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Cloning and Characterisation of Plasmid Encoded Catabolic Genes From *Rhizobium leguminosarum*

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cloning and Characterisation of Plasmid Encoded Catabolic Genes from *Rhizobium leguminosarum*" submitted by Laurie Ann Faas in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

From *Rhizobium leguminosarum* strains VF39 and W14-2 cosmids were isolated which contain genes allowing for the complementation of genes allowing for growth on adonitol, glycerol, rhamnose, and sorbitol. Further characterisation was carried out on the cosmid carrying the adonitol region isolated from strain W14-2 which demonstrated that the adonitol region is plasmid-located in 3 biovars of *Rhizobium leguminosarum* from different geographical regions; in some strains, the plasmid is the pSym plasmid. Sequence data confirmed that this region codes for a catabolic gene, ribitol dehydrogenase. Tn*5* mutagenesis of the adonitol catabolic region using Tn*5*B20 was carried out and re-introduction of the interrupted gene into strain W14-2 resulted in a mutant strain which was slightly less successful at nodule occupancy compared to the wild-type strain under laboratory conditions, thus suggesting a possible role of this plasmid-encoded catabolic gene in the competitive ability of strain W14-2.

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DEDICATION

To my family, Bill, Doreen, Lisa and Lynn Pacarynuk.

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LIST OF ABBREVIATIONS

ABBREVIATION	EXPLANATION
Amp	ampicillin
Amp^r	ampicillin resistant
bp	base pair
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EPS	exopolysaccharide
fix	fixation genes
Gm^r	gentamycin resistant
IPTG	isopropyl-ß-D-thiogalctopyranoside
Kb	kilobase
Km^r	kanamycin resistant
L	litre
LPS	lipopolysaccharide
M	molar
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
nif	nitrogen fixation genes
nm	nanometre
Nm^r	neomycin resistant
nod	nodulation genes
ORF	open reading frame
PEG	polyethylene glycol

LIST OF ABBREVIATIONS CONTINUED

pSym	plasmid containing genes necessary for symbiosis - *nod, nif, fix*
rpm	rotations per minute
SDS	sodium dodecyl sulfate
Sm^r	streptomycin resistant
SOD	superoxide dismutase
Tc^r	tetracycline resistant
µg	microgram
µL	microlitre
µm	micrometre
µM	micromolar
X-gal	5-bromo-4-chloro-3–indolyl-ß-D-galactoside
^oC	degrees Celcius
%	percentage

1.0 INTRODUCTION

The beneficial effects of the symbiotic association between members of the genus *Rhizobium* and leguminous plants are receiving a great deal of attention because of today's growing population of humans. Indeed, though, these benefits have been known for centuries; for example, Greek writings from the third century B. C. suggested that crops of broad beans (*Vicia faba*) were used to enrich the soil (Raven, 1986). This symbiotic association results in the reduction (fixing) of atmospheric nitrogen by the bacteria, which is then exported to the plant (Long, 1989). "Extra" nitrogen may even be released into the soil. Asymbiotic and symbiotic biological systems fix an estimated 100 - 175 million metric tonnes of nitrogen annually (Hardy, 1993). Significantly, the current annual worldwide expenditure for fertiliser nitrogen exceeds $20 billion. Seldom is more than 50 percent of fertiliser nitrogen assimilated, and often the efficiency of utilisation is much less (Hardy, 1993) The exploitation of this nitrogen fixing ability, once fully elucidated, could lead to the decreased use of chemical fertilisers which is becoming an increasing concern to an environmentally conscious world.

