, $2X10^{-3}$ for HB101 X MO 123 and $9X10^{-4}$ for HB101 X MO 124 and one colony of each of the progeny labelled respectively MO 301 (fig. 33, lane f), MO 302 (fig. 33, lane g), MO 303 (fig. 33, lane h) and MO 304 (fig. 33, lane i). The only previous study in which symbiotic genes were transferred to *E. coli* used cloned subsets

of the symbiotic genes such as nodABC in a mobilization vector and is not therefore comparable. This frequency is similar to the initial frequencies of transfer of the Sym-derivative to generate the 120 series. The E. coli 300 series strains were selected and maintained on Luria.Sm.Nm and appeared stable on subculture, even without antibiotic selection.

The cross between MO 121 (NR 41 X 2668::Tn5) and MO 800 (2668 Sm^R) was done in order to determine whether the smaller pSym of MO 121 was compatible with the full size version in 2668. The frequency of generating Nm^R MO 800 strains was $1X10^{-3}$, similar to that seen with the crosses yielding the 200 series. Eight isolates from the cross were examined and all had the same plasmid profile. The first four are shown (MO 810-813, fig. 35, lanes g-j). The 810 series strains were maintained on TY.Rif.Nm and by the criteria of plasmid profile, were stable under these conditions.

3.2 Identification of Soil Bacteria

The soil bacteria were isolated from a white clover-ryegrass paddock and had only been identified as gram negative rods unable to nodulate clover (but with homology to 2668). An attempt was made to further identify these organisms by subjecting them to a standard series of biochemical tests.

A motility test showed all four strains to be motile and they grew at 22°, 25°, 30° but not 37°C. The strains NR 41 and NR 64 show no color on TY medium, OR 168 was faintly yellow and NR 42 showed a faint green color in TY broth. Colony forms of NR 41 and NR 42 were punctate circles, with flat tops and entire edges. The colony surface was smooth and opaque with a butyrous consistency. OR 168 was similar, except the colonies were very small and faintly yellow. NR 64 was larger (1-3mm), irregular and raised with undulate edges and a smooth, glistening surface. The colonies were very mucoid and sticky. Descriptive terms were from a manual on identification compiled at Wallaceville Animal Research Centre. The biochemical tests on the API 20NE, as well as several supplementary tests such as litmus milk, gas production in sugars and sugar alcohols and growth on a variety

of solid media were performed. Incubation and inoculation of the strips was as suggested in the kits: a single colony was resuspended in the diluent provided and this was used to inoculate the cupules. Tests were read at 48 hrs, except were specified by the kit manufacturer. Supplementary tests were inoculated by a loopful of cells and read at 48, 72 and 96 hours. All strips and solid tests were incubated at 30°C under humidified conditions. All liquid tests were incubated at 28°C with shaking. The results of the tests are shown in table 4.

The key that accompanies the API 20NE lists a number of species and the various sugars and other compounds that comprise the test. With each test and for each species there is a percentage of strains of that species which are positive for that test after 24-48 hours incubation at 30°C. It promised to be extremely difficult to get a clear-cut identification as most standard tests relate to bacteria that are pathogenic for humans or domestic animals. A comparison of the soil bacteria to the chart showed that there was some degree of similarity to some of the organisms listed in the key.

TABLE 4. Biochemical analysis of the soil strains NR 41, NR 42,, NR 64, OR 168 and the inoculant 2668.

KEY

+ = positive reaction/growth/utilization. - = negative reaction/no growth/utilization. +/- = weak positive reaction/growth/utilization. a = acid produced. al = basic compounds produced. g = gas produced. p = peptonization of protein. ONPG and PNPG (ortho and para nitrophenyl β-D galactopyranoside) = β galactosidase produced. NAG = N acetyl glucosamine. PAC = phenyl acetate. nd = not done.

Other tests:

Nitrate: R = reduced, NR = not reduced to nitrite/nitrogen. EMB = eosin methylene blue. BG = brilliant green. SS = Salmonella/Shigella agar. These last three and MacConkey are tests for coliform-type organisms, with MacConkey and SS being much more specific and discriminating. O/F = oxidative or fermentative metabolism. The sugars are positive if they will support growth as sole carbon and energy sources.

TABLE 4.

TEST	2668	41	42	64	168
Gram reaction	-ve	-ve	-ve	-ve	-ve
Form	rod	rod	rod	rod	rod
An/aerobe	aerobe	aerobe	aerobe	aerobe	aerobe
Motility	yes	yes	yes	yes	yes
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+/-
ONPG	-	-	-	+	-
PNPG	+	+	+	+	+
Deaminase	+	+	+	+	+
Citrate sole carbon source	+	+	+	+	+
H_2S produced	-	-	-	-	-
Urease	-	-	-	-	-
Indole	-	-	-	-	-
V.P.	-	-	-	-	-
Gelatin	-	-	-	-	-
Litmus Milk	al/p	al/p	al/p	al/p	al/p
Nitrate	NR	NR	NR	NR	NR
O/F	O	O	O	O	O
MacConkey	-	-	-	-	-
SS	-	-	-	-	-
EMB	+	+	+	+	+
BG	+	+	+	+	+
S10	+	-	-	-	-
Amygdalin	nd	-	-	-	+
Adipate	-	-	-	-	-
Arabinose	nd	+	+	+	+
Caprate	-	-	-	-	-
Gluconate	+	-	-	-	-
Glucose*	+	+,g,a	+,g	+	+
Inositol*	nd	-	-	-	-
Lactose*	+	+,g	+,g	-	-
Malate	+	+	+	-	+/-
Maltose	+	+	+	+	+
Mannitol*	+	+	+	+	+
Mannose	+	+	+	+	+
Melibiose	nd	+	+	+	+
NAG	+	+	+	-	+/-
PAC	+	-	-	-	-
Rhamnose	nd	+	+	+	+
Sorbitol	nd	-	-	-	-
Sucrose	-	-	-	-	+

Only those sugars marked * were tested for acid and gas production.

2668 is very similar to OR 168, with the exception that 2668 will grow on lactose and PAC and OR 168 will not but does grow on sucrose which 2668 will not. The other strains show some differences in sugar utilization, which especially considering the discussion of cryptic genes and other rearrangements in section 1 cannot be regarded as definitively differentiating characteristics. Initial identification as Pseudomonas paucimobilis and Flavobacterium multivorum was probably erroneous and was due to attempting to use the criterion of best fit rather than a definitive identification. While it is likely that the soil bacteria are members of a known species, we do not have a sufficiently large data base to accurately identify them. There is some degree of DNA homology to 2668, but this hybridization data was based on a dot blot and is qualitative rather than quantitative (Jarvis et al, 1989). Reinterpretation of the biochemical test data has made two things clear. Firstly, one must be very careful using biochemical data that does not fit exactly. If two test profiles match exactly then there is a high probability that the two isolates are the same, but any variation reduces this probability dramatically. Secondly, although the information regarding biochemical tests on soil bacteria (as opposed to potential pathogens) may exist in the literature or in laboratories it is not readily available to be quickly used as a data base for identifying these soil bacteria.

In terms of the soil bacteria NR 41, NR 42, NR 64 and OR 168, the three most similar bacterial strains listed on the key or in the texts were Pseudomonas paucimobilis, CDC V E1 and 2668. The hybridization studies and the results of biochemical tests and colony morphology, although far from conclusive, suggest that these strains could be a species of Rhizobium lacking a Sym plasmid, as isolated and described by Soberon-Chavez and Najera (1989b). This remains speculative at present and the data available is not sufficient to properly identify the soil isolates. A comparison of the total genomic DNA restriction endonuclease (fig. 27) and plasmid (fig. 23) profiles of the four soil bacteria suggest that they are four

different strains, not four isolates of the same strain.

3.3 Antibiotic Sensitivity and Resistance

To isolate bacterial antibiotic resistant mutants approximately $5X10^7$ cells were plated on the selective medium. Mutants were isolated and treated as described in section 2.1. Antibiotic resistance appeared to be stable, subcultures maintained under non-selective conditions still displayed the appropriate resistance/s when tested. No obvious changes in the genome were detected as a result of selecting antibiotic resistant mutants.

Abbreviations used in the course of this project are as follows: **Ap** is ampicillin, **Cm** is chloramphenicol, **Km** is kanamycin, **Nm** is neomycin, **Nal** is nalidixic acid, **Rif** is rifampicin, **Sp** is spectinomycin, **Sm** is streptomycin and **Tc** is tetracycline. Unless stated, all concentrations are 50 ug/ml.

Sensitivities of the various strains used in this project are shown in table 5. Resistance level varied, with 2668 being highly sensitive to all antibiotics except Nal. The soil bacteria had relatively high intrinsic levels of resistance to Ap and Nal. Other resistances were as would be expected from mutations selected and markers known. MO 123 and 124 (and their parents NR 64 and OR 168) were very resistant to rifampicin, something not appreciated until after the 120 series X PN 104 cross. Although rifampicin was used to select against the donor strains (the 120 series) in this cross, the 120 series X PN 104 cross is not invalidated, as S10 still selects against the soil bacteria (table 4) and the plasmids of the 200 series include the two from PN 104 (fig. 30, lane j). High levels of resistance to Ap was observed in the soil bacteria, the 120 series, in MO 204 but not PN 104 and in the 300 series but not in HB101. It is unclear where the resistances seen in MO 204 and the 300 series arose, but they are not relevant to the theme of observed change in plasmid structure.

TABLE 5A. Levels of antibiotic resistance of the strains of bacteria generated in this project. All concentrations are in ug/ml and plates were made as described in section 2.3. Approximately 5 $X10^7$ cells were spotted on the plate and growth/no growth was scored after two days. Resistance to neomycin was assumed to imply resistance to kanamycin in this analysis and the ability of kanamycin to select for the pSym derivative in E. coli would suggest that they do cross react in this case.

Note: The RP4 plasmid is carried by HB101 and PN 302 is pSUP1011::Tn5.

TABLE 5B. Frequencies of isolation of the various antibiotic resistant mutants after plating 4±2 $X10^8$ cells on selective media.

TABLE 5A.

STRAIN	Ap	Cm	Nal	Nm	Rif	Sp	Sm	Tc
2668	6	<3	12.5	<3	<3	6	<3	<3
MO 103	50	6	12.5	50	3	3	3	50
MO 104	50	6	12.5	50	3	6	3	50
MO 110	25	3	12.5	6	3	6	3	3
MO 111	50	3	12.5	6	3	6	3	3
RP4*	50	3	3	50	<3	<3	50	50
PN 302	6	50	<3	50	3	50	<3	
NR 41	50	3	50	6	<3	50	<3	
MO 121	50	3	50	50	<3	50	<3	
NR 42	50	3	25	3	3	50	12.5	
MO 122	50	3	25	50	3	50	12.5	
NR 64	25	6	50	3	50	50	6	
MO 123	50	6	50	50	50	50	6	
OR 168	12.5	<3	12.5	<3	50	50	<3	
MO 124	50	<3	12.5	50	50	50	<3	
PN 104	3	<3	<3	<3	50	3	50	
MO 201	6	<3	<3	50	50	3	50	
MO 202	<3	<3	<3	50	50	3	50	
MO 203	6	<3	<3	50	50	3	50	
MO 204	25	<3	<3	50	50	3	50	
MO 300	12.5	<3	<3	50	50	<3	50	
MO 301	50	<3	<3	50	50	<3	50	
MO 302	50	<3	<3	50	50	<3	50	
MO 303	50	<3	<3	50	50	<3	50	
MO 304	50	<3	<3	50	50	<3	50	
MO 800	25	<3	12.5	<3	6	3	50	
MO 810	50	<3	12.5	50	6	3	50	
MO 811	50	<3	12.5	50	6	3	50	
MO 813	50	<3	12.5	50	6	3	50	

TABLE 5B. Frequencies of appearance of antibiotic resistant mutants.

STRAIN	DERIVATION	FREQUENCY OF ISOLATION
MO 800	Sm^R 2668	$2X10^{-7}$
MO 105	Cm^R MO 104	$4X10^{-7}$
MO 107	Rif^R MO 104	$1X10^{-7}$
MO 109	Sm^R MO 104	$6X10^{-8}$
MO 300	Rif^R HB101	$6X10^{-7}$

Frequencies of isolation of the various antibiotic resistant mutants are shown in table 5B. The antibiotic resistance of the soil bacteria to spectinomycin and of PN 104 to streptomycin and rifampicin were confirmed. Antibiotic resistant mutant isolation frequencies are slightly higher than others published for Rhizobium (Espuny et al, 1987; 1989). Strains were maintained on selective media.

Initially the three antibiotics chosen to act as markers for MO 104 (Cm for MO 105, Rif for MO 107 and Sm for MO 109) were thought to have little or no effect on nodulation effectiveness (Schwinghammer, 1967). There appeared to be some effect of antibiotic resistance on nodulation efficiency of some of the strains (discussed in 3.4)

3.4 Plant Tests and Isolation from Nodules

In each case approximately 10^8 cells were inoculated per tube and the tube incubated as described in section 2.5. Nodules appeared on the 2668 inoculated plants after about two weeks, but took as long as five weeks with some strains. Each trial consisted of two tubes per inoculant and each tube contained two plants. Generally from two to five nodules formed on each plant, but positive nodulation was scored as 1 nodule on 1 plant in either of the replicate tubes. Nitrogen fixation efficiency was not corrected for the number of nodules, it was based on that particular symbiotic association. For example, if there was only one nodule in a bottle the acetylene reduction efficiency is based on fixation by that nodule and if more nodules increased the efficiency then it was taken into account only in the increased level of acetylene reduced.

The nodules that formed were of two types in general appearance with those of 2668, the 200 and the 800 series being typically well-formed, rounded, large and pink-red. Those of MO 103/104, 110/111 and the 120 series were smaller and only slightly pink. It was commented by the electron microscopist (Mr. D. Hopcroft, DSIR, Palmerston North) that this was atypical for biovar trifolii and that it made this set (MO 103/104, 110/111, 120 series) difficult to section. Plants were often allowed to grow for periods of approximately two

weeks after nodules appeared to allow them to increase sufficiently in size to allow them to be readily fixed and sectioned. As a consequence the nodules may have begun to senesce and the decay and disruption observed in some electron micrographs may be due to senescence rather than characteristic of the particular plant-bacteria symbiosis under examination.

Reisolation of strains from nodules was performed a number of times as described in section 2.6. Inoculation of plants with either MO 103 or 104 or the mutants MO 105, 107 or 109 resulted in the isolation of MO 110/111-type strains (26 nodules in four trials, fig. 1). Inoculation with MO 105 (Cm^R), 107 (Rif^R) or 109 (Sm^R) resulted in the production of MO 110/111-type strains with the antibiotic resistances appropriate to the inoculant. For example, Cm^R MO 110/111-type nodule re-isolates were recovered from MO 105 inoculants. The appropriately marked nodule reisolates were observed in both trials in which antibiotically marked inoculants were used. Strains isolated from nodules inoculated with MO 110/111-type strains were also MO 110/111-type in the four nodules from two trials in which this inoculation was tested. Thus, this profile is stable on plant passage so far.

The various nodules generated by the strains discussed above were at least partly effective (table 6a and 6b, see sections 3.5 and 3.6) and nitrogen was fixed by all the strains. In part I, some variability was noticed in the four trials. 2668 nodulated 4 times in 4 trials, MO 103, 104, 110, 111 all nodulated 3 times in 4 trials. The antibiotically marked strains MO 105, 107 and 109 had a reduced nodulation efficiency (table 6a) and in the case of MO 109, this was seriously reduced to but one of the four nodulation trials being a success. There is some evidence to suggest streptomycin resistance affects the ability of a strain to nodulate red clover (Zelazna-Kowalska, 1971) and our results suggest that in strain MO 109 nodulation of white clover is also inhibited. Not all streptomycin resistant strains were inhibited, MO 800 (2668 Sm^R) and PN 104 (Rif^R Sm^R) nodulated satisfactorily in the trials in which they were used

and the poor nodulation of MO 109 may have nothing to do with streptomycin resistance, although there are no other known differences between MO 104 and MO 109. Spectinomycin resistant mutants are known to have the nodulation effectiveness reduced, but not all spectinomycin resistant mutants exhibit this inhibition (Schwinghammer and Dudman, 1973). Another factor which can affect nodulation efficiency is the presence of RP4 (Hynes and O'Connell, 1988).

The nodules of plants inoculated with 2668 appeared healthy and nodule sections showed normal development (fig. 2a). Electron micrographs showed normal bacteroids within the plant cells and the presence of other structures associated with nodules, such as mitochondria (fig. 2b). Similarly, the nodule formed by MO 105 (MO 104 Cm^R) showed normal development from plant cortical tissue (fig. 3a) and well-differentiated bacteroids in the plant cells (fig. 3b). By contrast, the nodule formed by MO 110 was white and small, with a disrupted and decaying internal structure (fig. 4a). An examination of sections from this nodule by EM showed disrupted and senescing nodules, suggesting this nodule was either too old when isolated or perhaps was damaged when removed (fig. 4b) or that there was a problem with the symbiosis. The difficulty often arose that some strains would nodulate well before others and the line between healthy and senescent was occasionally very sharp.

A comparison of nitrogen fixing ability of the strains derived in part I is shown in table 6a. None of the derivatives fix nitrogen as well as 2668 and the antibiotic resistant mutants of MO 104 are particularly poor. Generally, MO 103/104 and MO 110/111 were 60-80 % as effective as 2668, where nitrogen fixation effectiveness is measured as acetylene reduced per bottle.

There is insufficient evidence to conclude whether the poor nodulation and nitrogen fixation of MO 105, 107 and 109 is significant at this time.

Figure 1. Composite picture showing the nodule re-isolates, designated as MO 110/111-type, from plants inoculated with MO 103, 104, 105 and 107. Tracks: a=2668, b=MO 103, c=MO 104, d=MO 110, e and f=MO 110/111-type (MO 103 inoculant), g and h=MO 110/111-type (MO 104 inoculant), i and j=MO 110/111-type (MO 105 inoculant), k and l=MO 110/111-type (MO 107 inoculant).

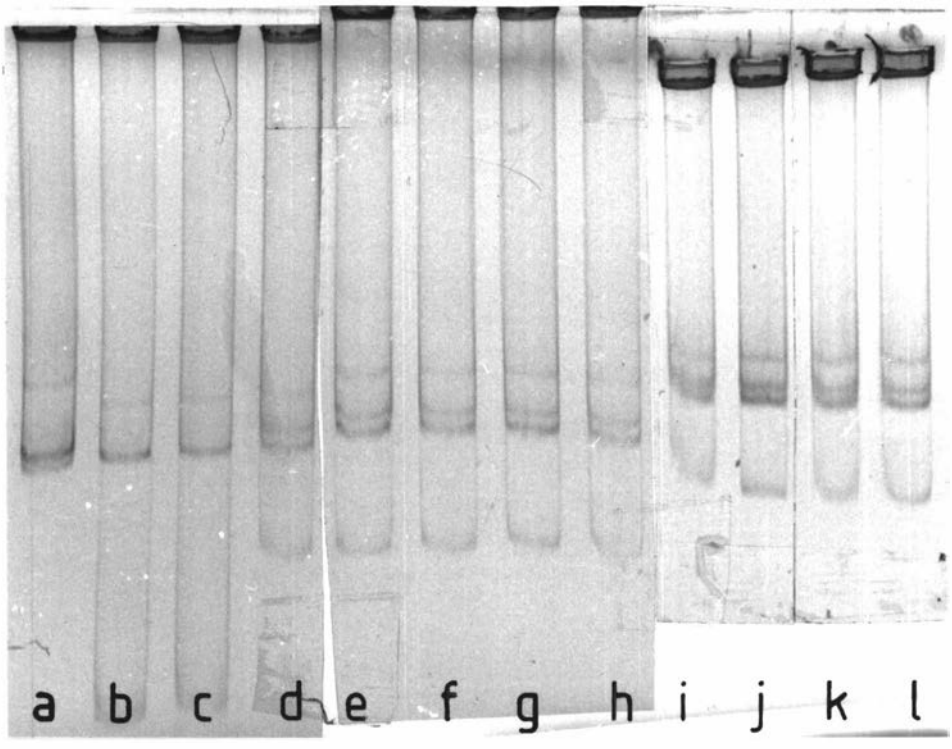

a b c d e f g h i j k l

TABLE 6a: Nodulation and Nitrogen Fixation Status Strains Derived in Part I.

All percentages of nitrogen fixed are in relation to (c.f.) 2668.

nd=not tested.

1. Scott and Ronson, 1982.

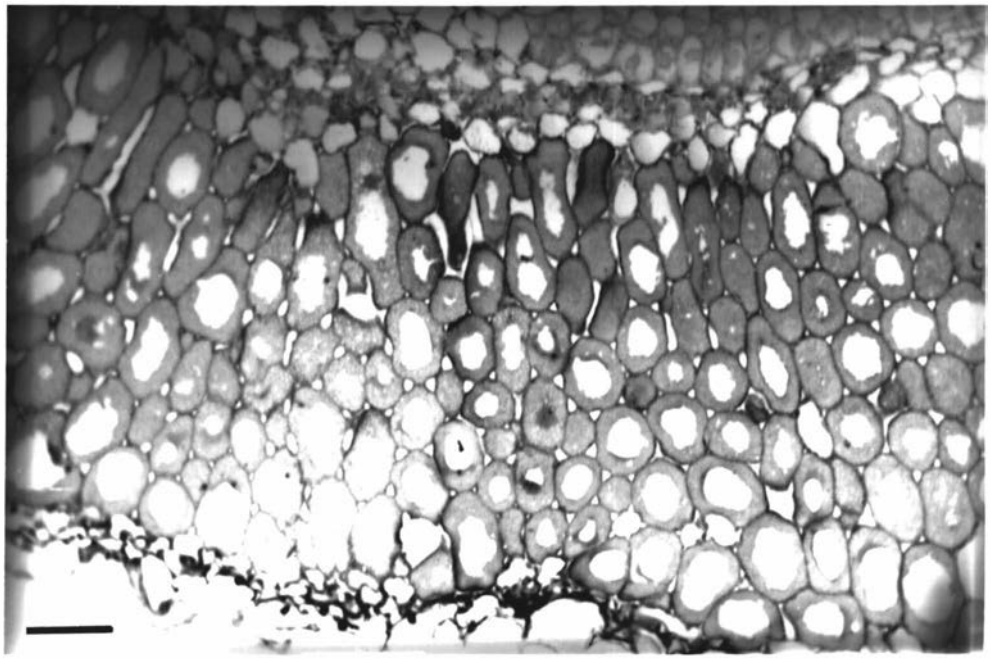
TABLE 6a.

STRAIN	STATUS	TRIALS NOD$^+$	%N FIXED c.f. 2668		DERIVATION
			TRIAL1	TRIAL2	
2668	Nod$^+$	4/4	100	100	inoculant
uninoc^d	Nod$^-$	0/4	0	0	sterile water
PN 104	Nod$^-$	0/4	0	0	Sym$^-$ mutant (1)
MO 103	Nod$^+$	3/4	71	77	this study
MO 104	Nod$^+$	3/4	59	62	this study
MO 110	Nod$^+$	3/4	65	0	this study
MO 111	Nod$^+$	3/4	69	63	this study
MO 105	Nod$^+$	2/4	nd	39	this study
MO 107	Nod$^+$	2/4	nd	44	this study
MO 109	Nod$^+$	1/4	nd	9	this study

Figure 2. Light and electron micrographs of nodule sections taken from a plant inoculated with 2668.

2a. Light micrograph, x160. Size bar is 70 um.

2b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, cw=cell wall, m=mitochondrion, v=vacuole.



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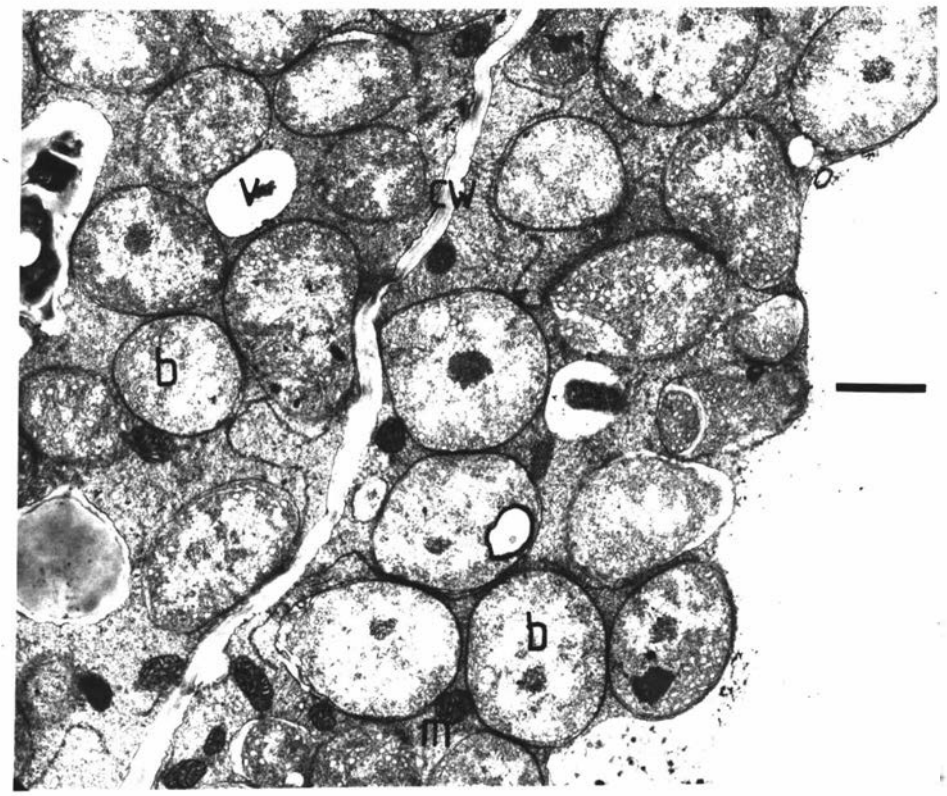
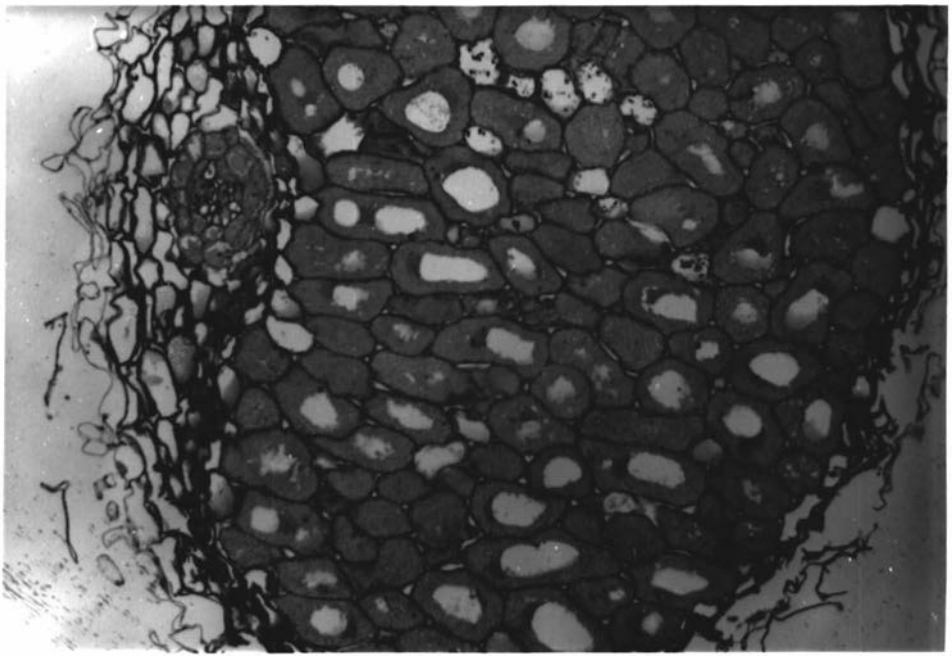


b

Figure 3. Light and electron micrographs of nodule sections taken from a plant inoculated with MO 105.

3a. Light micrograph, x160. Size bar is 70 um.

3b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, m=mitochondrion, pm=peribacteroid membrane.



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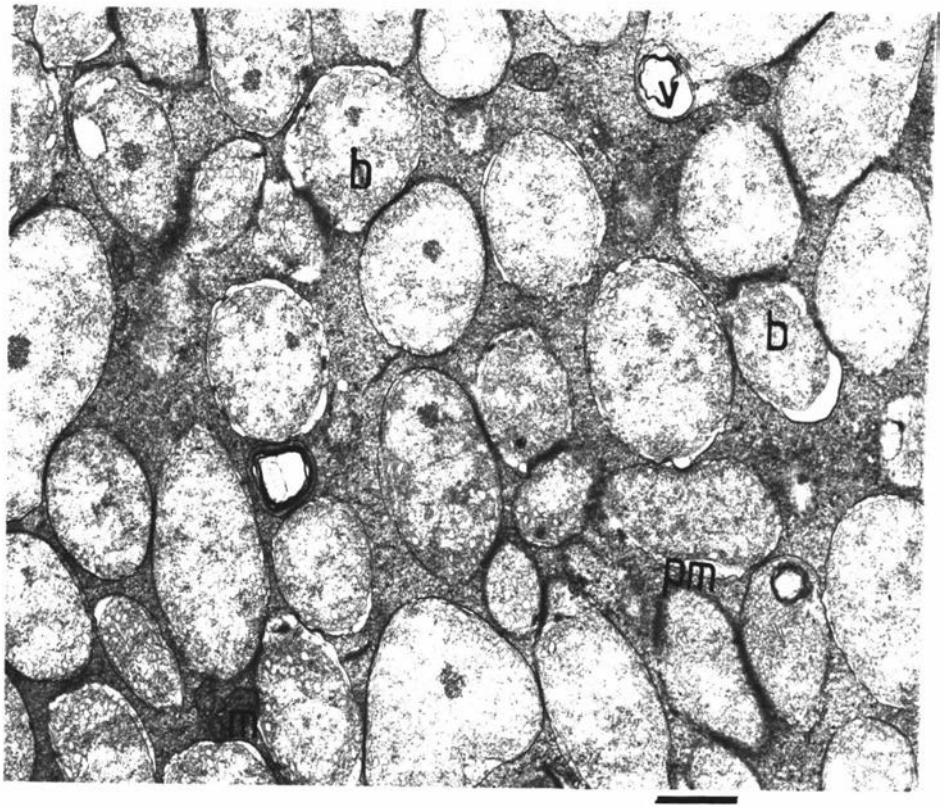
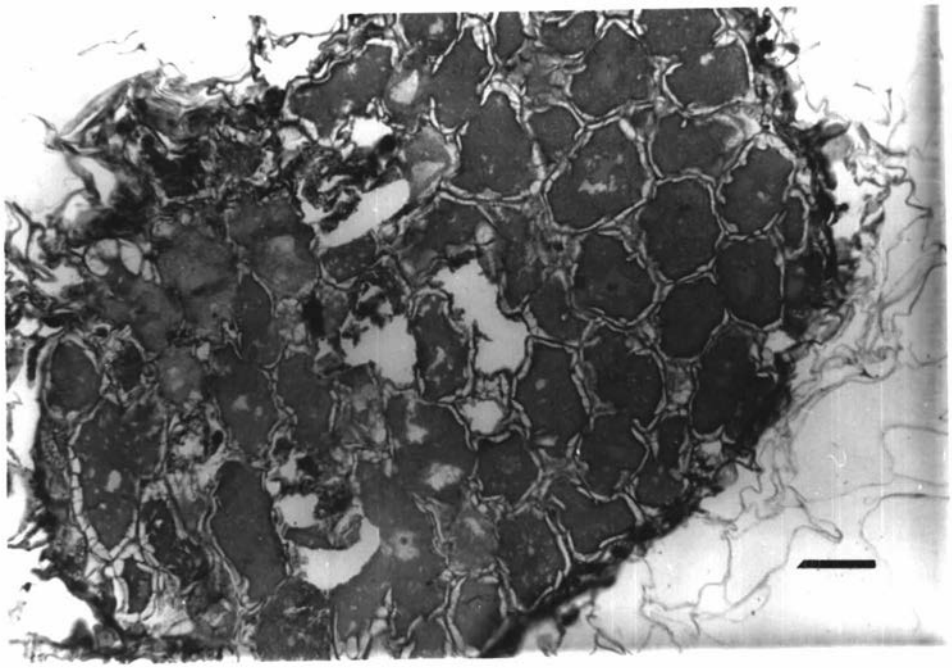


b

Figure 4. Light and electron micrographs of nodule sections taken from a plant inoculated with MO 110.

4a. Light micrograph, x160. Size bar is 70 um.

4b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, sb=senescent bacteroid, v=vacuole.



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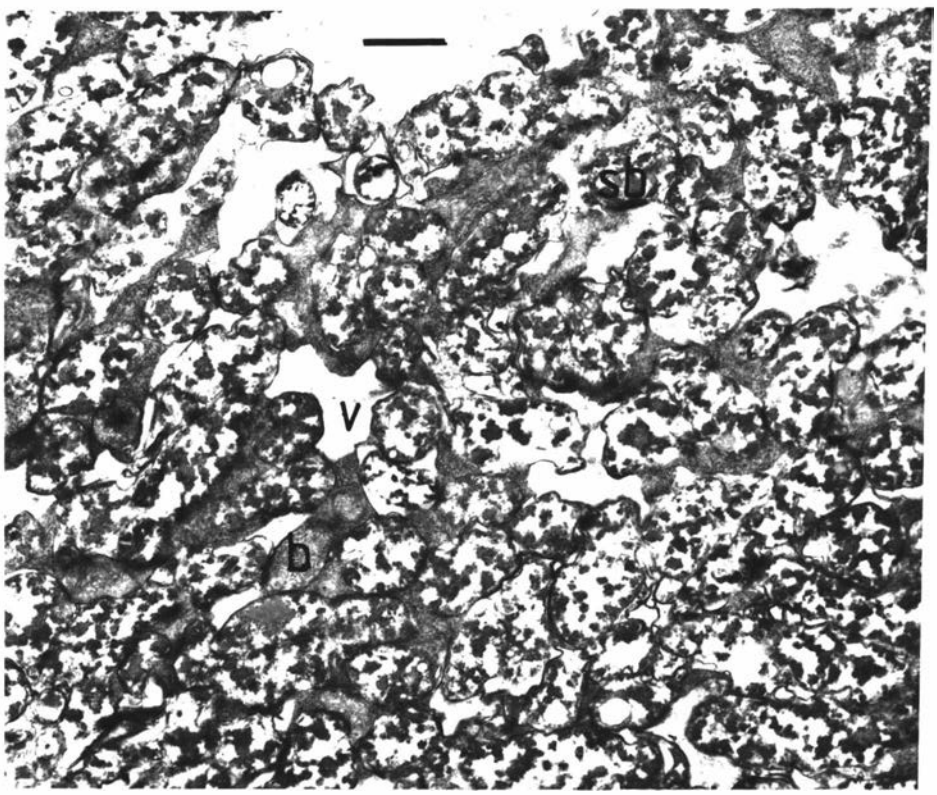


b

Reisolation from nodules inoculated with the 120, 200 and 810 series was only carried out once and only 1 nodule from each strain was examined. In all cases the reisolated strain was the same as the inoculant, in antibiotic resistance and plasmid profile. This examination is admittedly cursory, but in keeping with the rest of this section was intended as a survey, not a detailed investigation. There is much to be gained from a more exhaustive examination of the effect of plant passage, as there is evidence to suggest this can affect plasmid profile (Wang et al, 1986; Espuny et al, 1989).

The 120 series proved to be able to nodulate clover plants in the laboratory setting of a controlled environment (figs. 5 and 6). None of the soil bacteria were able to nodulate clover and it was only the exconjugant strains containing the pSym-derivative which could form nodules. However, some strains were much more efficient at inducing nodules, in terms of the number of trials in which a positive result was observed. MO 122 proved to be particularly poor, nodulating only once in five trials (table 6b). The low nodulation efficiency made measurements of nitrogen fixation efficiency very difficult and only one acetylene reduction assay was performed. As they are the results of only one trial, the acetylene reduction assay figures must be treated with some caution. However, it is possible to state with more confidence that the pSym-derivatives present in the exconjugant soil bacteria allow them to fix nitrogen at between 54-69% the level of 2668 in this trial. MO 122 fixes nitrogen the most poorly, which may be the result of the multiple symbiotic plasmid bands (figs. 23-26, lane f). It has been reported by Harrison et al (1988) that the presence of more than one copy of pSym can reduce nitrogen fixation efficiency.

The internal structure of the nodules formed by two of the exconjugant soil bacteria was relatively normal compared with that of 2668 (fig. 2) but a few anomalies were noted. In both cases some of the bacteroids appear fused (fig. 7a and 8a) as opposed to the normal discrete cells as in 2668 (fig. 2a) and MO 201 (fig. 10a). The nodule induced by MO 121 appeared only half infected and many of the

bacteroid-containing nodule cells of plants inoculated with MO 121 and 124 were beginning to senesce as shown by the clear zones between the core of the bacteroid and the peribacteroid membrane (figs. 7b and 8b). An example of a fully senescent bacteroid is labelled by the designation sb in fig. 8b.

Figure 5. Photographs of clover plants inoculated with parents and exconjugants from the cross between 2668 and the soil bacteria. The inoculant is shown to the right of the plant.

5a. The whole plant.

5b. Closeup showing root and nodules.

a

b

Figure 6. Photographs of clover plants inoculated with parents and exconjugants from the cross between 2668 and the soil bacteria. The inoculant is shown to the right of the plant.

6a. The whole plant.

6b. Closeup showing root and nodules.

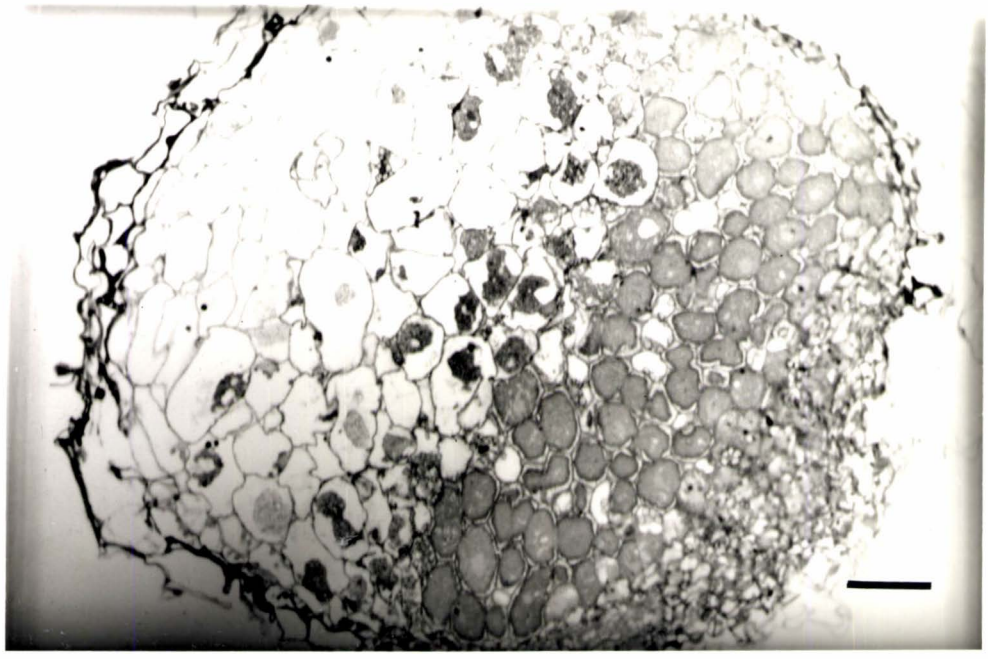
a

b

Figure 7. Light and electron micrographs of nodule sections from a plant inoculated with MO 121.

7a. Light micrograph, x160. Size bar is 70 um.

7b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, cw=cell wall, m=mitochondrion, pm=peribacteroid membrane.



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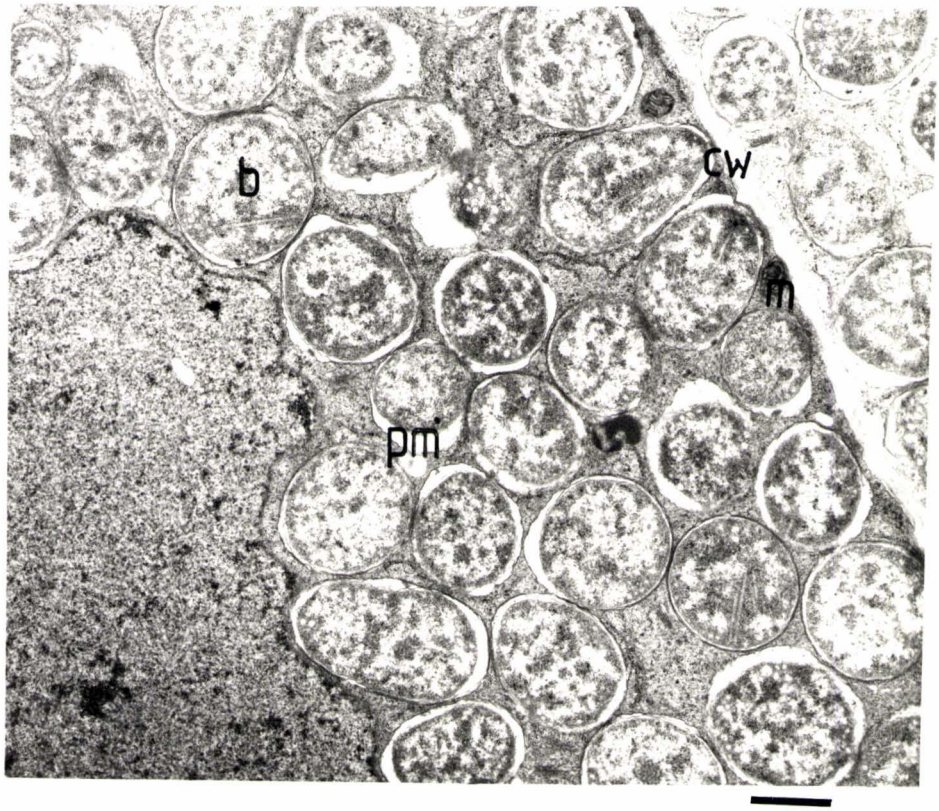
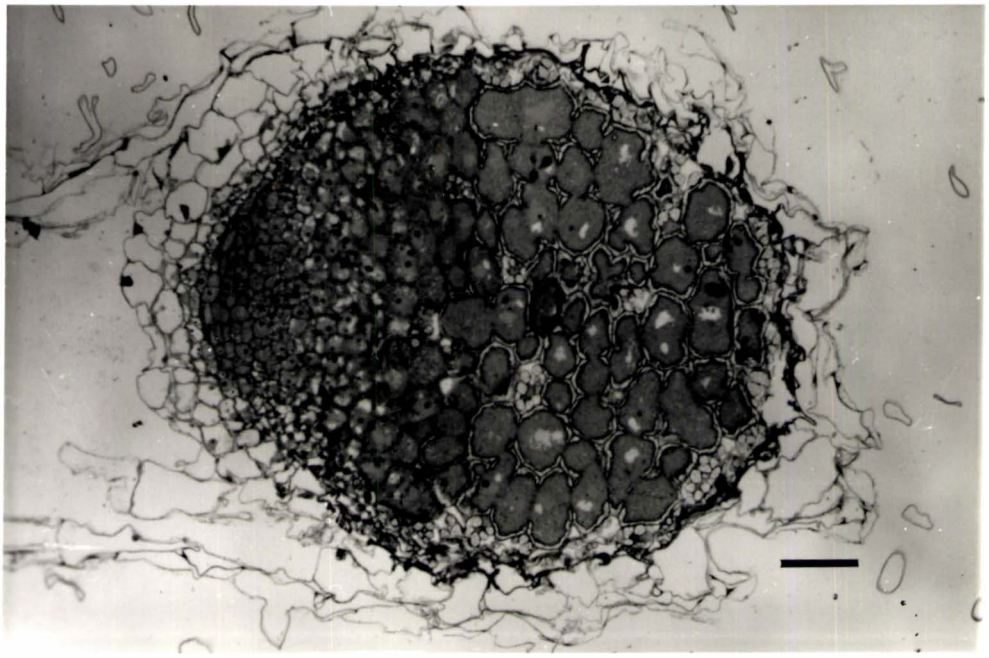


b

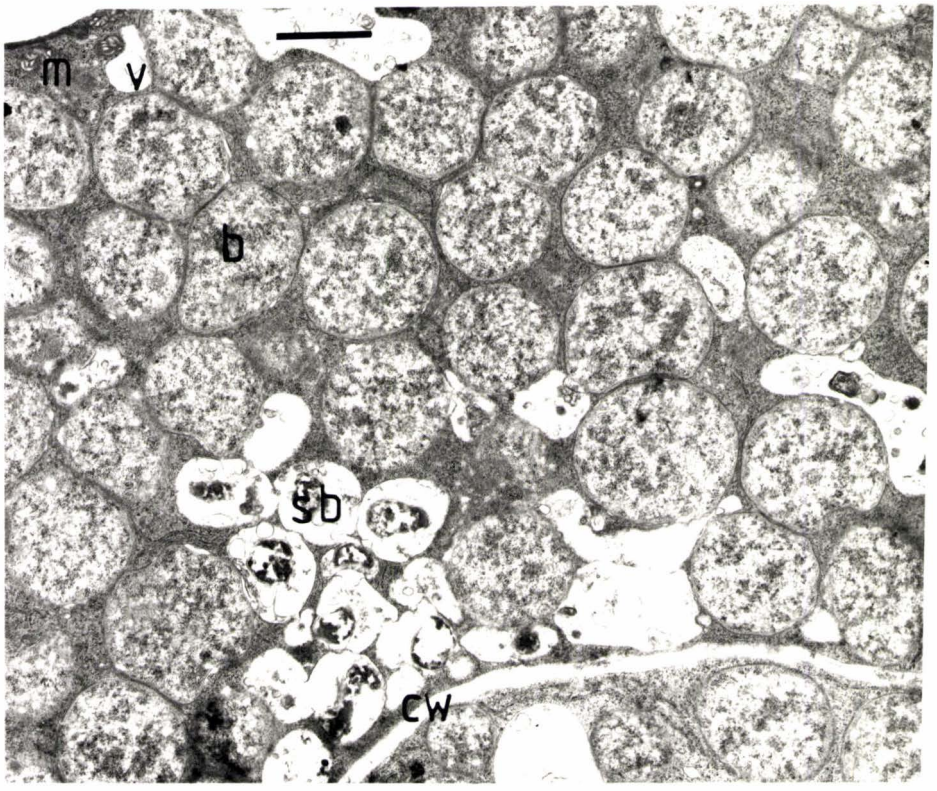
Figure 8. Light and electron micrographs of nodule sections from a plant inoculated with MO 124.

8a. Light micrograph, x160. Size bar is 70 um.

8b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, cw=cell wall, sb=senescent bacteroid, v=vacuole.



a



b

TABLE 6b. Nodulation and Nitrogen Fixation Status of Strains Isolated in Part II.

All percentages of nitrogen fixed are relative to 2668.

nd=not done.

TABLE 6b.

STRAIN	STATUS	TRIALS NOD$^+$	%N FIXED c.f. 2668	DERIVATION
NR 41	nod$^-$	0/5	0	soil bacteria
NR 42	nod$^-$	0/5	0	soil bacteria
NR 64	nod$^-$	0/5	0	soil bacteria
OR 168	nod$^-$	0/5	0	soil bacteria
MO 121	nod$^+$	4/5	69	2668::Tn5XNR 41
MO 122	nod$^+$	1/5	54	2668::Tn5XNR 42
MO 123	nod$^+$	3/5	62	2668::Tn5XNR 64
MO 124	nod$^+$	3/5	67	2668::Tn5XOR 168
PN 104	nod$^-$	0/2	0	pSym$^-$ rhizobia
MO 201a	nod$^+$	2/2	100	MO 121XPN 104
MO 201b	nod$^+$	2/2	54	MO 121X PN 104
MO 202	nod$^+$	2/2	85	MO 122XPN 104
MO 203	nod$^+$	2/2	73	MO 123XPN 104
MO 204	nod$^+$	1/2	nd	MO 124XPN 104
HB 101	nod$^-$	0/2	0	*E. coli* strain
MO 301	nod$^-$	0/2	0	MO 121XHB 101
MO 302	nod$^-$	0/2	0	MO 122XHB 101
MO 303	nod$^-$	0/2	0	MO 123XHB 101
MO 304	nod$^-$	0/2	0	MO 124XHB 101
MO 810	nod$^+$	2/2	107	MO 121X2668
MO 811	nod$^+$	2/2	54	MO 121X2668
MO 813	nod$^+$	2/2	36	MO 121X2668

The 200 series, with the plasmid/s in a known rhizobial chromosomal background, was much better at nodulating and MO 201-203 nodulated well in both trials. The nodules formed by the 200 series appeared to be like those of 2668 (large, pink and healthy) with the exception of MO 204 which formed small, white, sickly nodules (fig. 9). PN 104, the pSym$^-$ Rhizobium , did not form nodules at all and the 2668 control formed nodules in both trials. The structure of nodules formed in the 200 series was examined in more detail in MO 201. The internal structure of the nodule (fig. 10a) was very similar internally to the nodule formed by 2668 (fig. 2a) but a closer examination of the bacteroids (fig. 10b) showed that although the bacteroids appeared healthy, there were a number of vacuoles present in the strain.