Even though nitrogen fixation has yet to be understood fully, efforts to manipulate these nitrogen fixing processes by introducing superior nitrogen fixing strains of *Rhizobium* into soil have been made. Generally, however, these attempts have failed due to the presence of indigenous strains which are more efficient at nodulation of the host plant than the introduced strains. E. Triplett (1992), has termed this problem the "*Rhizobium* competition problem." Several bacterial phenotypes have been shown to play a role in nodulation competitiveness including antibiosis, cell surface

characteristics, motility, speed of nodulation, and nodulation efficiency (Triplett, 1992). As well, the host plant itself is involved in competition, and a recent study has proposed that *Rhizobium* strains carrying specific plasmids might be better suited to one host plant than another due to the presence of plasmid encoded catabolic genes (Hynes & O'Connell, 1990). To date, plasmids in different strains of *Rhizobium* have been shown to encode genes necessary for growth on compounds such as rhamnose, sorbitol, adonitol, lactose, trigonelline, homoserine, glycerol, inositol, ß-ketobutyrate, raffinose, melibiose, and catechol (Tepfer *et al.*, 1988; Charles *et al.*, 1990; Boivin *et al.*, 1991; Goldmann *et al.*, 1991; Baldani *et al.*, 1992; Hynes *et al.*, unpublished observations). It is hypothesized that these and as yet undiscovered catabolic loci make a significant contribution to the competitive ability of particulars strain of *Rhizobium.* The purpose of this study was the isolation of genes corresponding to plasmid encoded catabolic loci in *Rhizobium leguminosarum* biovar *trifolii* strain W14-2, which induces nodules on clover, and in *Rhizobium leguminosarum* biovar *viciae* strain VF39, which induces nodules on *Vicia*, and the investigation of the role of these loci in the competitive ability of the particular *Rhizobium* strain.

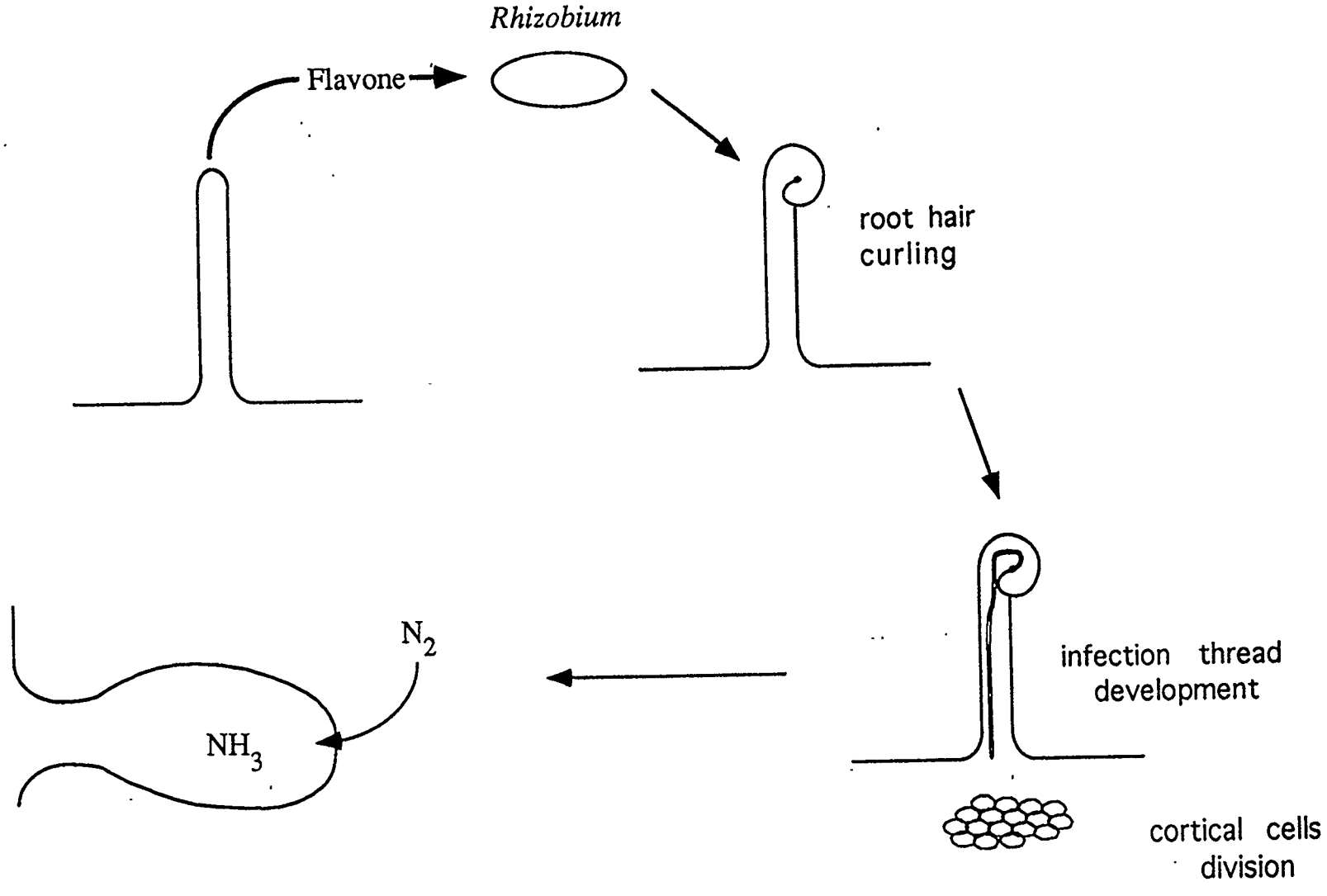
2.0 LITERATURE REVIEW

2.1 The Symbiotic Process

Organisms belonging to the genera *Rhizobium, Bradyrhizobium*, and *Azorhizobium* (collectively referred to as rhizobia) have the ability to exist in symbiosis with a host plant, and, in this relationship, fix dinitrogen into ammonia (Figure 1)(Long & Staskawicz, 1993; Fischer, 1994). The plant is provided with a form of nitrogen it can readily assimilate; in return, the bacteria receive carbohydrates (Long & Staskawicz, 1993). Rhizobia are attracted to the host plant by chemotaxis, and subsequently alter the growth of the epidermal hairs on the root, causing them to deform or curl (Long, 1989). Bacteria trapped in a curled root hair proliferate, and start to infect plant tissue inside a host derived infection thread (Fischer, 1994). At the same time, cells of the root cortex under the epidermis begin dividing, establishing the body of the nodule (Long, 1989). The infection threads penetrate into the root cortex, releasing the bacteria into the plant cytoplasm (Long, 1989). The bacterial cells will further divide and differentiate into bacteroids, where symbiotic nitrogen fixation begins (Fischer, 1994). At least three sets of bacterial genes, the *nod, nif* and *fix* gene clusters, are required in order for this process to occur (Long, 1989; Long & Staskawicz, 1993; Fischer, 1994).

Initially, the bacteria stimulate the plant to grow a nodule, with accompanying changes in the plant root such as altered cell morphology, cell growth and division, and gene expression. This is accomplished by the action of bacterial *nod* (nodulation) genes which can be divided into three sets: regulators such as *nodD*, common *nod* genes such as *nodABC*,

Figure 1. Overview of the nodulation process.


Rhizobium
Flavone
root hair curling
infection thread development
cortical cells division
N_2
NH_3

and host specific *nod* genes (Long & Staskawicz, 1993). Most *nod* genes are induced in the presence of the plant via plant secreted flavonoids (Figure 2)(Long, 1989; Long & Staskawicz, 1993). The gene-activating protein in the bacteria is NodD; evidence suggests that this protein is the direct receptor of the flavonoid inducer (Long & Staskawicz, 1993). Specifically, NodD is a 33 kDa protein (Long, 1989), and a member of the LysR family of prokaryotic gene activators, which interacts with a *nod* gene upstream region (*nod* box), causing a bend in the DNA in inducible *nod* genes although the exact method of biochemical activation is still unknown (Long & Staskawicz, 1993). Other *nod* genes are responsible for causing the synthesis of signals or Nod factors to the host plant. These factors cause events such as the initiation of host root cortical cell divisions, root hair cell growth, branching and deformation of root hairs, reorganisation of cortical cell cytoplasm, and membrane depolarisation (Long & Staskawicz, 1993). Nod factors themselves are ß-lipooligosaccharides having a ß-1-4-linked N-acetylglucosamine backbone with modifications which vary according to species and to host range (Figure 3)(Long & Staskawicz, 1993). The *nod* genes are required for synthesis of Nod factors; for example, host specific *nod* genes are responsible for modifications of the chitooligomer backbone (Long & Staskawicz, 1993).