Nitrogen fixation was somewhat more variable, with one isolate of MO 201 being as effective as 2668 and another only half as effective (table 6b). It is possible that this was due to technical difficulties as some minor intermittent malfunction was affecting the Gas Chromatograph during this trial. The effectiveness of the 200 series was 80-100% that of 2668, with the exception of the second MO 201 isolate.

Figure 9. Photographs of clover plants inoculated with the recipient and exconjugants from the cross between the 120 series soil bacteria and PN 104. The inoculant strain is listed to the right of the plant.

9a. The entire plants.

9b. Close-up showing the roots and nodules.


2668
PN 104
MO 204
MO 203
MO 202
MO 201

a

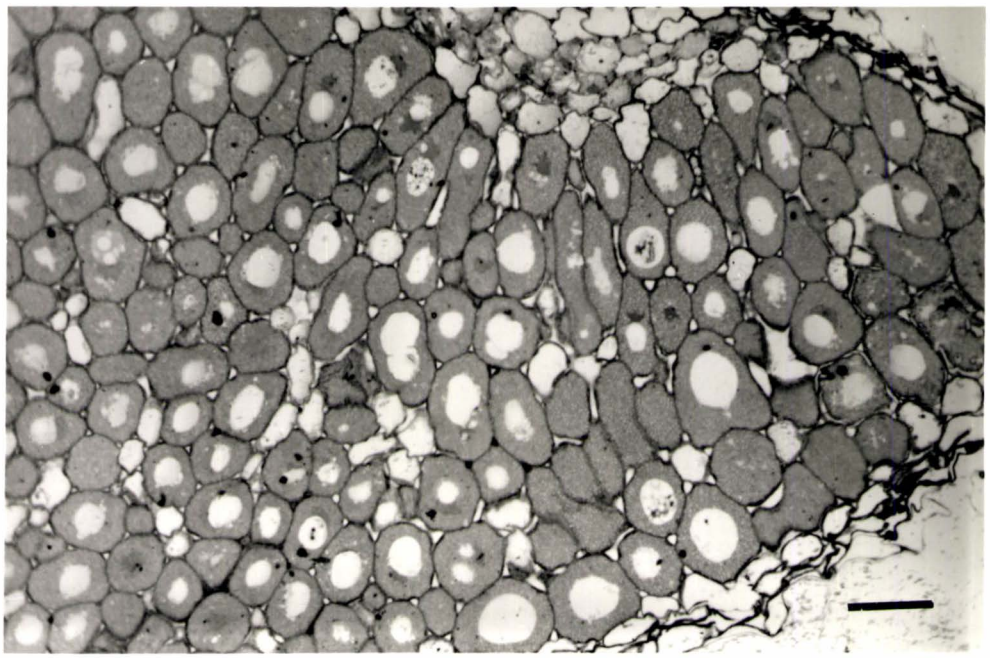

2668
PN 104
MO 204
MO 203
MO 202
MO 201

b

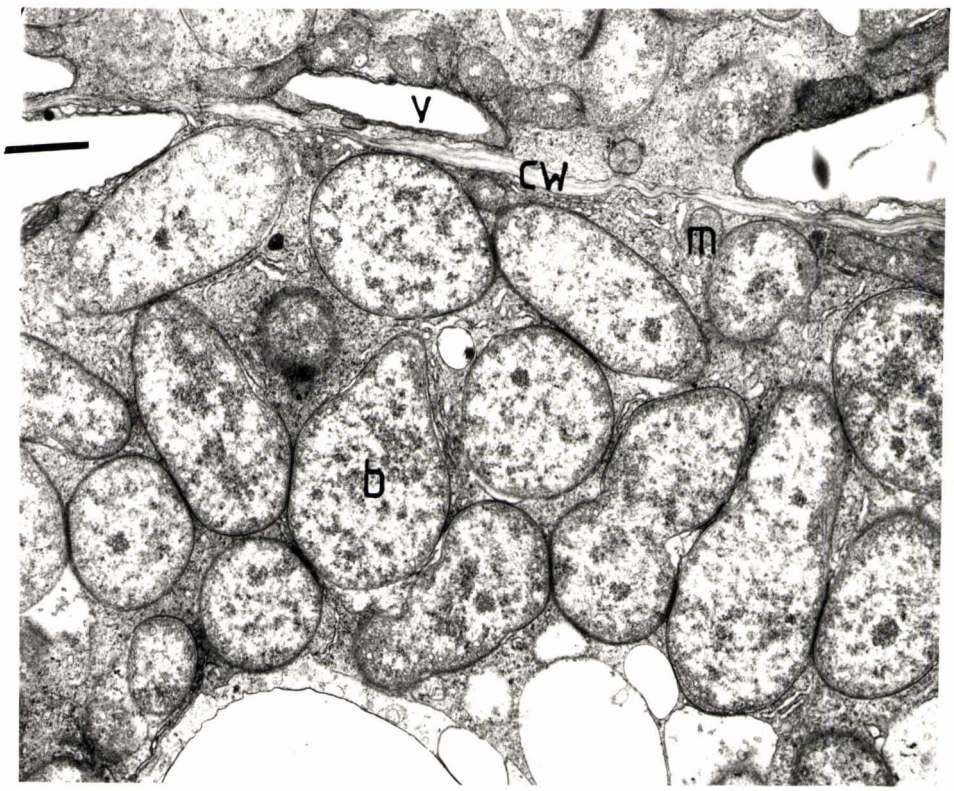
Figure 10. Light and electron micrographs of nodule sections from a plant inoculated with MO 201.

10a. Light micrograph, x160. Size bar is 70 um.

10b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, cw=cell wall, m=mitochondrion, v=vacuole.



a



b

Two plant nodulation trials, with the 300 series as inoculants, were carried out and no nodules were formed in either trial by any strain other than the 2668 control (table 6b). This was not surprising as most other researchers have been unable to generate nodules with pSym in an E.coli host. The only exception of which we are aware is the formation of pseudonodules by an E. coli host containing R. meliloti nod genes cloned into pLAFR1 (Hirsch et al, 1984).

The effect of two copies of the symbiotic plasmid of 2668 in 2668 were examined in the cross between MO 121 and 2668. The 810 series nodulated twice in two trials. The nodules appeared healthy and the plants grew as well if not better than plants inoculated with 2668 (fig. 11).

Examination of the internal structure of nodules induced by the inoculants MO 810 and 812 showed some signs of fusion of the bacteroids (figs. 12a and 13a) and that there were some signs of senescence in these nodules. There are some obvious differences in the structures of the bacteroids formed in response to inoculation by MO 810 (fig. 12b), MO 812 (fig. 13b) and 2668 (fig. 2b). The elongated cell structure visible in MO 810 and 812 may be attributable to the angle of the sections but the bacteroid-containing nodule cells of plants inoculated with MO 810 appeared to be distributed in a much less organized manner than the nodule cells of plants inoculated with 2668. The number of bacteroid containing cells appeared lower, particularly in the nodule formed by MO 812. It is not clear if the structure of the 810 series nodules is an effect of nodule age or multiple copies of pSym, but examination of other electron microscope fields from this nodule suggests that it is a general property. It would be useful to examine the nodule structure induced by other multi-pSym strains, such as MO 123 and 203, to see if the unusual organization is a feature of multi-pSym strains.

There was some variation in nitrogen fixing ability of the 810 series strains. Two of the isolates were only 35-55 % as effective as 2668, but one was more effective (table 6b). The reduced efficiency

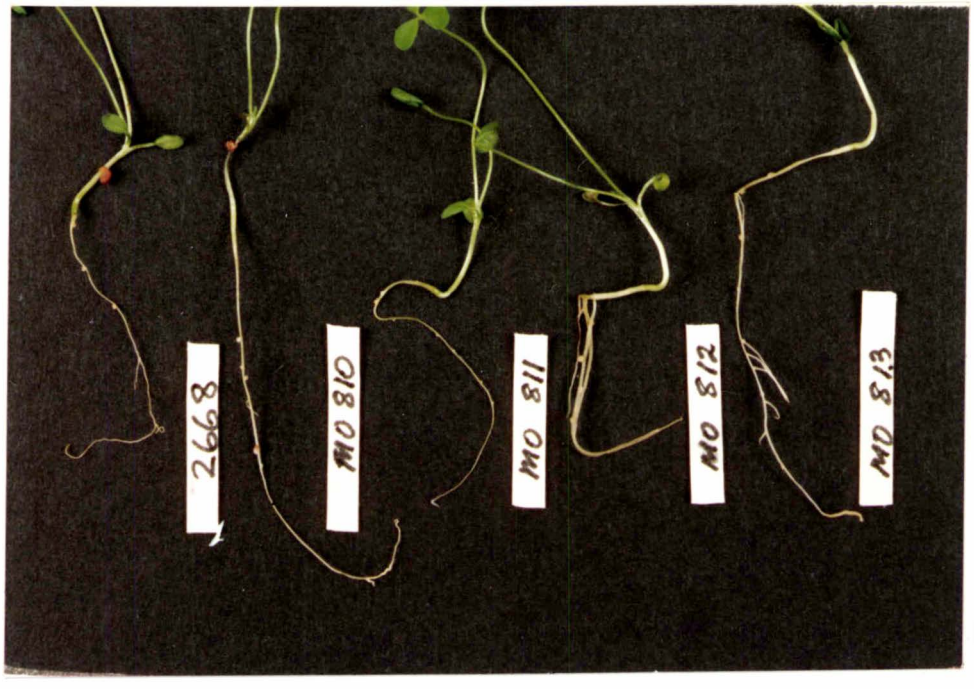
of nitrogen fixation of the two strains agrees with a study by Harrison *et al* (1988), which suggested that multiple symbiotic plasmids inhibited symbiosis. The high level of nitrogen fixation by plants inoculated with MO 810 does not agree with the results of Harrison *et al* but with only three samples and one trial, no firm comparisons between the two studies can be made.

Figure 11. Photographs of clover plants inoculated with one parent and the exconjugants from the cross between the soil bacterium MO 121 and the inoculant 2668. The inoculant strain is listed to the right of the plant.

11a. The whole plant.

11b. Close-up of the roots and nodules.

a

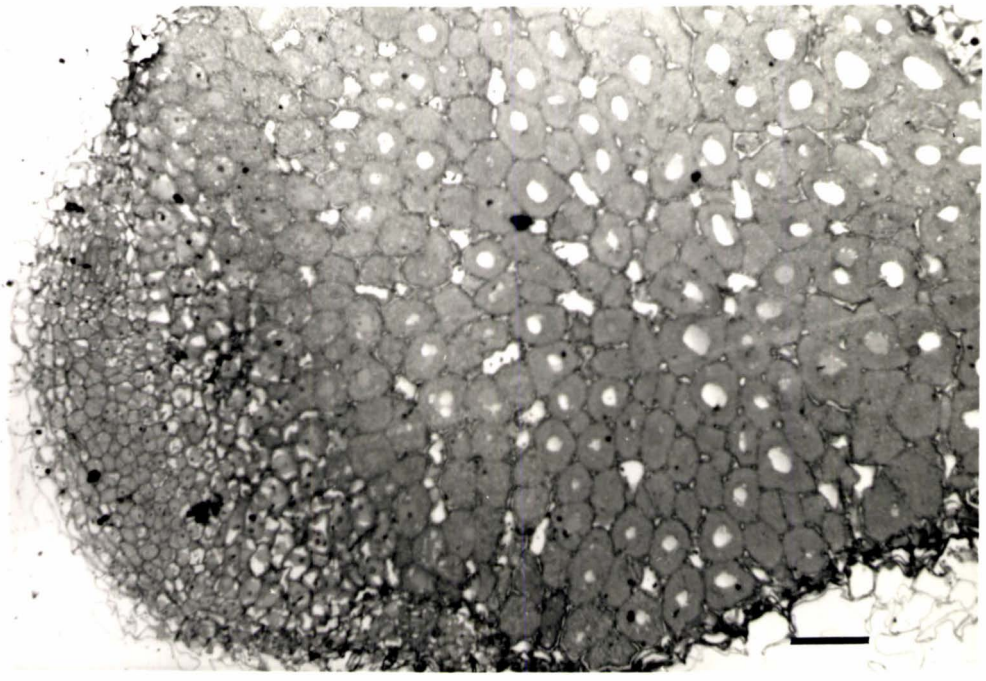


b

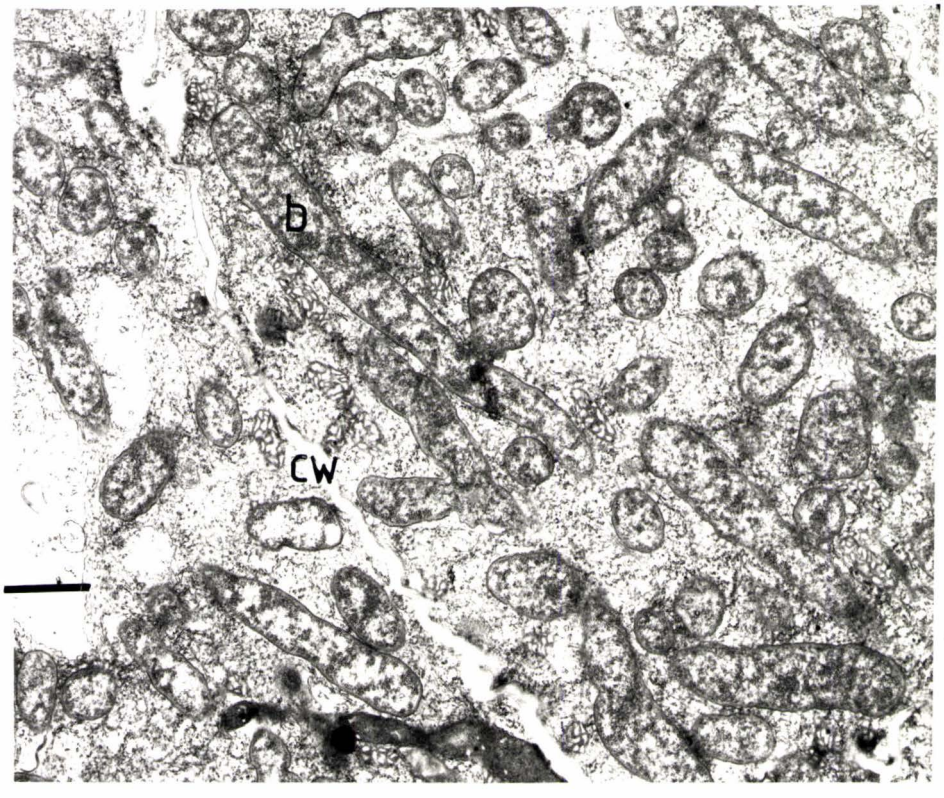
Figure 12. Light and electron micrographs of nodule sections from a plant inoculated with MO 810.

12a. Light micrograph, x160. Size bar is 70 um.

12b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, cw=cell wall.



a

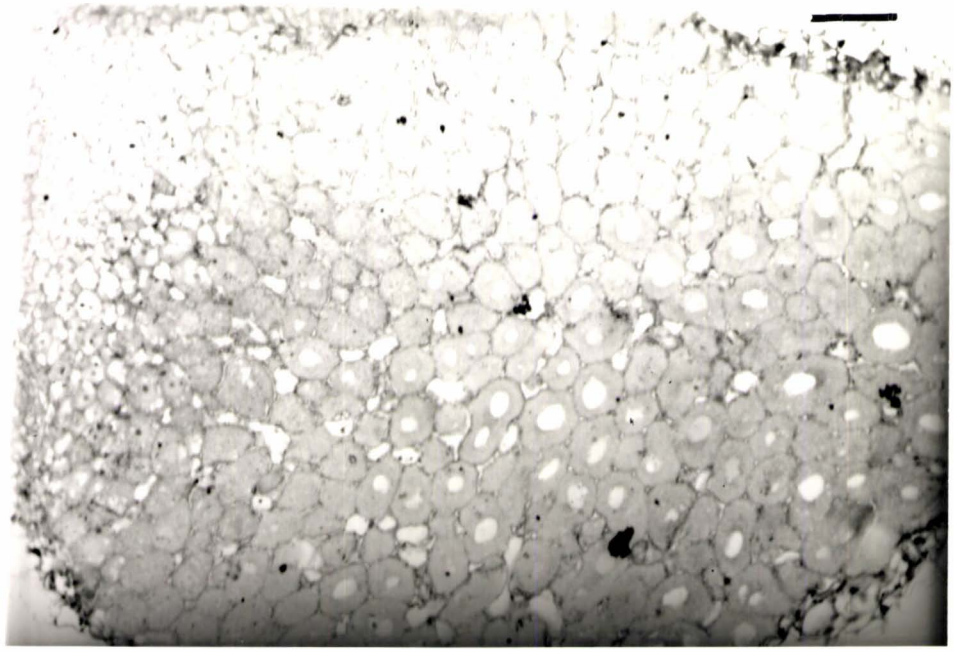


b

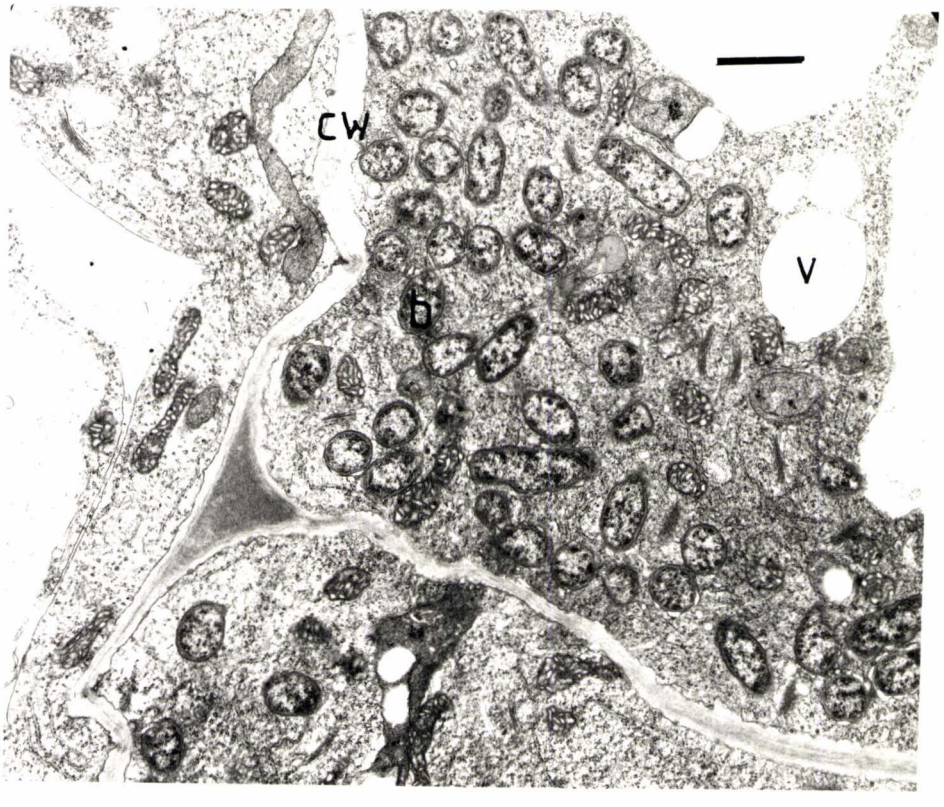
Figure 13. Light and electron micrographs of nodule sections from a plant inoculated with MO 812.

13a. Light micrograph, x160. Size bar is 70 um.

13b. Electron micrograph, x11,400. Size bar is 1 um. b=bacteroid, cw=cell wall, v=vacuole.



a



b

3.5 Rearrangement of a Strain of *R. leguminosarum* biovar *trifolii* carrying RP4

The use of recalcitrant carbon and nitrogen sources (in the form of dextrin, a highly cross-linked glucose polymer and NO_3^{2-} instead of NH_4^+) and selective solid agar plates enabled Heumann *et al* (1983; 1984) to identify a number of different DNA rearranged variants of an initial strain. In this study, a similar use of carbon and nitrogen stress media was used in an attempt to isolate rearrangements of 2668' which resembled strains isolated in a previous project (O'Hara, 1985). A graphic summary of the experimental design of the stress test is shown in diagram 5.

The broad host range plasmid RP4 was introduced into 2668 in an attempt to detect any pieces of DNA that were being translocated during rearrangement. Theoretically, if DNA was moving around the genome during rearrangement some might insert into RP4 (unless the rearrangements were site-specific only) and could then be detected by Eckhardt gel electrophoresis and isolated by crossing the modified RP4 back out of 2668 and into an *E. coli* strain.

One colony of fifty-two from the cross between 2668 and HB101(RP4) showed an altered plasmid profile (fig. 15, lane c). The discovery of this strain was entirely fortuitous but showed exactly the type of rearrangement we were looking for, albeit as an apparent consequence of introduction of RP4 rather than a consequence of stress. The strain, designated MO 103, was included in the stress experiment in an attempt to force it to undergo further rearrangement.

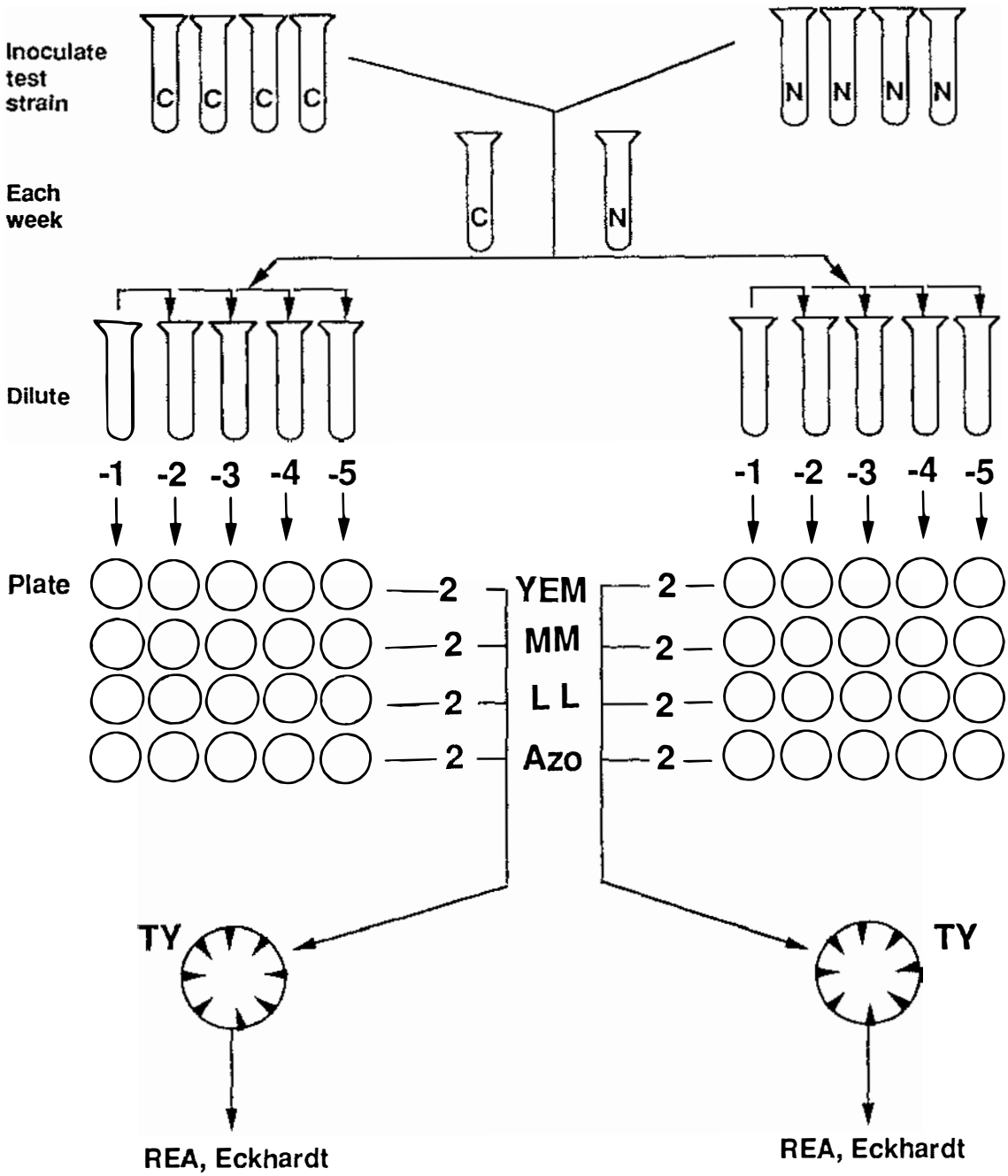


Diagram 5.
Flow chart depicting steps for sampling and analysis of carbon and nitrogen stress of bacterial strains.