Nitrogen fixation genes or *nif* genes are those genes which have been shown to share structural homology with *nif* genes of *Klebsiella pneumoniae* (Fischer, 1994). The majority of these genes are responsible for forming the structural components of the nitrogenase enzyme (Fischer, 1994). In addition, NifA is involved in regulation of nitrogen fixation; specifically, it is a transcriptional activator of the structural

Figure 2. Signaling molecules, Nod factors and flavonoids, involved in the nodulation process

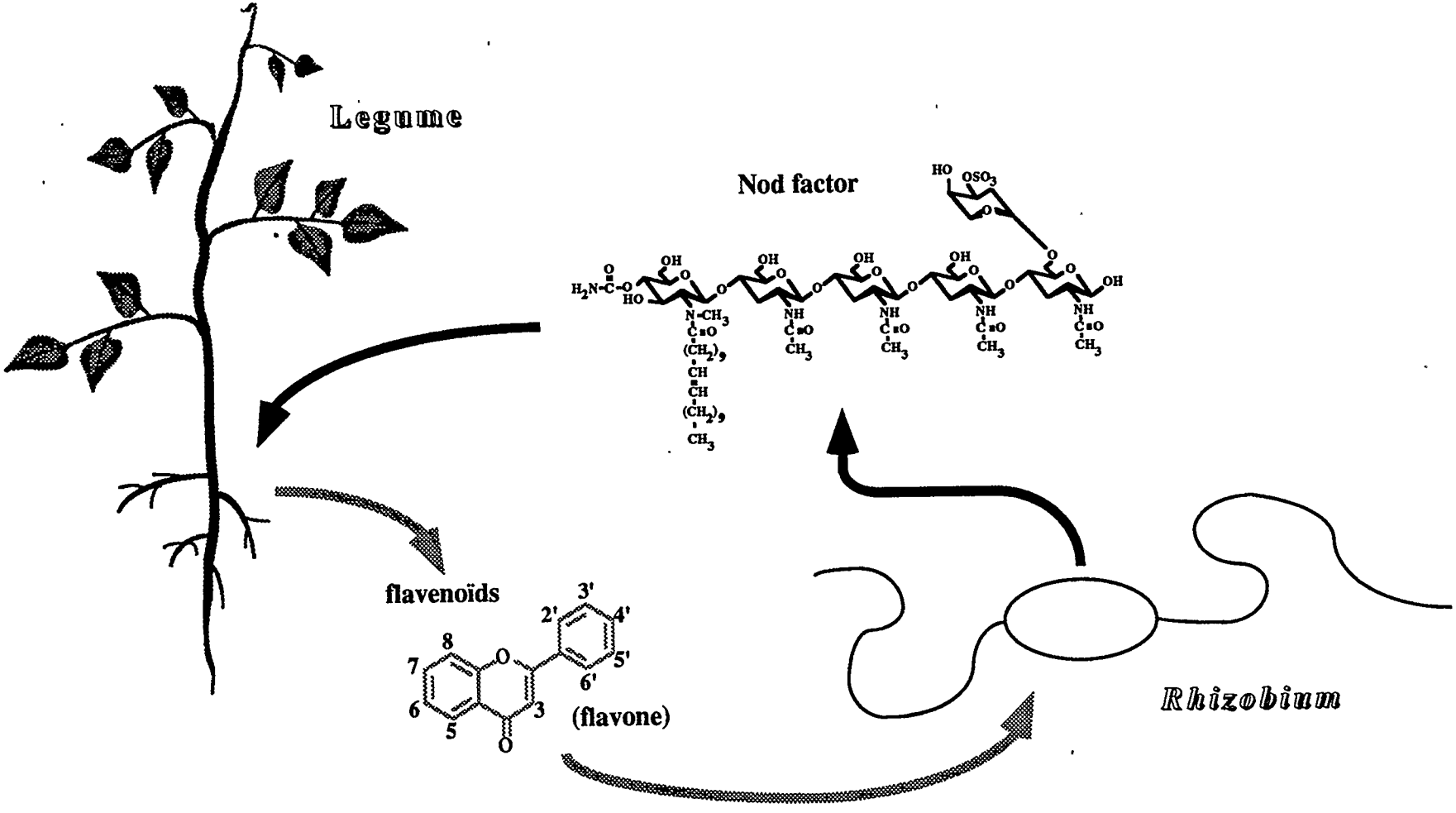
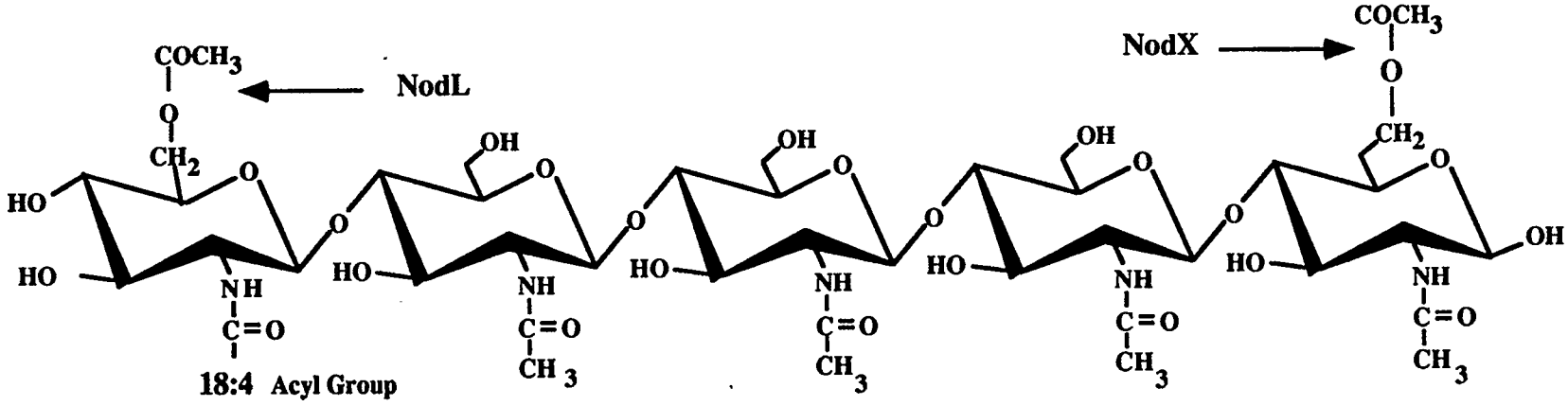