Three bacterial strains were grown in stress media containing a recalcitrant carbon or nitrogen source. 4 tubes of C-stress and 4 tubes of N-stress medium were inoculated with the test strains as follows:

1. 2668
2. 2668(RP4)
3. MO 103
4. MO 103 with neomycin at 50 ug/ml added to stress medium.

The inoculated tubes were incubated at 28°C with shaking. One tube of C-stress and one tube of N-stress were removed from each test line per week and plated onto four different solid media (YEM, MM, LL and Azo) designed to select for rearrangements, which were supposed to be detectable by color and colony morphology changes (Heumann et al, 1984). The composition of the media is described in section 2.2.

Each week thirty-two colonies from the plates (two from each stress medium, 4 selections, 4 strains) were examined by Eckhardt electrophoresis and subcultured onto TY (diagram 5). Of the 64 isolates from each test strain examined (2 stresses, 4 weeks, 4 selection plates, 2 colonies per plate) none appeared different from the original inoculant. Some examples are shown in fig. 14. A colony from the MO 103 test line that included neomycin (N stress, colony 6, week 4) grew more slowly than the other seven colonies isolated from that treatment and was designated MO 104. It proved to have the same plasmid (fig. 15, lanes b and c) and total genomic DNA restriction digest profile (fig. 19, lanes c and d) as MO 103 and subculture in TY alleviated the growth impediment. No difference between the initial strain and any subsequent isolate was observed and as confirmatory experiments have not been published by Heumann or colleagues, this approach to generating DNA rearrangement is now regarded with some scepticism. However, the rearrangement of 2668 to give MO 103 seemed clear cut and even though subsequent attempts to reproduce it have been unsuccessful, presumably because it is a low frequency event, rearrangements of this type are discussed in section 1.5 and Terzaghi and O'Hara (in press).

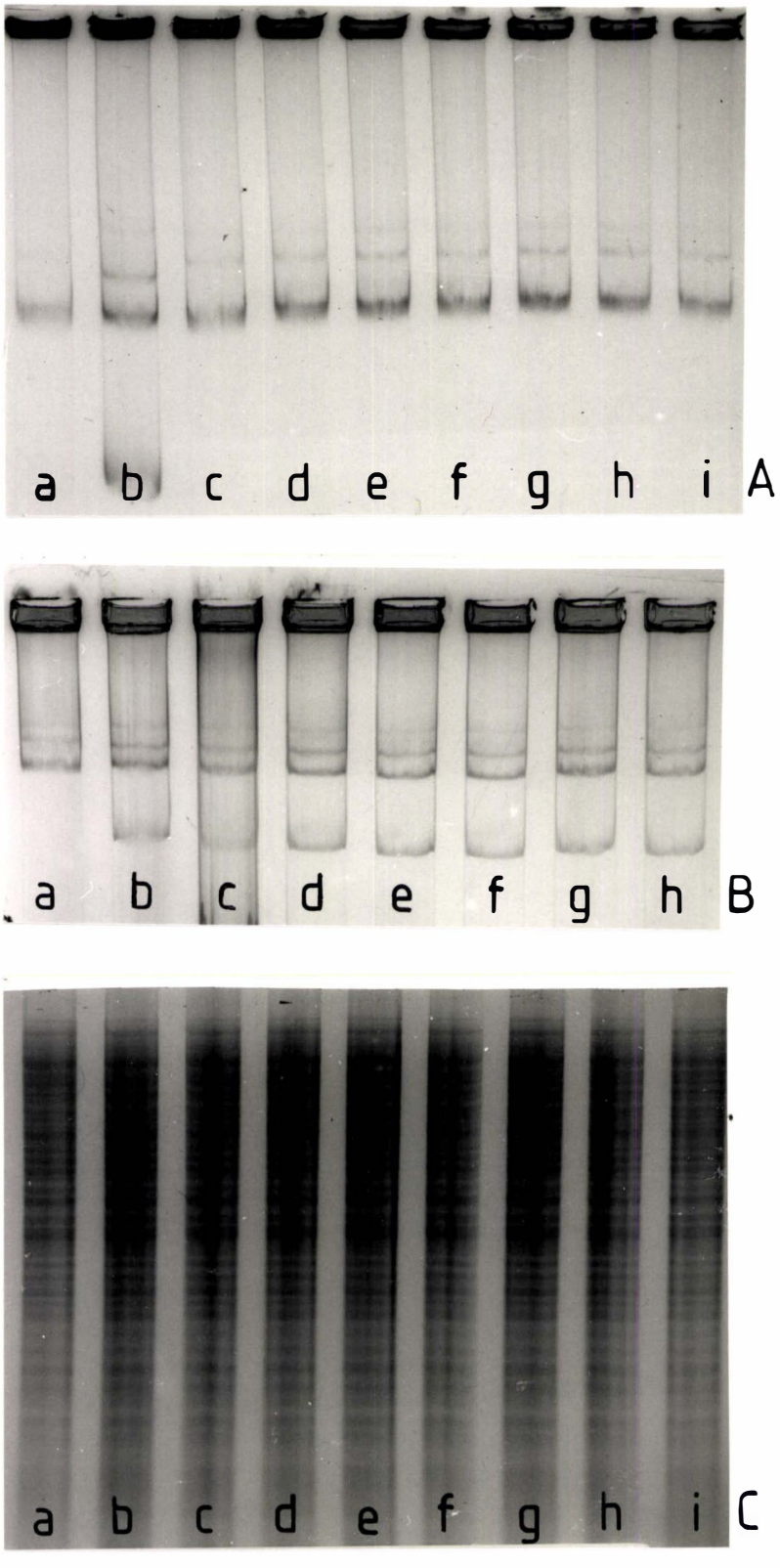
Comparison of the Eckhardt plasmid profile of 2668, MO 103 and MO 104 suggested that MO 103/104 had undergone a deletion in the second largest plasmid (band 2 of 2668 in fig. 15, lane a). The four bands of 2668 and the large bands in the other strains are shown more clearly in fig. 15A. The second plasmid of 2668 was measured as 356 kb by Harrison et al (1988), but the plasmid in MO 103/104 and MO 110/111 is approximately 300 kb, as measured from the other plasmids, which is a fairly substantial deletion. It also appears that one member of the doublet of plasmids (bands 3 and 4 of 2668 in fig. 15, lane a) has been completely lost. If the bands were running together one much heavier band would be visible and if there were a larger deletion the band would be seen further down the gel. It is difficult to tell if the missing band is band 3 or band 4 (fig. 15, lanes b and c). The RP4 plasmid crossed into the strain was present (band 6 in lanes b and c, fig. 15) and was expressing the appropriate antibiotic resistances (table 5).

Figure 14. Some of the strains isolated as a result of the stress treatment of the test strains listed above. The strains are labelled first by the stress treatment (C or N), secondly by the week of isolation (1-4) and finally by colony number (1-8).

Fig. 14A. 2668 test strain, C and N stress, weeks 1-4. a=2668, b=MO 103, c=N1-4, d=N2-4, e=N3-4, f=N4-4, g=C1-4, h=C3-4 and i=C4-4.

Fig 14B. MO 103 test strain, C and N stress, weeks 1-4. a=2668, b=MO 103, c=C1-2, d=C2-2, e=C3-2, f=C4-2, g=N1-2 and h=N3-2.

Fig 14C. Total genomic DNA. 2668 test strain, C and N stress, week 1-4. a=2668, b=N1-4, c=N2-4, d=N3-4, e=N4-4, f=C1-4, g=C2-4, h=C3-4 and i=C4-4.


a b c d e f g h i A
a b c d e f g h B
a b c d e f g h i C

A Southern blot of an Eckhardt gel was probed with nodABC, which hybridized to a plasmid in 2668 (figs. 16 and 17, lane a) and which did not hybridize to MO 103 or 104 (figs. 16 and 17, lanes b and c). The DNA probe suggests that the missing plasmid is pSym or some part of it, although if it is only part of pSym it is not clear where the rest is. Harrison et al (1988) have determined that the smallest plasmid of 2668 is pSym and assuming there is no difference between their pSym and ours, it is most likely pSym that has been lost from MO 103/104. Alternatively, only the symbiotic genes of pSym might have been lost and all of the other plasmid of the pair also lost. It is not clear from the Eckhardt gel which plasmid is missing, although it is likely that it is the smallest plasmid. However, the crucial observation is that nodABC is no longer detectable by hybridization in MO 103/104.

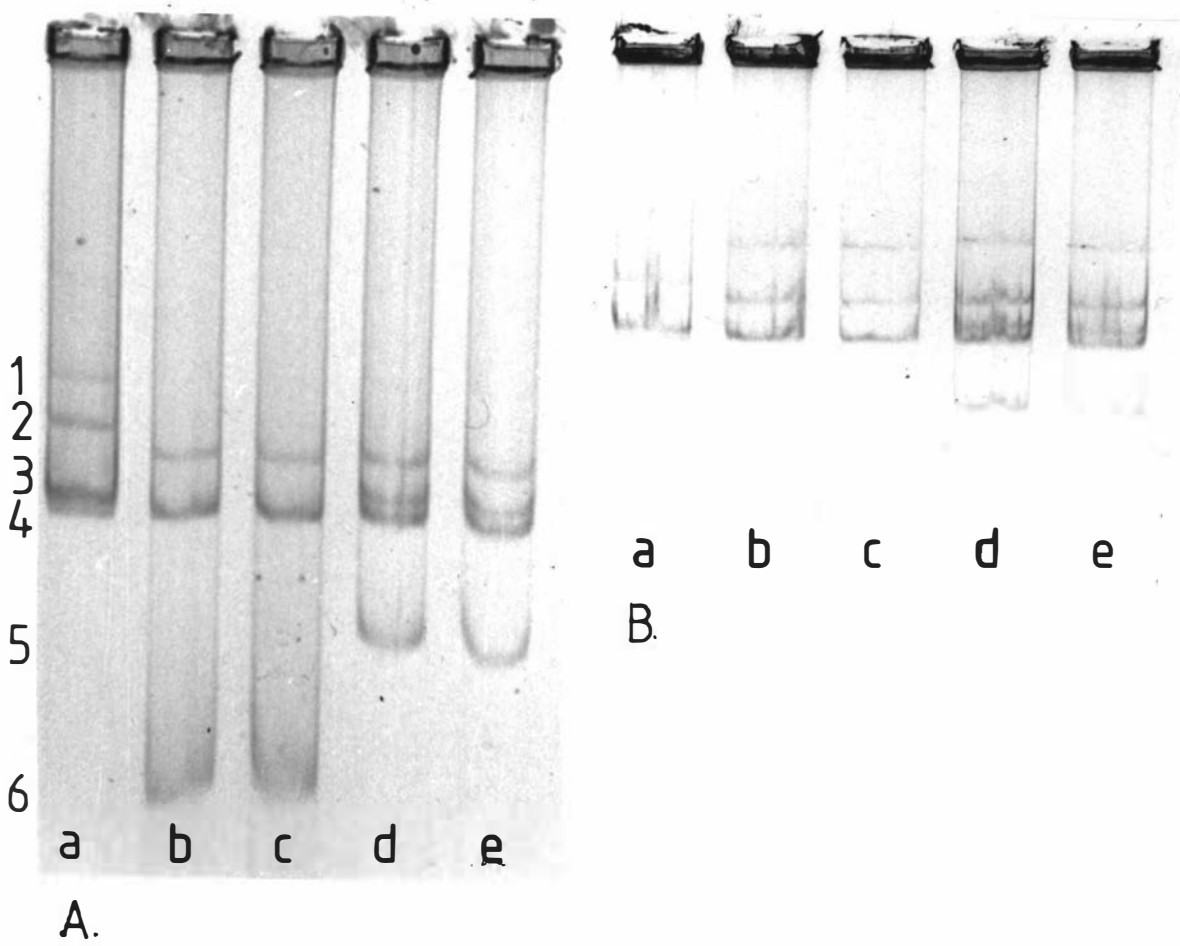
However, when MO 103 or MO 104 was inoculated onto plants, nodules were formed. The nodules were pink and healthy and appeared effective in the four trials when either MO 103 or MO 104 were inoculated onto plants. Strains with the appearance of MO 110 and MO 111 (fig. 15, lanes d and e) were recovered from all nodules in three trials and in the fourth no nodules were formed (see also section 3.4). MO 110/111 had the same size band 1 as 2668, the same size band 2 as MO 103/104, a doublet of bands (3 and 4) similar but not identical to 2668 and a band (band 5 in fig. 15, lanes d and e) of about 125 kb as estimated from the sizes of the bands of 2668 (Harrison et al, 1988). The fifth band is not found in the strains 2668, MO 103 and MO 104. This new band was initially assumed to be RP4 with a substantial insert but a probe consisting of the entire RP4 replicon failed to show any homology to the new band (fig. 15, lanes d and e, fig. 18, lanes d and e). The RP4 probe identified RP4 in MO 103/104 (fig. 15, lanes b and c, fig. 18, lanes b and c) and showed weak homology to some of the other plasmids in all five lanes except the 125kb plasmid in MO 110/111 (fig. 18). The most interesting result was with a probe of nodABC to a Southern blot containing MO 110/111. The nodABC probe (figs. 16 and 17) showed the

reappearance of pSym in MO 110/111 (figs. 16 and 17 lanes d and e). The pSym of MO 110/111 was of approximately the same size as pSym of 2668 (figs. 16 and 17, lane a).

Figure 15A. Eckhardt plasmid profile of the five strains derived in part I. The lanes are: a=2668, b=M0 103, c=104, d=110, e=111. The bands marked 1 to 4 and 6 are approximately (kb): 600, 356, 199, 188 and 60 respectively. From the sizes of these bands, the new band (5) is calculated at approximately 125 kb.

Figure 15B. As 15A except the largest band (approx. 600 kb in 2668) is visible in the strains on this gel. It is difficult to set conditions that will clearly show all of the plasmids because of the size range.

Figure 16. Southern blot of Eckhardt gel probed with nodABC. Lanes: a=2668, b=103, c=104, d=110 and e=111. The hybridizing plasmid is approximately 188 kb. The blot is of the gel shown in figure 15A.


1
2
3
4
5
6
a b c d e
A.
a b c d e
B.

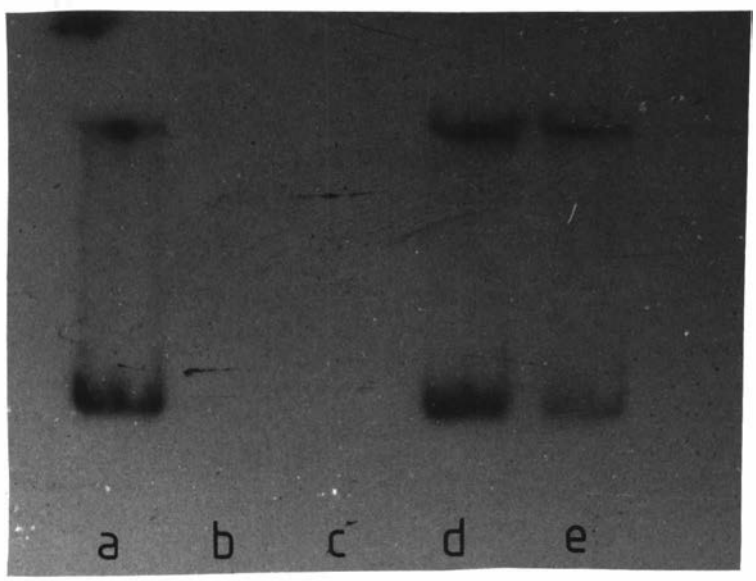
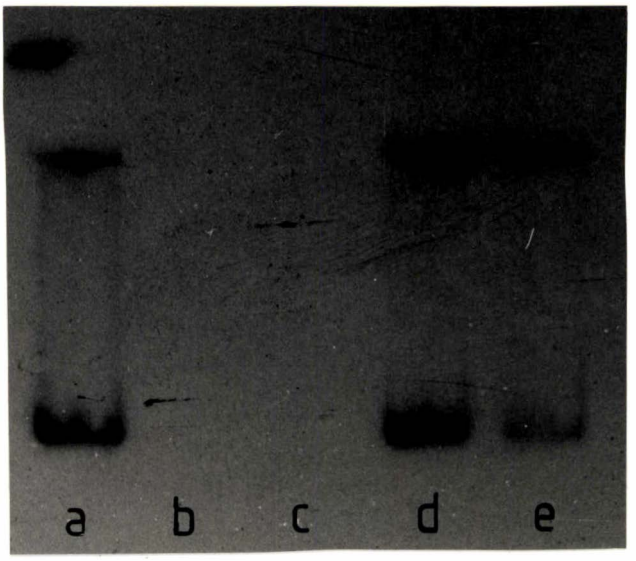
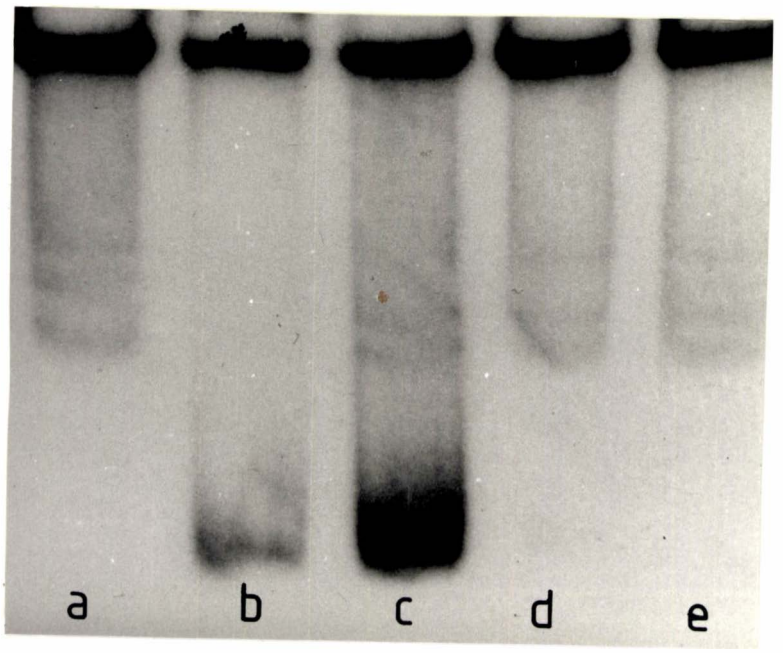

a b c d e

Figure 17. Southern blot of Eckhardt gel probed with nodABC. Lanes: a=2668, b=103, c=104, d=110 and e=111. The hybridizing plasmid is approximately 188 kb. The blot is of the gel shown in figure 15A.

Figure 18. Southern blot of Eckhardt gel probed with RP4. Lanes: a=2668, b=103, c=104, d=110 and e=111. The hybridizing plasmid (RP4) is approximately 60 kb. The blot is of the gel shown in figure 15A.


a
b
c
d
e

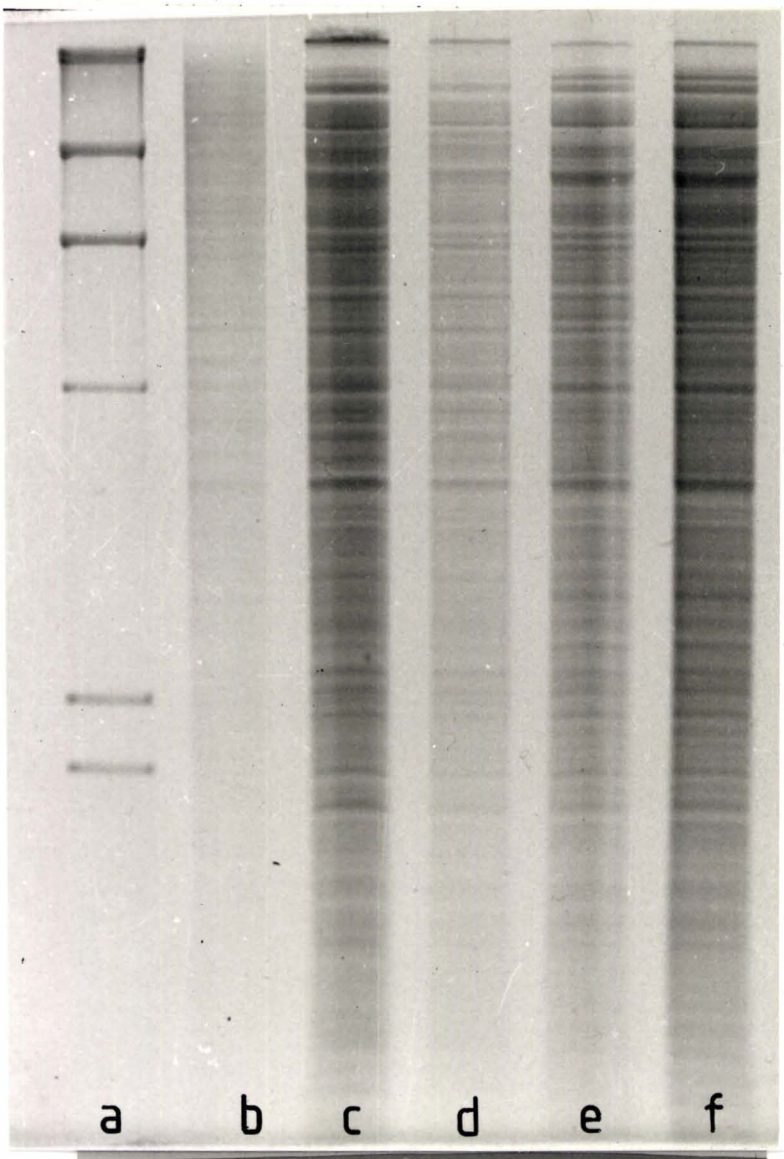

a
b
c
d
e

The total genomic DNA was isolated from the strains 2668, MO 103 MO 104, MO 110 and MO 111, digested with EcoRI and the fragments electrophoretically separated. The first difference observed was between the total genomic DNA restriction digest profile of 2668 and MO 103/104 (fig. 19, lanes b, c and d) which appear substantially different. Secondly, MO 103 and MO 104 appeared identical to one another (fig. 19, lanes c and d) and MO 110 and 111 likewise identical to one another (fig. 19, lanes e and f). The electrophoretic band patterns of MO 103/104 and MO 110/111 showed a high degree of similarity, suggesting that the strains were closely related.

Probing of Southern blots of the total genomic DNA digests of these five strains with the 5.2 kb nifKDH sequence identified a 5.2 kb band in 2668 (fig. 20, lane b) and in MO 110 and 111 (fig. 20, lanes e and f). No hybridizing band was observed in MO 103 or M0 104 (fig. 20, lanes c and d). The 7.2 kb nodABC probe detected bands of 7.2, 1.2 and 1.0 kb in 2668 (fig. 21, lane b), MO 110 (fig. 21, lane e) and MO 111 (fig. 21, lane f). No hybridizing bands were detected in MO 103 or 104 (fig. 21, lanes c and d). A probe consisting of the entire RP4 replicon identified a single very large band in MO 103/104 (fig. 22, lanes c and d) consistent with the single EcoRI site of RP4 and no bands of hybridization in 2668 (fig. 22, lane b) or MO 110/111 (fig. 22, lanes e and f). The lack of hybridization to RP4 by 2668 and MO 110/111 and the presence of a hybridizing band in MO 103/104 is consistent with the antibiotic resistances displayed by the five strains (table 5).

Figure 19. Electrophoretic profile of an EcoRI digest of the total genomic DNA of the five strains. Lanes: a=Lambda phage marker (Hind III cut), b=2668, c=103, d=104, e=110 and f=111. The sizes of the lambda fragments are 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb respectively.

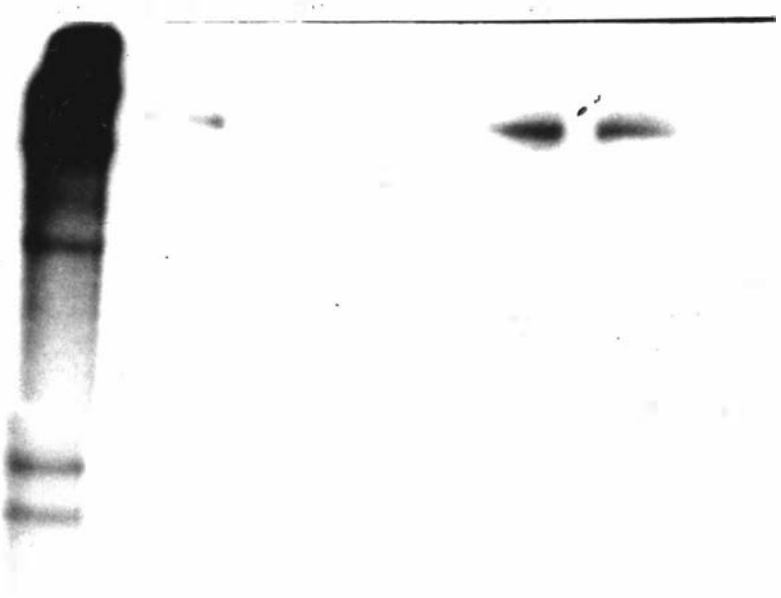
Figure 20. Southern blot of total genomic DNA digest probed with nifKDH. Lanes: a=Lambda, b=2668, c=MO 103, d=104, e=110, f=111. The hybridizing band is 5.2 kb. The sizes of the lambda fragments are 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb respectively. The blot is of the gel shown in figure 19, stripped and re-probed.

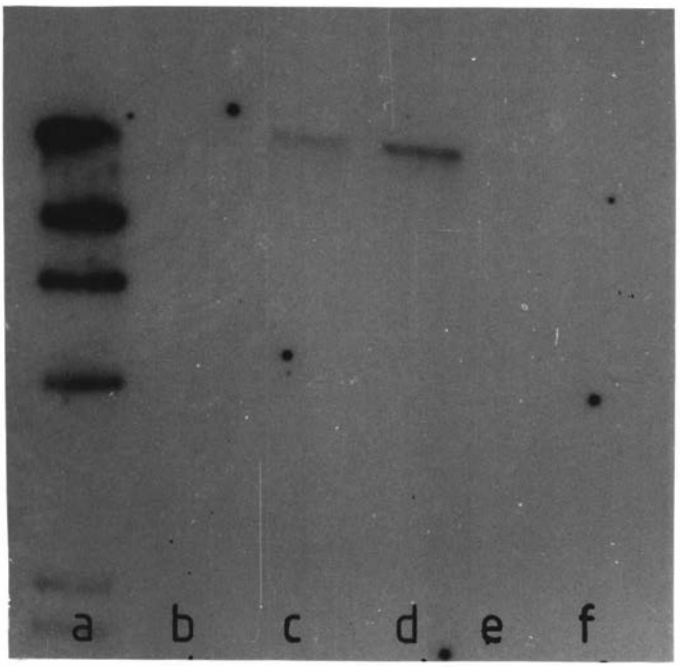

a
b
c
d
e
f


a
b
c
d
e
f

Figure 21. Southern blot of total genomic DNA digest probed with nodABC. Lanes: a=Lambda, b=2668, c=MO 103, d=104, e=110, f=111. The hybridizing bands are 7.2, 1.2 and 1.0 kb. The sizes of the lambda fragments are 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb respectively. The blot is of the gel shown in figure 19.

Figure 22. Southern blot of total genomic DNA probed with RP4. Lanes: a=Lambda, b=2668, c=MO 103, d=104, e=110, f=111. The hybridizing band is ≈ 60 kb. The sizes of the lambda fragments are 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb respectively. This blot is not from the gel shown in figure 19, but the track loadings are comparable


a b c d e f


a b c d e f

The lack of hybridization in MO 103/104 and the alteration of the plasmid profile is consistent with a loss of pSym or at least the symbiotic genes. However, it is not clear where the nodABC and nifKDH genes observed in MO 110/111 have come from. The apparent disappearance and reappearance of pSym (or parts of it) was treated with some scepticism, yet in four trials inoculation with MO 103/104 resulted in nodules from which were isolated MO 110/111. Inoculation of plants with MO 105 resulted in Cm^R MO 110/111 strains reisolated from the nodules. Similarly inoculation with MO 107 and MO 109 resulted in the reisolation of Rif^R and Sm^R MO 110/111 strains, respectively (fig. 1).

It must be remembered that although the plants are inoculated with MO 103/104 what is recovered is a strain with the plasmid/restriction profile of MO 110/111. This rearrangement includes the loss of RP4, but whether this occurs during the plant infection process or beforehand is unknown. Also unknown is whether the plant is acting to trigger the rearrangement of MO 103/104 to yield MO 110/111 which then nodulate or if the MO 103/104 rearrangement occurs spontaneously in culture and the plant selects the strains that can nodulate.

There is a clear contradiction observed with the results of this section. On the one hand, the restriction endonuclease and plasmid profiles of 2668, MO 103/104 and MO 110/111 are very stable in laboratory subculture and electrophoretic profiles of the strains made three years apart are indistinguishable. Yet, under appropriate conditions rearrangement can be recovered. Presumably the stimulus that causes the alteration of 2668 to yield MO 103/104 is the introduction of RP4, although the evidence for this is circumstantial and no mechanism has been proposed which would explain the observed rearrangements. In the case of the alteration of MO 103/104 to yield MO 110/111, the rearrangement seems to occur very readily, but it is unclear whether the stimulus is provided by the plant or whether it is some other endogenous or exogenous factor.

The hybridization studies with nodABC and nifKDH and the plant

inoculation tests suggest that pSym is not detectable by conventional hybridization (no bands in lanes b and c, figs. 16 and 17 nor lanes c and d, figs. 20 and 21) but is nevertheless present in MO 103/104 cultures in some hidden but retrievable form (they nodulate). If the symbiotic genes were translocated elsewhere in the genome, they should still be detectable in MO 103/104 by DNA hybridization with nodABC and nifKDH probes, which they are not. If the genes had been completely lost these strains (MO 103/104) could not produce nodules when they were inoculated onto plants.

Two explanations are suggested for these results, namely the trivial explanation of carry-through of a nod^+ contaminant and the more interesting possibility of DNA archivilization. Considering first the possibility of a contaminant, it could be hypothesized that a few members of the population of MO 103/104 bacteria maintain pSym normally and although they are not detected by electrophoresis or DNA probe, they are sufficient in number to nodulate plants. It is difficult to understand how this hypothesis could be correct as the MO 103/104 strains were subjected to several rounds of very careful single colony selection, including limit dilution in buffer containing detergent to separate the cells, as described by Martinez et al (1985, see section 2.4). The MO 105, 107 and 109 strains all went through further rounds of single colony purification in the course of selection of these antibiotic resistant derivatives. If a strain was selected and purified that was maintaining pSym normally it would have been detected by the probes. Even if the genes had been displaced to another site in the genome, the nodABC and nifKDH probes of the total DNA digests would have found them. This explanation is therefore very unlikely at best. Similarly unlikely, also because of the antibiotic markers, is the possibility of a contaminant strain from the laboratory environment.

The explanation at present considered the most likely, although mainly by default, is DNA archivilization. It is possible that the DNA is still present somewhere in the genome, therefore the strains can still nodulate, but is in a form which cannot be detected by

conventional hybridization. Such an archival form of DNA was proposed in order to explain the liberation of a P22 bacteriophage from a strain of Salmonella typhimurium which manifested none of the characteristics to be expected of a P22 lysogen, such as immunity or hybridization with P22 probes (Downs and Roth, 1987). If the symbiotic DNA is archived then either the interaction with the plant can trigger de-archivilization or a low frequency of bacteria per generation spontaneously undergo de-archivilization and they are selected by the plant for nodule initiation. The rearrangement of MO 103/104 to yield the MO 110/111 form is hypothesized to be a consequence of this process. However, what actually is involved in the initial archivilization of DNA, if it does occur at all, is not known.

At this stage there is insufficient evidence to propose a mechanism which might explain the apparent disappearance and subsequent reappearance of the symbiotic genes. A number of trials have been performed and all confirm the initial observation, namely, inoculation of a plant with a strain of bacteria that does not appear to carry symbiotic genes (MO 103/104) is followed by the formation of nodules from which are isolated strains (MO 110/111) derived from the Sym^- form which now carry and express the symbiotic genes. The inability to detect the symbiotic DNA and to devise experiments which could adequately test the hypothesis of archived DNA led reluctantly to abandonment of this section of the project, until such a time as rigorous and informative experiments could be devised. Some possible experiments are suggested in the conclusions.

3.6 Transfer of the Symbiotic Plasmid of **R. leguminosarum biovar trifolii** 2668 to a Range of Recipients and Expression of Symbiotic Genes

This section of the project was an attempt to label pSym with a selectable marker (Tn5) and to then see if other bacterial strains, in particular certain strains isolated from the soil, could accept

and express pSym. This section was designed to expand on the work of Jarvis *et al* (1989) by investigating the ability of pSym to transfer without the involvement of a broad host range carrier plasmid.

3.6.1 Conjugation of 2668::Tn*5* and Nod$^-$ soil bacteria

The recipients chosen for the initial cross between 2668::Tn*5* were four strains of soil bacteria which could express pPN1 (a biovar *trifolii* pSym Rtr5a/R68.45 cointegrate), the *nodABC*-carrying plasmid used by Jarvis *et al* (1989). The conditions of the cross and frequencies of transfer of pSUP1011::Tn*5* into 2668 and then transfer of a plasmid-borne Tn*5* from 2668::Tn*5* to the soil bacteria are discussed in section 3.1. An attempt was made to more clearly identify the soil microorganisms (discussed in section 3.2) but this was unsuccessful.

Many of the experiments listed in the following section appear to be cursory, but they were conceived more as a survey than a thorough evaluation of the consequences of transfer to a variety of hosts to the structure of pSym. For this reason very few isolates were examined at each step. In the cross between 2668::Tn*5* and the soil bacteria only one colony from each cross was examined and in the light of subsequent results this was insufficient. It is accepted that for this reason it is not possible to draw conclusions concerning the potential range of rearrangements. However, it must again be emphasized that the survey was designed to answer two general questions:

1. Is pSym of 2668 mobilizable to other strains without the presence of either a helper plasmid or a cointegrated self-mobilizable plasmid?

2. Is it expressible in the recipient strains?

The observation of the various plasmid rearrangements in the few isolates examined was not expected, and in hindsight a thorough investigation of the fate of pSym in one cross may have been the more interesting and perhaps more fruitful project. The reader is asked to bear in mind the small sample size in the following report but it should also be remembered that no special attempt was made to isolate

rearranged strains and yet such strains were observed regularly. This implies that DNA rearrangement is very frequent under these conditions of transfer between different strains or species.

In the crosses between 2668::Tn*5* and the soil bacteria it was possible to isolate neomycin resistant derivatives of the soil bacteria at about 1 per 10^4 cells. In all of the 120 series strains (where 2668::Tn*5* X NR 41 yields MO 121 etc, see section 3.1) one new plasmid was initially observed in the exconjugant soil bacteria (fig. 23). However, the plasmid profiles of the 120 series were unstable and subsequent Eckhardt plasmid electrophoresis profiles often showed multiple new bands. This was particularly obvious with MO 122 (compare fig. 23 lane f with fig. 30 lane d) and MO 124 (compare fig. 23 lane j with fig. 30 lane h). The source of the extra bands seen in tracks of MO 122 and 124 is not known but they could be artefactual open-circular forms of the normal covalent closed circular plasmid DNA, deletion derivatives (such as occurs in the initial transfer of the Sym-derivative from 2668 to the soil bacteria), rearranged variants of the transferred plasmid or multimeric forms (which might look like figs. 30 and 31, lane h).

Southern blots of the Eckhardt gels were probed with pSUP1011::Tn*5* in order to determine whether or not the new plasmid bands were derived from 2668::Tn*5* and without exception the pSUP1011::Tn*5* probe hybridized to the new plasmid/s (fig. 24 lanes d,f,h and j) in MO 121-124. In the case of MO 122 three bands hybridized to the pSUP1011::Tn*5* probe and there appears to be more than one hybridizing band in MO 123. Therefore all of these new plasmids were transferred from 2668 to the soil bacteria, or at least one plasmid was transferred and the rest generated through rearrangement within the recipient. Hybridization analysis with the PN 435 (*nifKDH* gene cluster in pBR328) probe identified the symbiotic plasmid of 2668 (fig. 25, lane a) and showed that all of the new plasmids of MO 121-124 also carried this sequence (lanes d,f,h and j) but that it wasn't present on any of the plasmids of the parent soil strains NR 41 (lane b), NR 42 (lane e), NR 64 (lane g) or OR 168

(lane i). Hybridization with the PN 291 (nodABC gene cluster in pBR328) probe identified the same plasmid band as the PN 435 (nifKDH) probe did in 2668 (fig. 26, lane a) and in the exconjugant 120 series (lanes d,f,h and j) with MO 122 showing three bands of hybridization and MO 123 possibly two. The PN 291 (nodABC) probe also identified all of these bands. No bands of hybridization are observed in the parent soil strains (lanes b,e,g and i). Taken together, the hybridization patterns of the 120 series with the three probes suggest that each of the new bands is a Tn5-marked derivative of pSym. It is clear from the relative sizes of the plasmids in 2668 and the exconjugants (e.g. compare fig. 23, lanes a and d) that only a portion of pSym has transferred to the recipients.

One other result of some interest is that of the four plasmids in 2668, only pSym appears to be mobile. All of the mobile bands were shown to be derived from 2668 by virtue of the Tn5 hybridization (fig. 24). The mobile bands also hybridized to nodABC (PN 291, fig. 26) and nifKDH (PN 435, fig. 25). Hybridization with nodABC (fig. 26) and nifKDH (fig. 25) to 2668 only identifies one pSym band, so there is only one possible donor. The hybridization data suggests that all of the new bands in the 120 series are derived from pSym and therefore that only pSym is mobile. It is not clear why only pSym should be mobile but the ecological implications are discussed in section 4.6.

The total genomic DNA was isolated from 2668, the soil bacteria and the exconjugants from the cross between 2668::Tn5 and the soil bacteria. These DNA samples were digested with EcoRI and subjected to gel electrophoresis (fig. 27). The soil bacteria and the exconjugants are basically identical in restriction profile with the exception of a single large band in each of the exconjugant strains (e.g. compare fig. 27 lane c and d). Presumably this large band is part of the transferred plasmid. However, this large band is not seen in 2668, from whence it is assumed to have come, which suggests there has been some rearrangement involving some of the EcoRI restriction enzyme sites. Alternatively, some methylation of the DNA might be protecting

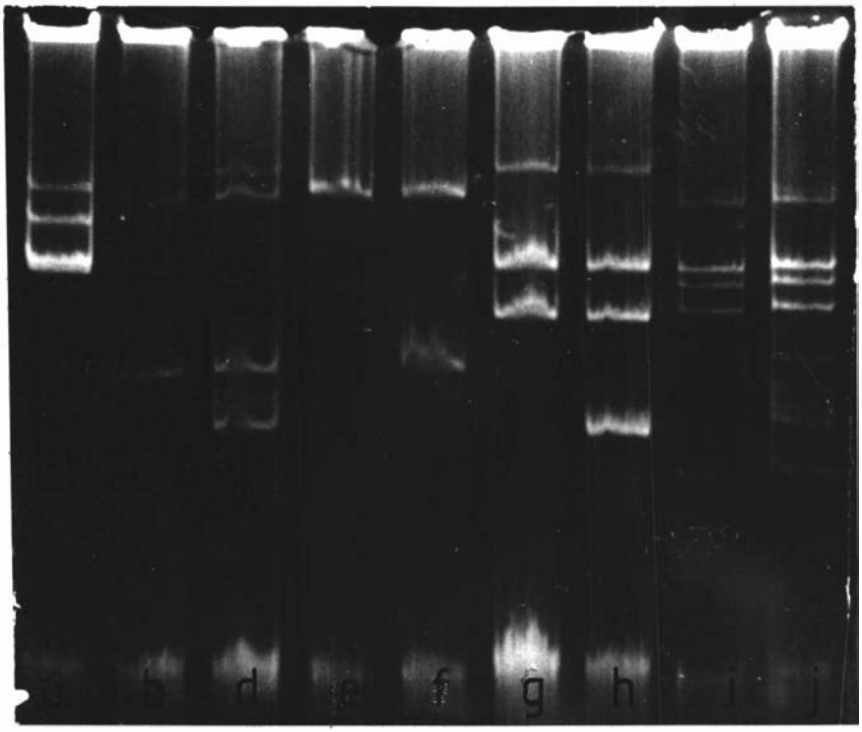
some of the sites.

The total genomic DNA restriction digest profiles of these bacteria were transferred to nitrocellulose and probed with Tn*5* and *nodABC*/*nifKDH*. The Tn*5* probe, which was pSUP1011::Tn*5*, identified single bands in MO 123 (fig. 28, lanes h and j), a poorly defined band in MO 121 (fig. 28, lane d) and nothing in MO 122 (fig. 28, lane f). The *nodABC*/*nifKDH*, which included the vector pBR328 DNA, detected single large bands in MO 121 and MO 123 (fig. 29, lanes d and h), the expected 5.2 kb band for *nifKDH* and 7.2 kb band for *nodABC* plus two bands at 1.2 and 1 kb in 2668 and MO 124 (fig. 29, lanes b and j), and nothing in MO 122 (fig. 29, lane f). Several independant blots showed the same results as are presented in figures 28 and 29. The blots are no where near as clear as those obtained with the Eckhardt plasmid gels (figs. 24-26). Despite the reproducibility of the total genomic DNA restriction digest profile blots, these results should be interpreted cautiously until clearer autoradiographs are obtained.

It should be noted that for this series of blots, in contrast to part I, fragments containing *nodABC*, *nifKDH* and Tn*5* in part II were not isolated and whole plasmids were labelled and used as probes. Despite the possibility of cross hybridization between pSUP1011::Tn*5* and pBR328, we have no reason to believe our results are invalidated, mainly because these strains form nodules. It was considered possible that pSUP1011 integrated *in toto*, rather than acting as a suicide vector and serving only as a delivery vehicle for Tn*5*. This is thought to be unlikely for two reasons. Firstly, pSUP1011 is not reported to do so (Simon *et al*, 1983; numerous other studies using Tn*5* marking). Secondly, the strains do not show any resistance to chloramphenicol (see table 5), which is carried by pSUP1011.

Figure 23. Eckhardt plasmid profile of the parents and exconjugants from the cross between 2668 and the soil bacteria. Lanes:a=2668, b=NR 41, d=MO 121, e=NR 42, f=MO 122, g=NR 64, h=MO 123, i=OR 168, j=MO 124. The 2668 band sizes are approximately 600, 356, 199 and 188 respectively. Track label c has been left out to correspond with the Southern blots.

Figure 24. Southern blot of the Eckhardt gel probed with pSUP1011::Tn5. Lanes: a=2668, b=41, c=blank, d=121, e=42, f=122, g=64, h=123, i=168, j=124. This blot is not from the gel shown in figure 23 and has been stripped and re-probed from the blot shown in figure 26.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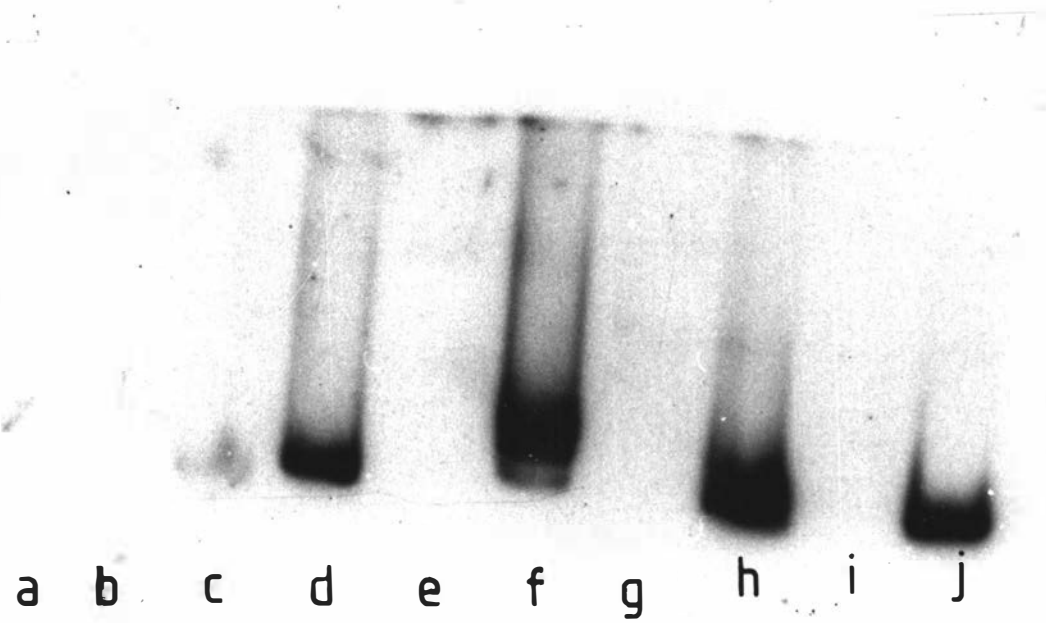
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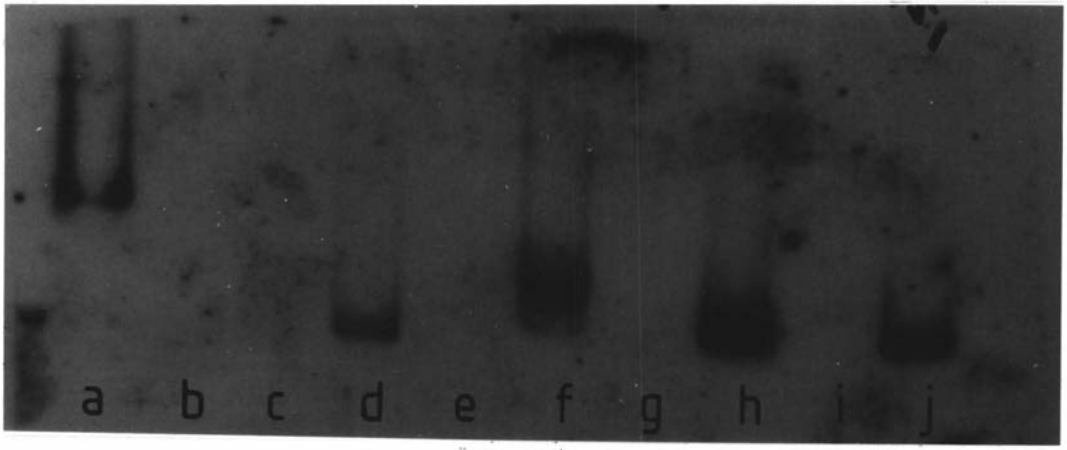
Figure 25. Southern blot of an Eckhardt gel probed with nif KDH in pBR328. Lanes: a=2668, b=41, c=blank, d=121, e=42, f=122, g=64, h=123, i=168, j=124. The 2668 band is approximately 188 kb. This blot is not from the gel shown in figure 23 and has been stripped and re-probed from the blot shown in figure 26.