Legume
Nod factor
flavenoïds
(flavone)
Rhizobium

Figure 3. The Nod factor from *Rhizobium leguminosarum* bv. *viciae* strain TOM. Acyl groups attached by NodX and NodL are indicated (adapted from Firmin et al., 1993).


NodL
NodX
18:4 Acyl Group

genes for the nitrogenase enzyme and for genes encoding accessory functions (Fischer, 1994).

The *fix* genes are also necessary for nitrogen fixation; however, some have no homologues in *K. pneumoniae* (Fischer, 1994). It is hypothesised that Fix gene products may be involved in electron transport to the nitrogenase enzyme complex (Fischer, 1994). The *fixNOXP* operon amino acid sequence suggests that this encodes a membrane-bound, cytochrome c containing heme/copper cytochrome oxidase which may support bacteroid respiration under conditions of low oxygen present in root nodules (Fischer, 1994). The *fixGHIS* operon amino acid sequence suggests that this operon encodes for transmembrane proteins (Fischer, 1994), with a possible role in copper transport (Mitsch, 1995). In terms of regulation, in *R. meliloti, nifA* expression depends on two regulatory genes, *fixL* and *fixJ* which belong to one operon, *fixLJ* (David *et al.*, 1988). The *nifA* gene in turn activates other *nif* and *fix* genes such as *nifHDK*, *fixABCX*, and *nifN* (David *et al.*, 1988). It is thought that FixL, a transmembrane protein senses low oxygen present in the symbiotic environment and transduces this signal to FixJ by covalent modification, enabling the bacteria to respond to environmental or cytoplasmic signals with specific cellular activities (David *et al.*, 1988; Fischer, 1994).