Figure 26. Southern blot of of an Eckhardt gel probed with nodABC in pBR328. Lanes: a=2668, b=41, c=blank, d=121, e=42, f=122, g=64, h=123, i=168 and j=124. This blot is not from the gel shown in figure 23.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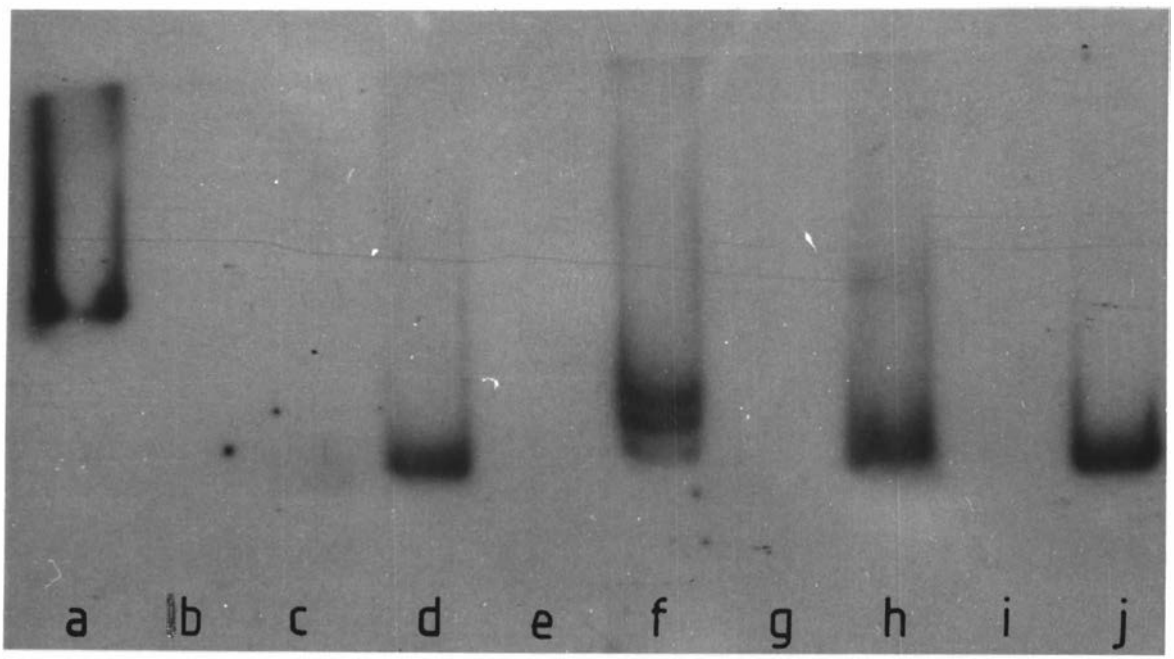
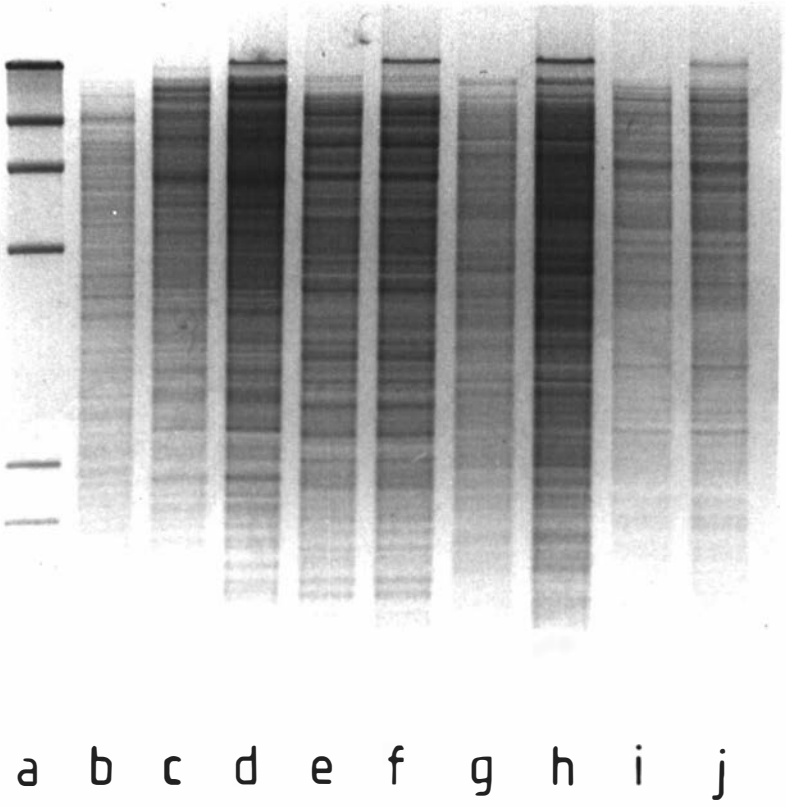

a b c d e f g h i j

Figure 27. Total genomic DNA restriction digest profile using the enzyme EcoRI of DNA isolated from the parental and exconjugant strains from the 2668/soil bacterium cross. Lanes: a=lambda HindIII standard, b=2668, c=NR 41, d=MO 121, e=NR 42, f=MO 122, g=NR 64, h=MO 123, i=OR 168 and j=MO 124. The sizes of the lambda pieces are 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb respectively.

Figure 28. Southern blot of total genomic DNA restriction digest profile using the enzyme EcoRI of DNA isolated from the parental and exconjugant strains from the 2668/soil bacterium cross. Lanes: a=lambda HindIII standard, b=2668, c=NR 41, d=MO 121, e=NR 42, f=MO 122, g=NR 64, h=MO 123, i=OR 168 and j=MO 124. The sizes of the lambda pieces are 23, 9.4, 6.6, 4.4, 2.3 and 2.0 kb respectively. This blot is not from the gel shown in figure 27. The probe is pSUP1011::Tn5.


a b c d e f g h i j

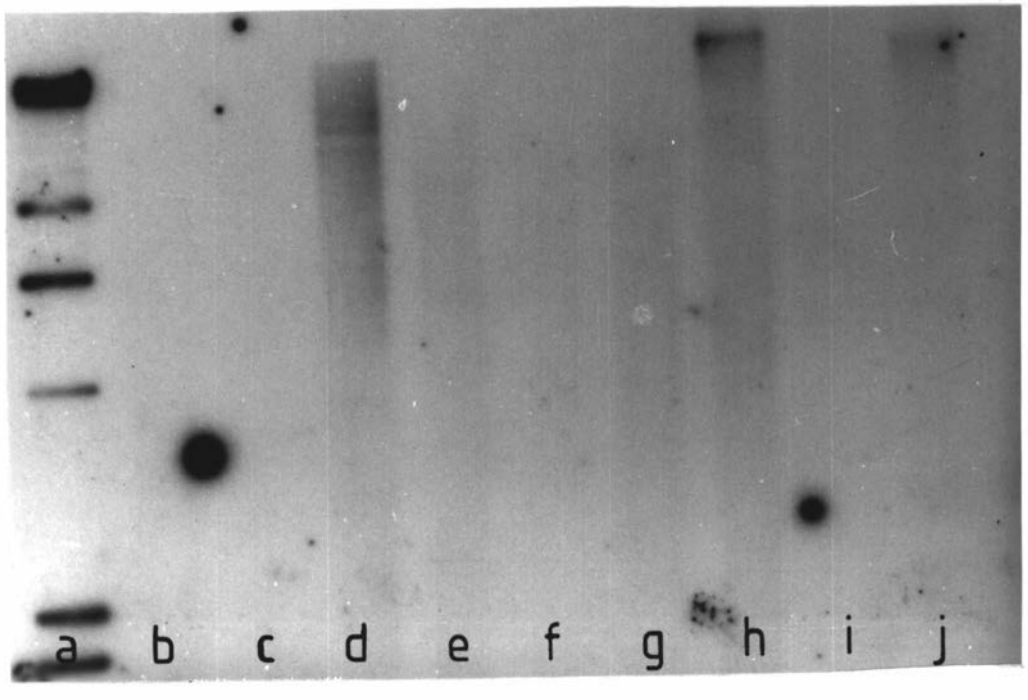
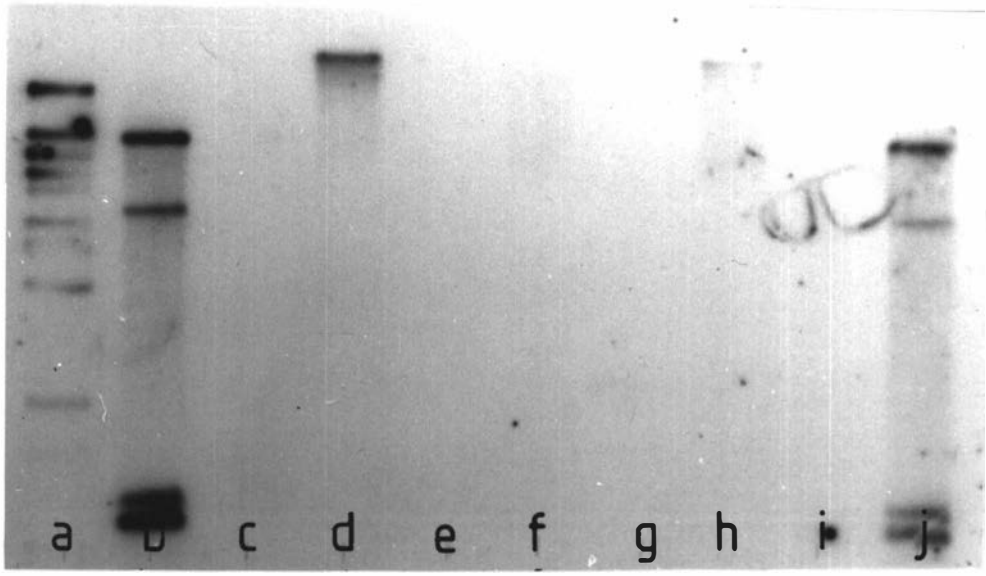

a b c d e f g h i j

Figure 29. Southern blot of total genomic DNA restriction digest profile using the enzyme EcoRI of DNA isolated from the parental and exconjugant strains from the 2668/soil bacterium cross. Lanes: a=lambda BstEII standard, b=2668, c=NR 41, d=MO 121, e=NR 42, f=MO 122, g=NR 64, h=MO 123, i=OR 168 and j=MO 124. The sizes of the lambda pieces are 8.4, 7.2, 6.7, 6.4, 5.7, 4.8, 4.3, 3.6, 2.3 and 1.9 kb respectively. This blot is not from the gel shown in figure 27. The probes are nodABC in pBR328 and nifKDH in pBR328 combined.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3.6.2 Conjugation between the 120 series and a Nod$^-$ biovar trifolii strain, PN 104

The second cross series was between the four 120 series bacteria and the pSym$^-$ rhizobial strain, PN 104. The selection and frequency of transfer of the pSym-derivative from the 120 series to PN 104 is discussed in section 3.1. There was some interest in the ability of the 200 series (where MO 121 X PN 104 yields MO 201 etc) to nodulate and fix nitrogen as PN 104 is a known R. leguminosarum biovar trifolii strain (discussed in section 3.4).

The pSym-derived plasmids observed in the 120 series were able to transfer to PN 104 (fig. 30). It is still not clear whether or not these plasmids are self-transmissable as any of the plasmids of the 120 series donors might be providing the transfer functions. A single band of hybridization with both pSUP1011::Tn5 and PN 291 (pBR328 with a nodABC insert) is observed in MO 201 (figs. 31 and 32, lane c) corresponding approximately in size to the band in MO 121 (figs. 31 and 32, lane b). MO 123 shows two hybridizing bands (figs. 31 and 32, lane f) but transfers only one to yield MO 203 (figs. 31 and 32, lane g). MO 124 shows four or five bands of hybridization (fig. 31) and transfers two bands to yield MO 204 (figs. 31 and 32, lane i). MO 122 shows three bands of hybridization (fig. 31, lane d) and transfers all three to yield MO 202 (figs 31 and 32, lane e). PN 104 does not hybridize to either probe (figs. 31 and 32, lane j).

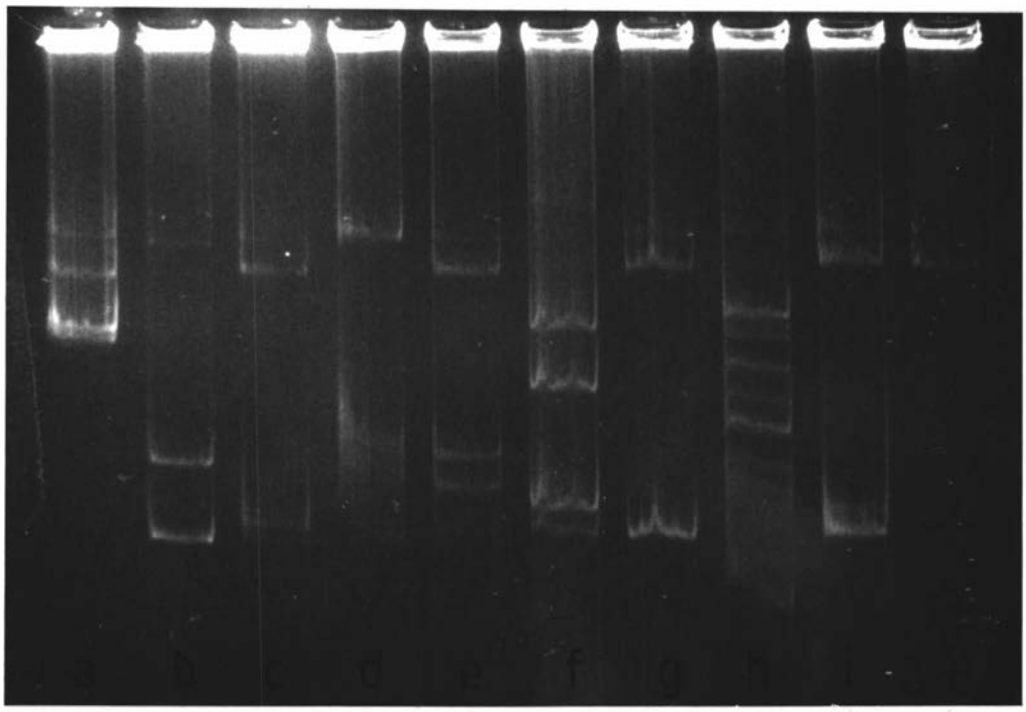
At present it is not possible to state the origin of the multiple bands in the 200 series with any certainty. They may have all arisen in the donor 120 series and transferred during conjugation or a single band may have transferred and undergone some unknown changes to generate the multiple bands. The question is therefore either one of transfer of multiple bands or of alteration/rearrangement of the plasmid after transfer or possibly a combination of the two. However, the plasmid profile of the 200 series remained stable on laboratory subculture over a six month period. Secondly, the bands in the 200 series appear very similar in size to their counterparts in the 120 series and unless the type of

rearrangement occurring is very specific and only yields certain plasmid sizes, it could cautiously be suggested that the multiple plasmid bands observed in the 200 series arose as the result of conjugation.

The symbiosis of the 200 series and its results for plants was discussed in section 3.4. The poor nodulation and appearance of MO 204 (fig. 9) could be due to the loss or inactivation of symbiotically important genes (other than nodABC) as a result of passage through the soil bacterium OR 168 but with the small sample size no firm conclusions can be drawn. In general, the efficiency of the symbiosis is better in the 200 series than in the 120 series.

Figure 30. Eckhardt plasmid profile of the parents and exconjugants from the cross between the 120 series of modified soil bacteria and R. leguminosarum biovar trifolii PN 104. Lanes: a=2668, b=MO 121, c=MO 201, d= MO 122, e= MO 202, f=MO 123, g=MO 203, h=MO 124, i=MO 204, j=PN 104. The sizes of the plasmids in 2668 are approximately 600, 365, 199 and 188 kb respectively.

Figure 31. Southern blot of an Eckhardt gel probed with pSUP1011::Tn5. Lanes: a=2668, b=MO 121, c=MO 201, d= MO 122, e= MO 202, f=MO 123, g=MO 203, h=MO 124, i=MO 204, j=PN 104. The blot is not derived from the gel shown in figure 30.



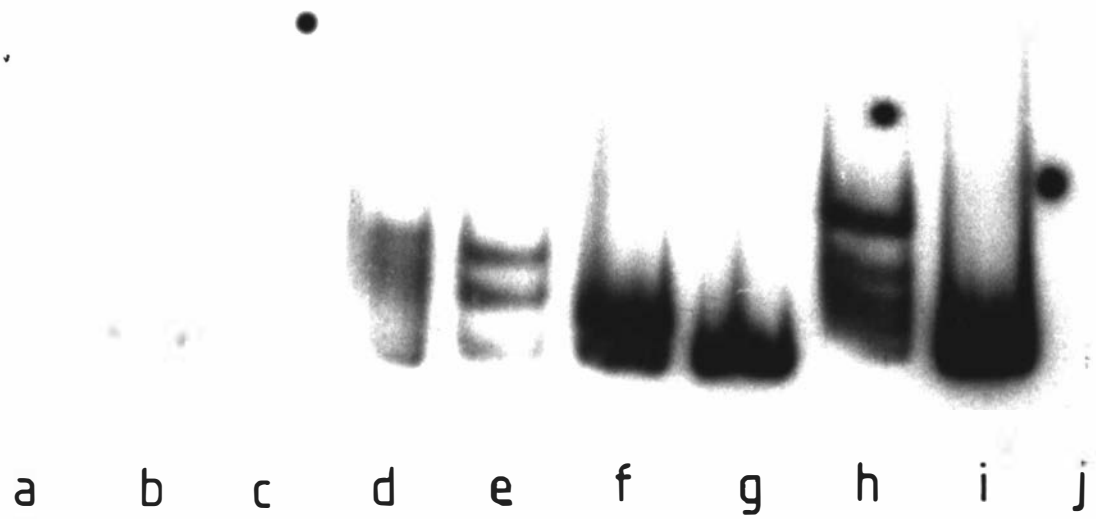

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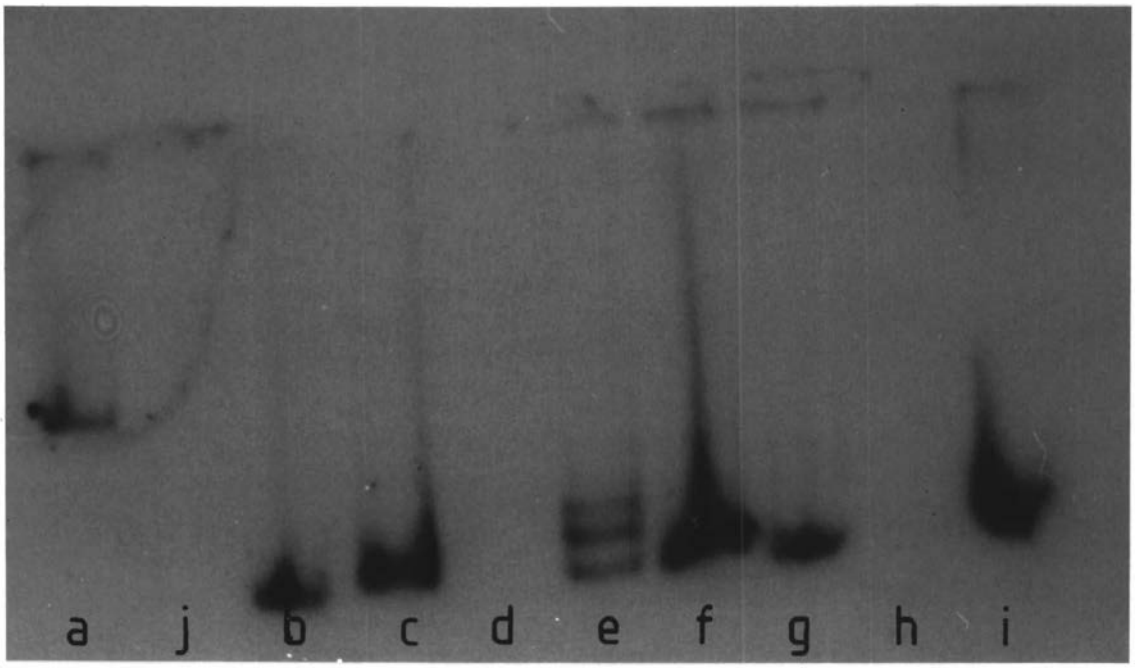
Figure 32. Southern blot of an Eckhardt gel probed with nodABC in pBR 328. Lanes: a=2668, b=MO 121, c=MO 201, d= MO 122, e= MO 202, f=MO 123, g=MO 203, h=MO 124, i=MO 204, j=PN 104. The blot is not derived from the gel shown in figure 30.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3.6.3 Conjugation between the 120 series and *E. coli* HB101

One aim of this project was to develop a laboratory model which could explain the results from an earlier field trial. This aim has been uppermost in priority throughout this project and especially in the cross between the 120 series and *E. coli* HB101. This cross was designed to act as a test of the pSym to cross intergeneric barriers and to transfer pSym to a host which had no other plasmids in order to facilitate characterization of pSym.

The Tn*5*-marked pSym transferred from the 120 series to HB101 to yield the 300 series. The 300 series exconjugants were examined by the Eckhardt gel electrophoresis procedure (fig. 33) and a Southern blot of the Eckhardt probed with PN 291 (*nodABC* in pBR328, fig. 34). In all but one case (MO 302, figs. 33 and 34, lane g) only a single plasmid is transferred from the 120 series donor, despite multiple bands of hybridization in the donors MO 123 (figs. 33 and 34, lane d) and MO 124 (figs. 33 and 34, lane e). The PN 291 (*nodABC*) probe hybridizes to pSym in 2668 (fig. 34, lane a), to the single bands in MO 301 (lane f), MO 303 (lane h) and MO 304 (lane i), the two bands of MO 302 (lane g) and the various bands of the 120 series donors (lanes b, c, d and e), but not to HB101 (lane j). This indicates that the *nodABC* genes are carried on the pSym derivative transferred from the 120 series.

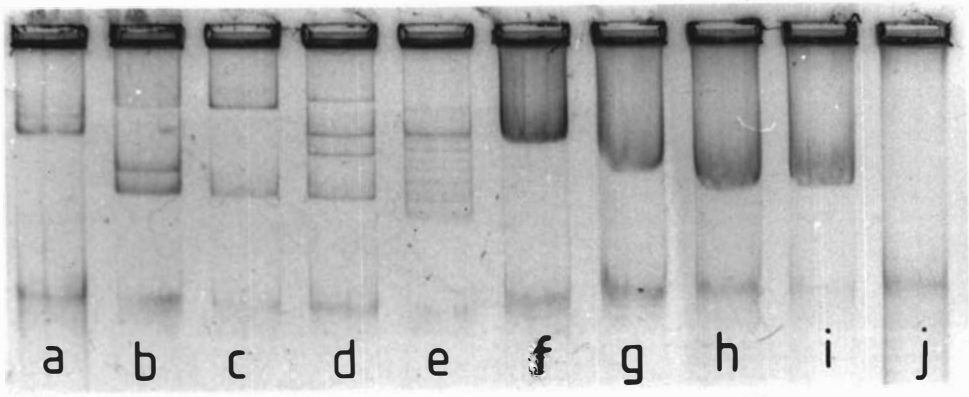
However, in MO 301, 302 and 303 (Figs. 33 and 34, lanes f, g and h) the new band in HB101 which hybridizes to *nodABC* is not the same size as the hybridizing band/s in the donor. This is particularly obvious when the donor MO 121 (figs. 33 and 34, lane b) and exconjugant MO 301 (figs. 33 and 34, lane e) are compared. The plasmid band observed in MO 301 is of similar size to pSym of 2668 and considerably larger than the pSym derivative of MO 121. At least one of the bands of MO 302 (figs. 33 and 34, lane g) is larger than the bands of the donor MO 122 (figs. 33 and 34, lane c). Similarly the band seen in MO 303 (fig. 33 and 34, lane h) seems larger than either of the bands in MO 123 (figs. 33 and 34, lane d). Some dramatic rearrangements involving size changes are occurring during

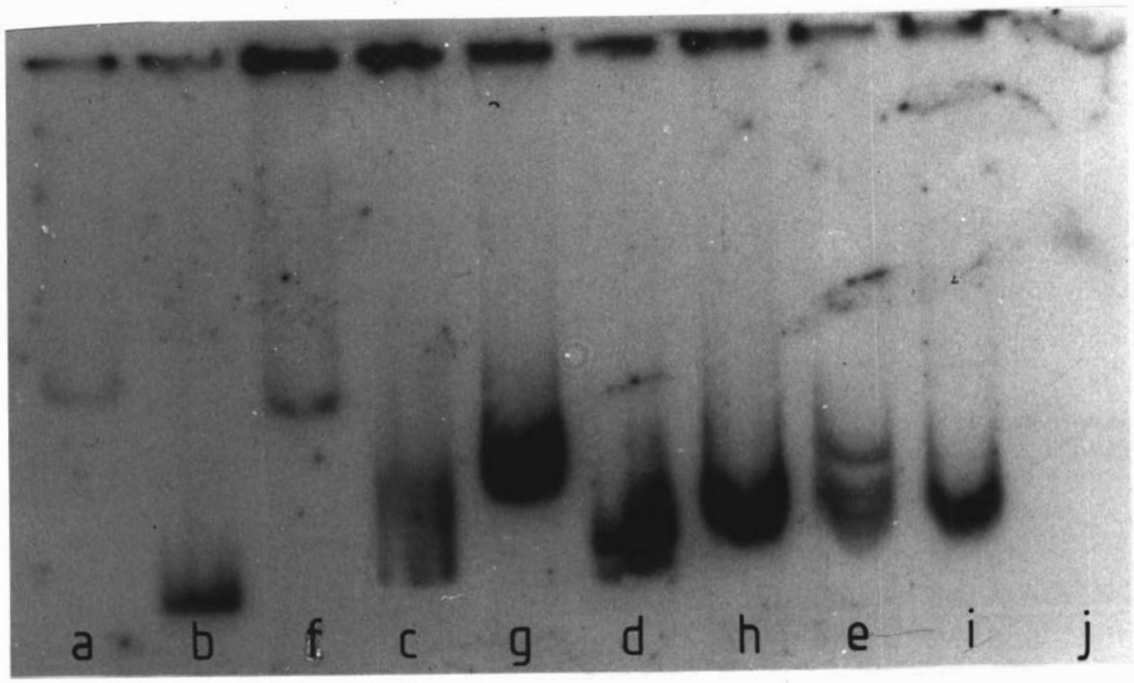
or as a result of this cross (see section 3.6.5).

The 300 series cross demonstates that the pSym-derivative of the 120 series is a broad host range plasmid. It is still not clear whether the pSym of 2668 is a broad host range plasmid. All of the plasmids involved in transfer are deletion derivatives of pSym of 2668 and the deletion may be required to "activate" the plasmid for transfer. Earlier, unpresented data involving conjugation of 2668 with other rhizobia suggested that the transfer frequency of pSym was 20-50 times lower than was observed for the cross between the 120 series and PN 104. The activation of the pSym-derivative could involve either the reducton of the plasmid size to an appropriate level or switching on specific genes by deletion of intervening DNA, as is seen with nifKDH of Anabaena (Golden et al, 1987).

Figure 33. Eckhardt plasmid profile of the parents and exconjugants from the cross between the 120 series of modified soil bacteria and E. coli HB101. Lanes: a=2668, b=MO 121, c=MO 122, d=MO 123, e=MO 124, f=MO 301, g=MO 302, h=MO 303, i=MO 304, j=HB101. The 2668 plasmids are approximately 600, 356, 199 and 188 kb respectively.

Figure 34. Southern blot of an Eckhardt gel probed with nodABC. Lanes: a=2668, b=MO 121, c=MO 122, d=MO 123, e=MO 124, f=MO 301, g=MO 302, h=MO 303, i=MO 304, j=HB101. The 2668 band is approximately 188 kb. The blot is not derived from the gel shown in figure 33.


a b c d e f g h i j


a b f c g d h e i j

3.6.4 Conjugation between MO 121 and a Nod$^+$ biovar *trifolii* strain, 2668

The final cross between MO 121 and 2668 was done in order to answer two questions:

1. Does the small plasmid of MO 121 still carry the incompatibility determinants and what will happen to pSym if it is transferred back into 2668?

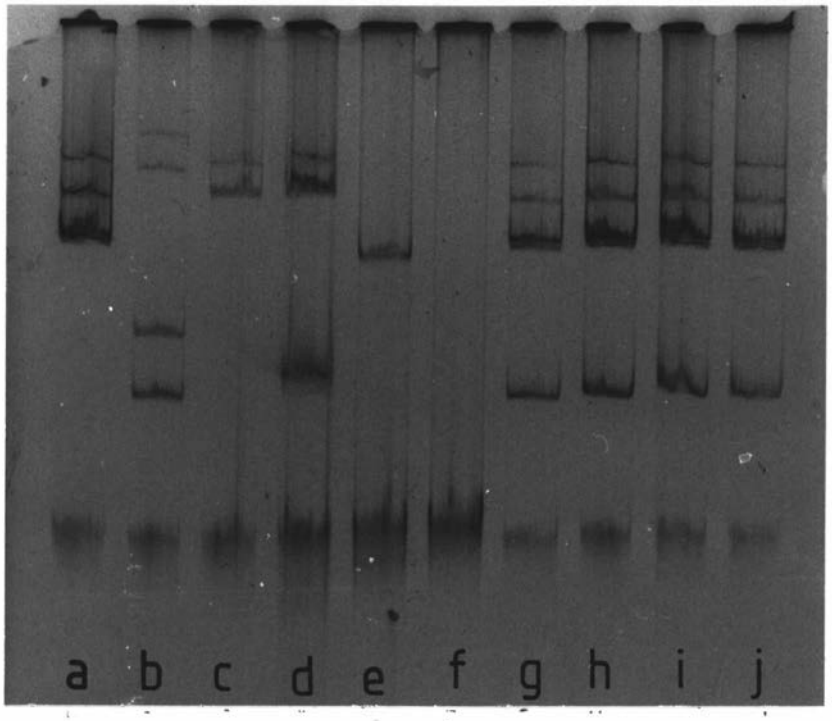
2. If the pSym of MO 121 is compatible with pSym of 2668, what will be the effect of two copies of the symbiotic plasmid?

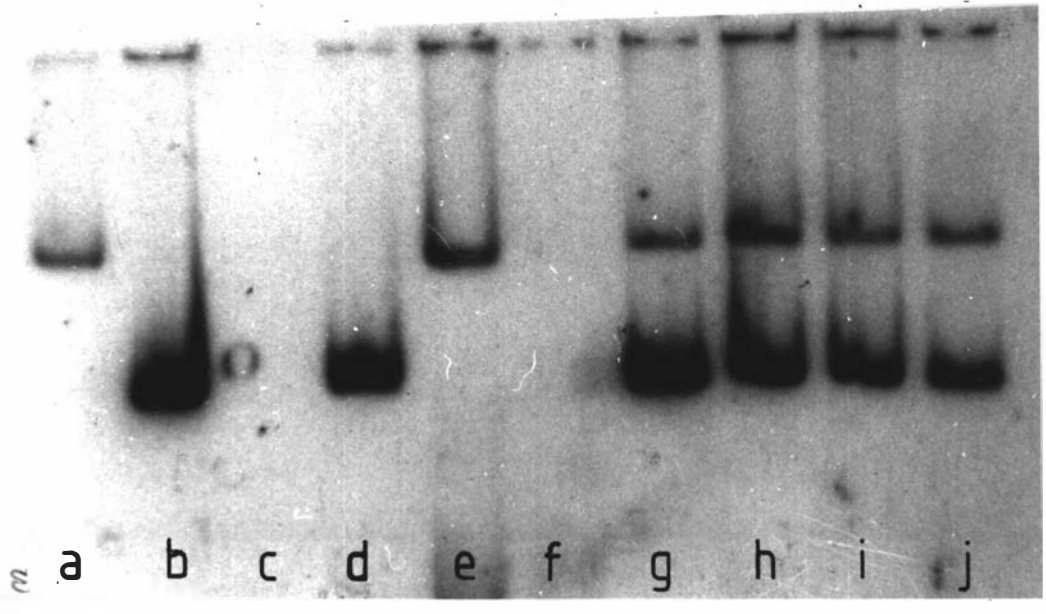
The answer to question 1 is shown clearly in figure 35, which shows the entire lineage of the pSym-derivative throughout one series of crosses. Lane a is 2668 (the original donor), lane b is MO 121 (the exconjugant from the cross 2668::Tn*5* X NR 41), lane c is the pSym$^-$ *Rhizobium* PN 104, lane d is MO 201 (the exconjugant from the cross MO 121 X PN 104), lane e is MO 301 (the exconjugant from the cross MO 121 X HB101), lane f is HB101 and lanes g-j are exconjugants from the cross MO 121 X 2668 (the 810 series). The 810 series show the presence of pSym of 2668 and the pSym derivative from MO 121. This band is confirmed as a pSym plasmid when a PN 291 (*nodABC* in pBR328) probe is hybridized to a Southern blot of MO 810-813 (fig. 36, lanes g-j). This suggests that the plasmid in MO 121 no longer has the same incompatibility determinants as pSym of 2668 and clearly answers question 1.

The answer to the second question was answered by plant tests and is discussed in detail in section 3.4. After this work was performed we became aware of the work of Harrison *et al* (1988) who suggested that multiple copies of pSym inhibited nitrogen fixation efficiency. We do not have enough samples to provide a clearcut agreement or rebuttal of the proposal of Harrison and colleagues. A more detailed survey with greater numbers of plants and several different crosses would prove useful.

Figure 35. Eckhardt plasmid profile of the parents from the cross between the soil bacterium MO 121 and the original donor 2668. Lanes: a=2668, b=MO 121, c= PN 104, d= MO 201, e=MO 301, f=HB101, g=MO 810, h=MO 811, i= MO 812, j=MO 813. The 2668 plasmids are approximately 600, 356, 199 and 188 kb respectively.

Figure 36. Southern blot of an Eckhardt gel probed with nodABC in pBR328. Lanes: a=2668, b=MO 121, c= PN 104, d= MO 201, e=MO 301, f=HB101, g=MO 810, h=MO 811, i= MO 812, j=MO 813. The blot is derived from the gel shown in figure 35.


a b c d e f g h i j


a b c d e f g h i j

3.6.5 Discussion of transmissability and variation of structure of pSym.

The initial host for pSym was a group of gram negative soil bacteria isolated from a white clover-ryegrass pasture. These bacteria had some chromosomal homology to 2668 based on a colony hybridization test using the genome of a pSym$^-$ 2668 as a probe. The soil bacteria were able to accept and express pSym and were primary hosts in this study. The soil bacteria containing the pSym derivative were designated the 120 series. The 120 series strains were used as donors to the secondary hosts which were the pSym$^-$ rhizobia PN 104 (200 series), HB101 (300 series) and 2668 (810 series). The 120, 200 and 810 series bacteria showed full expression of pSym, nodulating clover and fixing nitrogen in clover nodules.

The rationale behind these crosses was to demonstrate, at least in the laboratory, that pSym could transfer to common soil bacteria and from the soil bacteria to other potential hosts. This linking of bacterial strains by plasmids is one of the central themes of the "linked gene pool" hypothesis of Reanney *et al* (1983). The demonstration that pSym has a broad host range indicates that it is a good candidate for maintaining links among bacteria in the rhizosphere by transfer among species resident there.

The variation in plasmid profile (in terms of size and number of plasmids) was observed first in the 120 series and later in the other recipients. A comparison of sizes and numbers of bands among a series of exconjugants is presented in the discussion of each cross series. In this section we will recap on these results, but instead of a horizontal comparison (among one exconjugant series e.g. 200 series) we will examine the variability vertically (e.g. 2668 to MO 121 to MO 201, MO 301, MO 810-813). The results of all the crosses are summarized in diagram 6, which shows the relative sizes of the new bands in the exconjugants. The origin of the bands is discussed below.

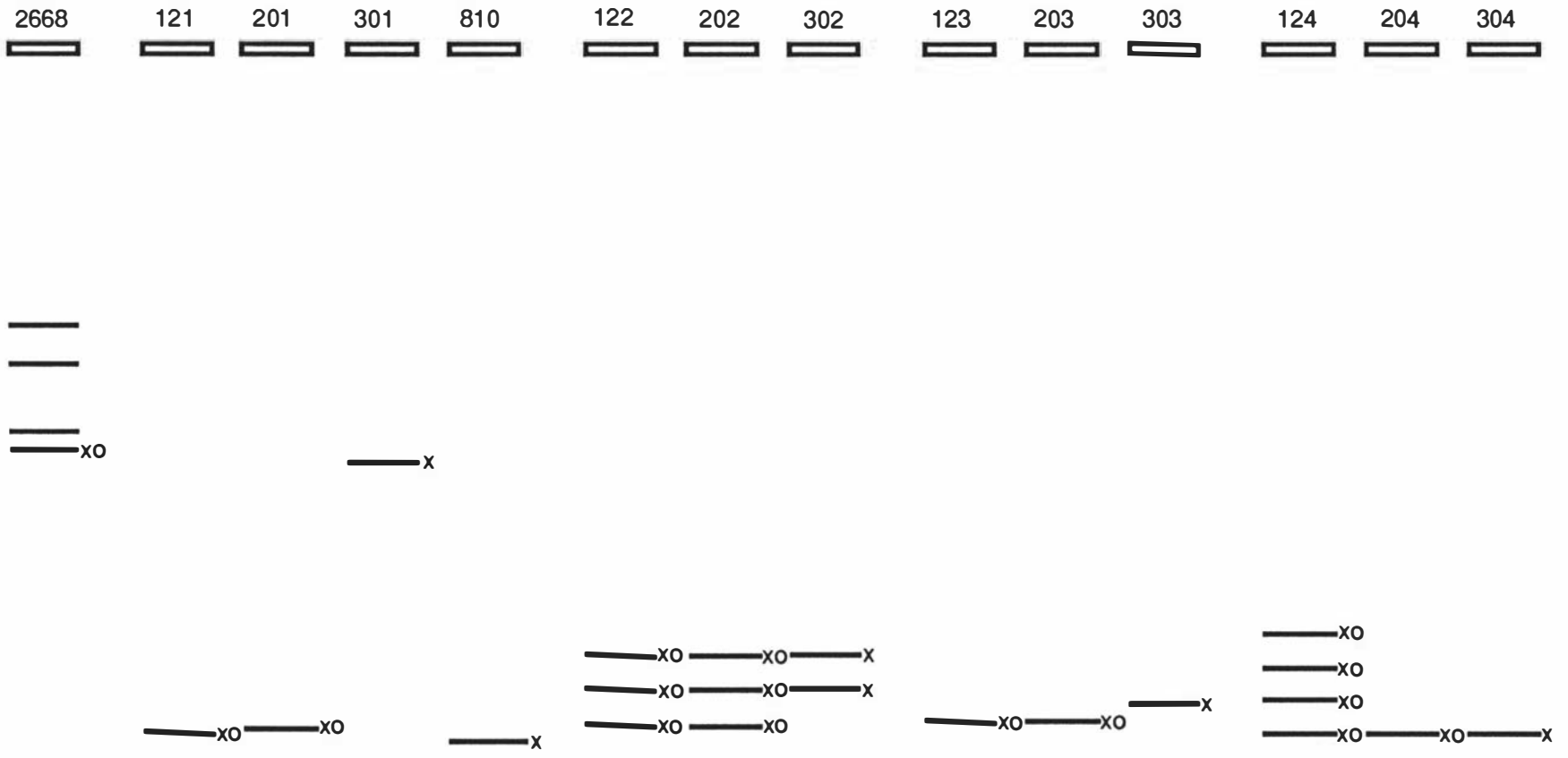


Diagram 6. Summary of plasmid profiles obtained from the crosses in part II.

x=probed with PN 291 (nodABC). o=probed with pSUP1011 (Tn5).

The pSym seen in MO 121 is smaller than the pSym of 2668, but hybridizes to Tn*5*, *nodABC* and *nifKDH* and is therefore derived from it. The pSym plasmid in MO 201, donated by MO 121, is of similar size to the pSym of MO 121. This is also true for the new pSym plasmids in MO 810-813. These plasmids all carry the *nodABC*, as demonstrated by DNA hybridization. However, the pSym in MO 301, donated by MO 121, is considerably larger than the pSym in MO 121 and more similar in size to pSym of 2668. Only a single band of hybridization with PN 291 (*nodABC* in pBR328) is seen in all of the exconjugants in this line (MO 121, 201, 301 and 810-813) but the size of the hybridizing band is variable.

The pSym seen in MO 122 is smaller than the pSym in 2668, but hybridizes to *nodABC*, *nifKDH* and Tn*5* and is therefore derived from it. Three bands are visible in MO 122, the smallest of which is about the same size as the band in MO 121. Three bands of very similar, if not identical size, are seen in MO 202. In this line the band size seems stable, but the band number varies, with initially one band in MO 122 (fig. 23, lane f) which later was observed to be three (fig. 30, lane e), three bands in MO 202 and two in MO 302. All of these bands carry *nodABC*, as shown by DNA hybridization.

The pSym seen in MO 123 is smaller than the pSym in 2668, but hybridizes to *nodABC*, *nifKDH* and Tn*5* and is therefore derived from it. The size of the band in MO 203 appears to be the same size as the band in the donor, MO 123, but the band in MO 303 appears to be slightly larger. This version of pSym, after the initial deletion from the pSym of 2668, appears to be the most stable in terms of size and number.

The pSym seen in MO 124 is smaller than the pSym in 2668, but hybridizes to *nodABC*, *nifKDH* and Tn*5* and is therefore derived from it. The strain MO 124 initially appeared to have only gained a single new plasmid band from 2668 (fig. 23, lane j) but hybridization data (figs. 24-26, lane j; 31, lane h; 34, lane e) suggested that there was four or five bands present. Two bands of similar size to the smallest bands of MO 124 were visible in MO 204 and a single band in

MO 304. This vertical series (MO 124, 204, 304) and the series of MO 122, 202, 302 were the most variable in terms of size and the numbers of plasmid bands.

Despite the variation of numbers of bands and sizes of bands seen in the Eckhardt gels and that hybridized in Eckhardt gel blots withPN 291 (nodABC in pBR328) and pSUP1011::Tn5, all of the exconjugants bar the 300 series were able to effectively nodulate plants. It is unclear whether the size and number variation seen in the various exconjugants is functionally significant or due to random rearrangement as a result of some unknown process. Investigation of the structure of the pSym derivatives in the 300 series may enable us gain some understanding of the process that formed them.

CONCLUSIONS

As a preface to this conclusion, it must be stated that we feel the information gained in the course of this project is incomplete. The first part of the project, namely the seeming disappearance and reappearance of *nod* and *nif* DNA, has been confirmed in a number of trials but no mechanism or readily testable hypothesis has been generated. The second part of the project aimed to characterize the host range of the pSym of *R. leguminosarum* biovar *trifolii* by conjugation between 2668 and a number of potential recipient strains. A variety of plasmid DNA rearrangements were observed during the analysis of the exconjugants, but the structural detail and the mechanism of these rearrangements is not yet understood.

The conclusions derived from the experimental work of this project can be summarized very loosely into four statements:

1. The genome of rhizobia can undergo rearrangement.

2. Symbiotic information is present in MO 103/104 and can be expressed under appropriate conditions.

3. The symbiotic plasmid of 2668 can transfer to and be expressed in a variety of hosts.

4. The size of the symbiotic plasmid and the number of plasmids showing homology to the symbiotic genes can vary.

These statements are used as subject headings and are expanded on in the following text. A comparison is made between our results and studies and from related work in the literature.

1. The genome of rhizobia can undergo rearrangement.

Strains of rhizobia isolated from the nodules of field grown clover plants showed a great diversity of plasmid profiles, despite inoculation with a homogeneous population of rhizobia and the lack of an already existing population of clover rhizobia in the soil. It was hoped that by using laboratory-based procedures, we could generate variants similar to those seen among the field nodule isolates. Stress of various kinds was used by Heumann *et al* (1983: 1984) to generate an enormous number and variety of variants and it was hoped that by using

the methods published by this group, we could derive variants similar to the rhizobia isolated from the previous field study (O'Hara, 1985). It was reasoned that some form of stress might cause genomic rearrangement and be responsible for the variant plasmid profiles observed. No rearrangements were observed using Heumann's methods. The one rearrangement observed was MO 103 which was isolated from among the exconjugants of a cross between 2668 and HB101(RP4). The RP4 plasmid had been introduced to act as a genetic sponge in the stress experiment as discussed in section 3.5.

The rearrangement of 2668 to produce MO 103 is assumed, for the lack of evidence to the contrary or an alternative explanation, to be due to the presence and possibly active involvement of RP4. The frequency of this rearrangement is not known but is thought to be 10^{-6} or lower on the basis of published data from similar experiments (see section 1.6). While no other experiments reported in the literature are directly comparable to this study, as they were performed for different purposes, some figures for the frequency of rearrangement and chromosomal mobilization associated with RP4 have been published. The frequency of chromosomal transfer in R. leguminosarum mediated by RP4 and R68.44 was observed to be 10^{-9} and for R68.45 to be 10^{-7} (Beringer and Hopwood, 1976). Mutation involving loss or rearrangement of RP4 due to the insertion sequence ISR1 of R. lupini has been reported to be as high as 10^{-1} (Priefer et al, 1980). Broad host range plasmids have been used as vectors and mobilizing replicons but a systematic study of frequencies of rearrangement and mutation induced by the IncPα plasmids has not been carried out.

Rearrangements involving the symbiotic genes of Rhizobium species have been reported by a number of workers and include rearrangements and recombinations of two different Sym plasmids in the same strain (Christensen and Schubert, 1983) and evidence for rearrangements in a series of natural isolates (Schofield et al, 1987). However, frequency data and systematic examination designed to elucidate a mechanism for rearrangements is only available for R. phaseoli. Symbiotic plasmids were shown to lose nod and nif genes at a high (62%) frequency but not

alter in size. Probes of nod and nif genes failed to detect homologous sequences on the rearranged plasmids (Soberon-Chavez et al, 1986). The three copies of the nif genes that are lost are spread over 120 kb of DNA and the authors suggest that the lost DNA is replaced but no source for the replacement DNA is identified. Another possibility is that the rearrangements only involve nod and nif, perhaps recombining at specific sequences associated with the symbiotic genes. It is known that reiterated sequences are associated with symbiotic genes in biovar trifolii (Scott et al, 1984). Close examination of the Sym plasmid by Soberon-Chavez and others (Soberon-Chavez and Najera, 1989b: Ollero et al, 1989) have led to suggestions of repeat sequence involvement in the Sym plasmid rearrangements.

The lack of nod and nif genes in MO 103/104 and apparent deletion of pSym was at first regarded as a rare type of rearrangement or an RP4-promoted deletion, as in most strains pSym and RP4 could coexist with no apparent alterations. Frequencies of pSym rearrangement involving loss of nod and nif can be as high as 1% in R. phaseoli (Soberon-Chavez et al, 1986). Although the actual rearrangement was of interest in regard to providing some explanation of the numerous field isolate plasmid profiles, the field isolates were all extracted from nodules and were therefore Nod^+. The Nod^- (with reference to the nodABC radioactive probe data) MO 103/104 strain was regarded, at least until the nodulation tests were performed, to be of interest but not of significance in the context of the altered plasmid profiles of the field isolated strains from the earlier study.

2. Symbiotic information is present in MO 103/104 and can be expressed under appropriate conditions.

In this section the crux of the first part of this study is reviewed but to set this discussion in context, the reader is reminded of some types of DNA reorganization, discussed in more detail in the introduction.

The most well studied set of "cryptic" genes are the bgl (β-glucoside utilization) genes of E. coli investigated by Hall and co-workers (Hall, 1983; Kricker and Hall, 1984; Parker and Hall, 1988;

Hall, 1988). Most interesting is the reactivation of an operon which codes for the utilization of salicin (a β-glucoside). The operon contains two mutations which upon reversion to wild type restores the operon to full activity at a frequency much higher than would be expected based on the reversion frequency of each mutation separately (Hall, 1988). The reactivation of the gene was only observed when the organism was grown on salicin, as if the presence of the substrate was stimulating the reactivation of the gene. Activation of some silent genes, notably promoterless genes of the histidine operon, has been shown to be considerably stimulated by the insertion of transposons (Wang and Roth, 1988). The expression of polysaccharide production genes in a marine Pseudomonas sp is controlled by the insertion and excision of a 1.2 kb DNA element (Bartlett et al, 1988). Studies of regulation and differentiation in old colonies suggest that a colony is not necessarily a homogenous accretion of bacterial cells expressing the same genes (Shapiro, 1985: 1988). Many of the studies of bacteria that have been published recently, some of which are listed above and more of which are reviewed by Terzaghi and O'Hara (in press), suggest that the bacterial genome is much more plastic than hitherto believed.

How can the apparent existence of the nod and nif genes in MO 103/104 (as manifested by the production of nodules), but their apparent indetectability (as shown by the lack of hybridization signal), be explained? At present, this contradiction (genes present but not detectable) cannot be resolved but a plausible hypothesis can be suggested, namely that the Sym genes are still present in the genome (somewhere) but are in a form of DNA which either is lost during conventional DNA preparation or which does not hybridize to probes. Such DNA has been designated "archival" by Downs and Roth (1987) and in the case they describe a Salmonella P22 bacteriophage can be induced and isolated in the supernatant of a culture but hybridization to the genome with a P22 bacteriophage probe does not detect the lysogenic sequences (see section 3.5). The authors suggest that the DNA is modified so that most restriction sites are not available for cutting and therefore the DNA does not separate electrophoretically into a form

which is accessible to DNA probes. We have no evidence to suggest that the Sym genes are archived, but we have no other plausible explanation at present for the contradictory observations. We are satisfied with the precautions we have taken to exclude the possibility of contaminant strains forming the nodules by the use of appropriate negative controls and antibiotic markers. If archivilization of the DNA is the reason that we are unable to detect Sym genes, then this raises a further question, namely what is the signal to "de-archivilize" the DNA? It is possible that the process is triggered by a signal of plant origin, such as the flavones that initiate the plant-microbe interaction, in a manner analogous to the de-cryptification of the salicin genes in the presence of salicin (Hall, 1988) but it is impossible to so state at this early stage. Alternatively, a few bacteria may spontaneously revert to the "non-archival" state and it is these bacteria which induce nodule formation. Some experiments which might shed light on the process are discussed later.

3. The symbiotic plasmid of 2668 can transfer to and be expressed in a variety of hosts.

The Sym plasmid of 2668 was labelled with Tn5 to provide a selectable marker and was shown to transfer, in a modified form, to some unclassified soil-isolated bacteria (120 series), from the soil bacteria to a pSym$^-$ Rhizobium (200 series), to E. coli HB101 (300 series) and back to 2668 (810 series). In all strains, with the exception of E. coli, the plasmid-bacteria combination is able to induce nodulation of white clover and fix nitrogen to a greater or lesser extent.

Once it became known that genes important in the plant-legume symbiosis were carried on plasmids, there was interest in the transfer of these Sym plasmids to other rhizobia and in their ability to function in the new host. Initially the transfers were between species of Rhizobium (Johnston et al, 1978) and then rhizobia and Agrobacterium, also of the *Rhizobiaceae*, (Hooykaas et al, 1981; 1982; Pankhurst et al, 1983). The nodules formed by A. tumefaciens and some

of the other rhizobia were ineffective, but the Sym plasmid of R. phaseoli could generate nitrogen-fixing nodules on plants when carried by A. tumefaciens (Martinez et al, 1987). Investigation of transfer of pSym to taxonomically more distinct recipients has shown that the Sym plasmid of biovar trifolii can transfer to a strain of Rhizobium which nodulates the tropical legume Hedysarum coronarium and allow this host to form ineffective nodules on white clover (Espuny et al, 1987). Other bacterial species have been the recipients of symbiotic genes. The nod genes of R. meliloti allowed E. coli to form pseudonodules on alfalfa (Hirsch et al, 1984) and the nod genes of biovar trifolii were able to induce the formation of nodules on clover when present in a Lignobacter sp host and nodule-like structures on clover when a Pseudomonas sp carried the nod genes (Plazinski and Rolfe, 1985). Bacteria isolated from the soil which show chromosomal DNA homology, based on an unquantified dot blot, to a biovar trifolii strain were able to form nodules on white clover if supplied with the nodulation genes (Jarvis et al, 1989). A possibly related observation was the isolation of bacteria resembling R. leguminosarum, but lacking symbiotic genes, which could nodulate peas if provided with the appropriate genes (Soberon-Chavez and Najera, 1989a). There is ample evidence presented there and from our studies to suggest that the symbiotic genes can be maintained and expressed in a variety of host backgrounds. The data from this study would further suggest that pSym (or a reduced portion of pSym) has a broad range of hosts in which it can function.

In this study, as in most others, attention has focused on pSym. It is not clear from our study whether or not pSym is self-transmissable or whether another plasmid supplies the **Tra** genes. In a few cases where the general effect of other plasmids has been examined, it seems that large numbers of plasmids in a strain can have a deleterious effect on symbiosis (Thurman et al, 1985; Harrison et al, 1988). A few functions have been attributed to the cryptic plasmids, including transmissability (Bedmar and Olivares, 1980) and an unspecified helpful effect on symbiosis (Bromfield et al, 1985) but in most cases, including this project, the function of the other plasmids

is unknown.

In most studies to date Tn*5* has been used to mark the symbiotic plasmid prior to transfer (e.g. Beringer *et al*, 1978). However, once present in a strain the Tn*5* element does not necessarily become quiescent and transposition frequencies from one site to another on the *E. coli* chromosome can be as high as 10^{-2} (Berg, 1977). Given the known mutagenic properties of transposons and other evidence suggesting that Tn*5* can activate silent genes (Wang and Roth, 1988), what is the effect of Tn*5* insertions on the symbiotic plasmids? It is possible that some (or all) of the rearrangements observed in this study (and others) are due to the effects of Tn*5*. Two copies of Tn*5* on the same molecule could provide homologous sequences for the formation of inversions, assuming no other factors prevent inversion (Segall and Roth, 1989), deletions and cointegrations. Furthermore, the actual insertions can activate or inactivate a variety of genes.

In most strains examined, the symbiotic plasmid of the rhizobial strain could be mobilized or was a self-transmissable replicon. The significance of this is discussed later in answer to the question of why the symbiotic genes should be located on a transmissable replicon.

4. The size of the symbiotic plasmid and the number of bands showing homology to the symbiotic genes can vary.

The pSym-derivative seen in the 120 series was much smaller than pSym of 2668 and remained approximately the same size in the 200 and 810 series exconjugants. Some of the plasmids of the 300 series strains had increased in size. Where tested for by hybridization the markers which for our purposes define pSym (Tn*5*, *nod*, *nif*) were still present. Variation in symbiotic plasmid size and loss of pSym, apparently as a result of conjugation, has been observed. Loss of transferred symbiotic function is observed in *R. phaseoli* either by incompatibility or as a result of plant passage (Espuny *et al*, 1989). Deletions involving the *nod* genes of pSym (*H. coronarium*) were observed after this plasmid was transferred to biovar *trifolii* (Ollero *et al*, 1989). Total loss of pSym as a result of conjugation has been observed when biovar *viceae* pJB5JI

is transferred to a biovar trifolii strain, as well as smaller scale deletions (Djordjevic et al, 1982). Interactions which result in deletion have been observed between Sym plasmids (Christensen and Schubert, 1983) as well as deletions due to some type of unspecified functional incompatibility apparently selected for by the host plant. The loss or deletion of incorrect host nodulation plasmids was frequently observed and successive plant selection resulted in more extensive deletion (Wang et al, 1986).

This is not meant to imply that deletions of pSym always occur as a result of transfer, as this is certainly not the case (Hooykaas et al, 1981; 1982) but rather that there are precedents for deletions such as those observed in this project. Several authors have suggested that repeat sequences are involved in these deletions (Soberon-Chavez and Najera, 1989b; Ollero et al, 1989). It is possible that there is an optimum plasmid size for transfer into a new host such that plasmids are trimmed either before or after transfer. We have no evidence for any mechanism or functional necessity which might account for the deletions at present, although it has been shown by Innes et al (1988) that not all of pSym is required for effective nodulation.

All of the pSym-derivatives identified by Eckhardt gel electrophoresis and DNA hybridization were initially derived from 2668. Yet, the number of bands in Eckhardt gels that hybridized and transferred in the various crosses was very variable. The variation was more similar to the variability observed between field isolated strains of rhizobia, than between laboratory isolates. The number of plasmids in as few as 21 isolates has been shown to be as disparate as 2 and 10, but how many of these were pSym bands was not determined (Thurman et al, 1985). Variation was also observed in the rhizobia nodulating chickpeas, although the nif genes of this strain are chromosomally located one isolate also had a plasmid that hybridized to a nif probe (Cadahia et al, 1986). In the 192 strains examined by Brockman and Bezdicek (1989) a number of different plasmid profiles were identified. Interestingly, a study by Harrison et al (1988) of strains from all around the world demonstrated a huge range of plasmid profiles and a

number of strains with multiple Sym plasmid bands. Admittedly, all of these studies examine field isolates, which might be expected to show variation, and this project deals with variation in the laboratory, but it was designed to attempt to reconstruct elements of the natural field environment and it is therefore very gratifying to find natural isolates with characteristics similar to the laboratory generated variants.

The difference between laboratory cultures, which exist in safe, nutrient rich and unchallenged environments and the field isolates which may exist in very marginal environments must be kept in mind, however. While there is no direct selection for stability in the laboratory cultures, at least not in this study, there is certainly not the need for metabolic flexibility which might be needed in soil dwelling rhizobia.

This study, unlike most others of which we are aware, did not use rhizobia as the donors of pSym in subsequent crosses. It is therefore diffficult to make direct comparisons with other transfer studies. It will prove interesting to further examine the multiple bands in an attempt to determine whether they are artefactual open circular forms, which have never been observed in 2668, or semi-stable derivatives of the original pSym-derivative. At present the second possibility is more plausible as bands of the same size are seen in donor and recipient and open circular plasmid forms are not known to transfer. A systematic study of a series of the size-reduced pSym plasmids may also contribute to our understanding of the minimal requirements for a functional pSym under laboratory and/or field conditions. Furthermore, it may be possible to determine whether or not the pSym deletions are random or occur at defined places on the plasmid, to yield some form of "sym cassette".

The initial purpose of this project was to devise laboratory experiments that could provide plausible simulations of processes that occur in the soil and examine the effect on the genome. The end result was a different set of experiments, which while perhaps not directly applicable to what had been observed in the field isolates from the

earlier study, nevertheless provided us with further evidence of microbial genomic plasticity. The summation of this project is therefore divided into several sections:

5. How might the various DNA transactions (archivilization, rearrangement and transfer) affect the structure and function of the bacterial genome?

6. What are the ecological consequences of bacterial plasticity for Rhizobium?

7. How can laboratory simulations help us understand microbial responses to the field environment?

8. Future directions for investigation.

There is considerable (and intentional) overlap between parts 5 and 6, but the two parts are considered separate as part 5 is focused on the bacterium and part 6 is focused on the action of the bacterium in the environment.

5. How might the various DNA transactions affect the structure and function of the bacterial genome?

A discussion of this question has recurred throughout this thesis and has been central to the experiments we have performed and the literature we have reviewed.

The apparent archivilization of DNA reported here for pSym and discussed in detail for P22 by Downs and Roth (1987) is the most unusual form of storage of genetic information we have identified in the literature. DNA archivilization could be considered part of a hierarchy of DNA management strategies involving expression and regulation of genes. It is possible to divide expression and regulation into four categories and assign genes to each category on the basis of their frequency and mechanism of regulation of expression. These hypothetical categories can be labelled the constitutive ("housekeeping"), inducible, cryptic and archival. The housekeeping functions include those genes that are always expressed in the cell such as the glycolytic pathway. Inducible genes include the various catabolite-repressed operons such as lac and gal and the amino acid synthesis pathways. The cryptic genes, such as the bgl operon (Parker

and Hall, 1988), are genes which may be useful to the cell on a more intermittent basis. The final category, the archived genes, could be functions that may disadvantage the cell if expressed inappropriately or functions that may be only rarely required. While it is clear that the first two categories of genes exist in the cell, the presence of the other two categories as organized and regulated mechanisms of gene storage, remains conjectural.

What might be the value to the cell of archival DNA? If we accept the thesis of Jacob (1977) that evolution works like a tinkerer, using whatever tools and materials are available, then it would be foolish to waste potentially useful "ingredients". Therefore, genes not immediately required by a bacterium can perhaps be stored in a way which will not tax the cell by expression of currently unnecessary functions, but these functions are not lost. Why exactly this should occur in this case with the symbiotic functions is not known, but it is possible we have managed to isolate a rare event.

A great diversity of plasmid profiles were observed in this project and was similarly observed with R. meliloti (Adachi et al, 1983), chickpea rhizobia (Cadahia et al, 1986), R. leguminosarum biovar phaseoli (Martinez et al, 1985; Soberon-Chavez et al, 1986; Palacios et al, 1987; Soberon-Chavez and Najera, 1989b), biovar trifolii (Watson and Schofield, 1985; Thurman et al, 1985; Harrison et al, 1988) and biovar viceae (Young and Wexler, 1988; Brockman and Bezdicek, 1989). Plasmid rearrangements similar to those observed in this study have been reported elsewhere (Quinto et al, 1982; Martinez et al, 1985; Flores et al, 1987; Soberon-Chavez and Najera, 1989a). What is not clear from this study, nor from published material is whether the observed rearrangements are the result of random recombinations or purposeful (even if this purpose is unknown) reorganization of the genome. Two examples of apparently directed mutation have been reported in the literature (Cairns et al, 1988; Hall, 1988). In both cases the implication seems to be that the potential exists within a bacterium to direct adaptation to a changing environment. Further study will be required before the extent to which a bacterium can control its own

evolution is known.

6. What are the ecological consequences of bacterial plasticity for Rhizobium**?**

The point of departure of this project was to isolate variants in the laboratory that are like those isolated from the field. The question posed above is the next one that must be addressed once similar variants can be shown to arise in the laboratory and overlaps considerably with the question posed in the previous section. Assuming that the niche exploitation is of primary importance for microbial survival, then the ability to alter genome structure and function could be especially useful for adaptation to environmental variation.

The rearrangements we have observed in the laboratory may reflect a bacterial system which enables the bacteria to direct alterations in response to the appropriate stimulus. Reports of "directed mutation" have been made by Cairns et al (1988) and implied by Hall (1988), but we do not yet have sufficient information to compare our study with theirs. Alternatively, the rearrangements may reflect a "last-ditch" effort of a genome under stress to cope with adverse conditions, as proposed by Heumann et al (1983) or a random variation as a result of conjugation. At this stage it is difficult to tell. Certainly, the entire symbiotic plasmid is not absolutely required for effective symbiosis in R. leguminosarum biovar trifolii (Innes et al, 1988) and suggested by the results of Part II.

There appears currently to be two schools of thought regarding the variability of soil bacterial strains. One group proposes that the strains are essentially genotypically fixed and while gene transfer and DNA reorganizations do occur, it is on a sufficiently small scale that in the short term there is little influence on the stability of a population (Young, 1985; Young et al, 1987; Young and Wexler, 1988; Harrison et al, 1988). The second group suggests that the population is much more plastic and that bacteria can readily adapt to challenges provided by the environment (McArthur et al, 1988; Brockman and Bezdicek, 1989), that transfer of plasmids can occur at an appreciable

frequency (Schofield et al, 1987; Demezas et al, 1988) and that genomic rearrangement could play a major role in bacterial variability (Palacios et al, 1987; Soberon-Chavez and Najera, 1989b). Indeed, it has been suggested that rather than distinct lineages of symbiotically-competent bacteria, there is a continuum of symbiotically proficient strains in the soil (Broughton et al, 1987; Mozo et al, 1988).

If the Sym plasmid can transfer to many different bacteria, then the limiting step of the bacteria-plant symbiosis will be the efficiency with which the bacterial recipient can exploit the symbiotic genes in competition with other Nod^+ bacteria. This would imply that the bacteria which are the most saphrophytically competent and which could most competitively nodulate a legume would be the first to gain access to the protected nodule environment. The actual nitrogen fixing ability of these strains might be relatively unimportant to the bacteria in terms of its survival.

Therefore, the ecological consequences of bacterial variability in Rhizobium could be an increased gene pool from which Nod^+ strains can be isolated and the ability to alter genotype, and possibly phenotype, in response to certain stimuli. This would make the strains very adaptable and the isolation of nonsymbiotic strains, which can nevertheless be made Sym^+ (Jarvis et al, 1989; Soberon-Chavez and Najera, 1989a) means that not all rhizobia live in nodules and models of the life strategy of Rhizobium must take that into account.

7. How can laboratory simulations help us understand microbial responses to the field environment?

The initial approach of science to a problem is to reduce it to its component parts and then gradually reconstruct the framework via simple interactions of a few components of the system until it is possible to understand the essential features of the system in their totality.

With the reductionist approach, which we employed in this project, it becomes a matter of combining data from a variety of sources in an attempt to generate a coherent and internally consistent picture. The

strictly theoretical models of genome interaction in the soil (Knudsen et al, 1988) combine with more practical studies (Weinberg and Stotzky, 1972; Brockwell et al, 1982; Trevors and Starodub, 1987; Saye et al, 1987; Richaume et al, 1989) to give a clearer understanding of what is possible. With Rhizobium the effect of the plant is another important variable which must be taken into account (Demezas and Bottomley, 1986a and 1986b, Wang et al, 1986). Other factors which must be borne in mind include variation in symbiotic properties that can arise within a culture (Weaver and Wright, 1987) and most importantly, we must be aware that what we observe in the laboratory may not necessarily translate directly to what occurs in the soil (Demezas and Bottomley, 1987). Yet, until soil-based experiments have been performed we must proceed cautiously, for if the laboratory results do not truly reflect the field environment we are experimenting in a vacuum.

From the results of this study, the following conclusions can be drawn. Firstly, that R. leguminosarum biovar trifolii 2668 has the ability to undergo rearrangements involving chromosomal and extrachromosomal elements. Secondly, the symbiotic plasmid can transfer/be transferred to a variety of hosts and provide symbiotic functions sufficient to allow some of these hosts to nodulate white clover. There is support in the literature for these conclusions, from both laboratory and field studies which have been discussed previously in this thesis, but will be briefly reviewed here.

The existence of a recombination system would allow the rhizobia to accept DNA from other sources and integrate it into the genome and also give the genome the ability to respond to stress by gene shuffling. Large scale genomic changes are observed in R. phaseoli which are believed to be due to recombination between repeat elements (Palacios et al, 1987; Ollero et al, 1989; Soberon-Chavez and Najera, 1989b). There is potential for the rhizobia to accept DNA from taxonomically distant sources in the soil (Richaume et al, 1989) and from other rhizobia (Schofield et al, 1987; Harrison et al, 1988; Young and Wexler, 1988). The acceptance of DNA from other strains and transfer of pSym might allow the bacterial populations to optimize the

plant-bacteria symbiosis by accretion of functions from other strains.

A transmissable symbiotic plasmid could spread to bacteria in the soil other than those bacteria identified as rhizobia (Jarvis et al, 1989; Soberon-Chavez and Najera, 1989a) and so allow the symbiotic phenotype to spread. However, this poses the dilemma of soil bacteria which can accept and express nodulation functions, but that are not normally recovered from nodules in the field, and are accordingly not classified as rhizobia. Several solutions suggest themselves. Firstly, if the organism was similar enough to the rhizobia in all criteria except the possession of a Sym plasmid, then it is unlikely to be detected in the non-symbiotic form. If it gained a symbiotic plasmid and nodulated then it is likely to be accepted as a field adapted variant already present. The non-symbiotic form would be unlikely to be detected explicitly unless looked for (as was done by Jarvis et al, 1989 and Soberon-Chavez and Najera, 1989a). Secondly, the plant itself may tend to select more specifically for a particular strain, based on characteristics not necessarily carried on pSym. Thirdly, there will be competition among Nod^+ strains, in which the most competitive will occupy the majority of nodules and the symbiotically-adapted rhizobia are likely to have an advantage here. This may not be the case, as recent analyses in laboratory trials suggests that the strain which reaches and invades the plant first will occupy the majority of the nodules (Bianchin, personal communication).

To discuss the value of laboratory studies in a more general way, two major advantages of controlled environments suggest themselves. Firstly, analysis of the many components that make up the ecology of the Rhizobium-legume interaction in the laboratory allows us to build a picture of what might occur in nature. Secondly, the laboratory study can allow the researcher to propose a testable hypothesis, from which experiments can be designed and applied to the natural environment (Wimpenny et al, 1983). Results from controlled natural studies, such as small scale field trials, and from sampling existing natural populations allow hypotheses to be refined until a clearer picture of what occurs in nature is obtained. However, without the initial

framework obtained from laboratory studies into which field data can be fitted, it is extremely difficult to draw any logical conclusions regarding the importance of each component in the overall plant-bacteria interaction under field conditions.

8. Future directions for investigation.

The test of the utility of a laboratory simulation is its ability to predict events which can then be detected in the real system which was being modeled. With the possible exception of the archived DNA, there is nothing completely new that has been suggested in this project. The contribution of this project has been the attempt to link some of the information in the literature, particularly the work of Soberon-Chavez and colleagues on DNA rearrangement and soil rhizobia with the studies of Young and colleagues of field isolates of rhizobia and those of Jarvis and colleagues on the isolation of soil bacteria that can accept pSym, all of which are discussed in more detail in section 1.8. However, as was stated at the beginning of this conclusion it is felt that this work is incomplete and the following experiments are suggested as means of obtaining a more complete picture of what has happened to 2668 and its pSym.

In the case of the "disappearing DNA" we are faced with two problems: what triggers the loss and reappearance of the DNA and by what mechanism does it occur? At this point it is felt that the more information we obtain about what triggers the rearrangement, the easier it will be to propose and test for a mechanism. The easier portion of the disappearance/reappearance sequence to examine is the reappearance as the plant provides either a useful trap for a relatively low frequency event or is a source of some compound that triggers the reappearance. It should be possible to detect the MO 103/104 to MO 110/111 rearrangement by transfer of the DNA from a number of colonies of MO 103/104 cells on a plate onto nitrocellulose by the colony blot procedure. The filters can be probed for nif and nod, and any colonies that hybridize can be examined more closely by isolation of that colony from a replicate plate. Furthermore, one series of plates could contain root exudate in an attempt to determine whether or not the plant and/or

the compounds it produces is a factor in the rearrangement to MO 110/111-type strains. The frequency of the rearrangement from MO 103/104 to MO 110/111 is not known but it is envisaged that more than 10^4 colonies will have to be screened. With appropriate dilutions about 500 colonies can be screened per plate and this should make the screening procedure feasible.

It is not clear from the results to date exactly what sequences are not hybridizing. The nodABC and nifKDH probes suggest that neither of these two sequences are present in a physically detectable form and the results of plant nodulation trials suggest that they are. It seems that at least some of the symbiotic genes are archived, but despite the apparent disappearence of the entire pSym from MO 103/104 there is insufficient evidence to state that the entire symbiotic plasmid has been archived. Furthermore, although a plasmid of similar size to pSym is visible in MO 110/111 there is no evidence to suggest that this is the same plasmid as was "lost" from 2668 originally. The symbiotic genes make up only a small portion of pSym and it will be necessary to use a wider selection of pSym to determine exactly what DNA is being archived. If in fact only portions of pSym are archived, then it will be interesting to determine whether or not there is anything special about the border regions of the archived DNA. Similarly, it is important to know if the rearrangements of pSym observed in Part II occur at specific sites on pSym and in fact whether the DNA in the pSym derivatives of Part II is contiguous or is the result of DNA splicing. A great deal more needs to be known about the structure of pSym and of the fine detail of DNA sequence.

Isolation and restriction mapping of the pSym-derivatives of the 300 (E. coli) series will allow the physical structure of the pSym-derivatives to be determined. Probes to the digested plasmids will allow the localization of nodABC, nifKDH and the Tn5 insertion site. It may prove possible to determine whether the deletions we observe are random or have defined endpoints by isolation of further 300 series strains from a cross and subjecting them to restriction mapping and DNA sequencing of appropriate portions. If all isolates from separate

crosses had the same physical map, this would be strong evidence for a Sym cassette. Secondly, the use of the 300 series as donors will enable us to determine if the pSym-derivatives are all self-transmissable, as there are no other plasmids in E. coli. If it is self-transmissable it will be able to move to a new host.

Other work envisaged is more in the nature of confirmation. Only a very few samples of the exconjugants at each step are available in part II. It would probably prove useful to choose two of the soil bacteria as pSym recipients and examine many more exconjugants of a soil bacteria X 2668::Tn5 cross and more exconjugants in the subsequent crosses in order to determine whether the events described in this thesis represent single most probable events or whether they are one set of events among a large number of possible events.

In summary, in this project we have acheived the following:

1. We have demonstrated gross modifications of pSym in the form of DNA archivilization and DNA rearrangements and have isolated bacteria which are similar to field-isolated bacteria.

2. We have generated material with which we can examine the details of rearrangements, in particular the 110/111-type strains and 300 series where the pSym derivative is in an E. coli strain.

3. Related to 2. above we have generated a pSym derivative which can be mapped and characterized.

4. We have outlined a further set of experiments for checking the generalizability of the results.

This study was described as an attempt to generate variants of 2668 in the laboratory that resembled variants isolated from the field and could at this stage be said to be a qualified success. We obtained evidence to suggest that rearrangement and DNA transfer on a fairly large scale was possible in the laboratory. There was sufficient evidence in the literature (referred to earlier in this section) to indicate that our results were understandable within the present context of molecular biology and microbial ecology. There is no reason to believe that this laboratory-based study is mere artefact with no relevance to what is occurring in nature and good reason to believe that further work may enable us to make some fundamental contributions to the study of the effect of DNA rearrangement on genome structure, bacterial interaction and bacterial adaptation.

5.0 BIBLIOGRAPHY

Journal abbreviations are as used by the Science Citation index.

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