Regulation in the nitrogen fixation process has been most extensively studied in *R. meliloti.* The two main regulatory components of the nitrogen fixation process are NifA and RpoN (Fisher, 1994). The activation of gene expression by NifA requires RNA polymerase plus the σ^{54} encoded by *rpoN* (Ronson *et al.*, 1987). In *R. meliloti,* transcription of *nifA* is controlled by a FixJ dependent promoter (Ronson *et al.*, 1987). Upon FixL sensing low oxygen conditions in the environment, a

regulatory cascade occurs, resulting in the binding of NifA to a conserved sequence motif , the Upstream Activator Sequence (UAS) in NifA dependent promoters; for instance, those contained in *nifHDKE*, *fixABCX,* and *nifN* (Ronson *et al.*, 1987). Then, bound NifA and σ^{54} RNA polymerase interact, resulting in loop formation of the DNA region between the UAS and the core promoter resulting in transcription of genes necessary for the nitrogen fixation process (Fischer, 1994).

One of the key features of *Rhizobium* species is that most strains contain one or more large plasmids (Hynes *et al.*, 1988). In many species, the genes necessary for nodulation and some for nitrogen fixation are found on plasmids, which, due to the necessity of these plasmids for symbiosis, are termed 'pSyms' (as cited in Hynes & McGregor, 1990). For example, *R. meliloti* contains pSyms, termed megaplasmids; these are, very large extrachromosomal replicons, 1200 to 1700 kb in size (Charles & Finan, 1991). *R. leguminosarum* biovars *viciae, trifolii,* and *phaseoli,* (where biovar refers to the type of host plant the organism is capable of nodulating) *R. fredii,* and other *Rhizobium* spp. also have been shown to carry their nodulation and nitrogen fixation genes on large plasmids (as cited in Hynes *et al.*, 1989). Consequently, host range of *Rhizobium* is plasmid determined. Generally though, the majority of these plasmids remain uncharacterised (Baldani *et al.*, 1992), although they do contain catabolic genes which may play a role in competitive ability of the organism.

2.2 The Competition Problem

Triplett (1992) suggested that the main problem facing the introduction of superior nitrogen fixing strains into soil in order to decrease the use of chemical fertilisers is the presence of indigenous strains which are more efficient at nodulation of the host plant than the introduced strains. Several factors have been discovered which contribute in some way to nodulation competitiveness.

2.2.1 Antibiosis

The production of bacteriocins by *Rhizobium* may contribute to increased competitive ability (Triplett & Sadowsky, 1992). Hirsch (1979) examined 97 strains of *Rhizobium leguminosarum,* and found that all but 5 produced small (able to diffuse through cellophane), medium sized, or both kinds of bacteriocin, and, it was proposed that production of the medium sized bacteriocins was a plasmid encoded characteristic. If such bacteriocins were produced by a soil-dwelling microorganism, and were inhibitory to closely related strains, this would provide the organism producing the bacteriocin with a competitive advantage.

The best characterised of *Rhizobium* produced bacteriocins is trifolitoxin, which is produced by *Rhizobium leguminosarum* bv. *trifolii* strain T24, isolated from the nodules of *Trifolium dubium* (Triplett & Barta, 1987). Trifolitoxin is a peptide bacteriocin with a molecular mass of 1 037 Da (Breil *et al.*, 1993), which is bacteriocidal at low concentrations to most strains of *R. leguminosarum* bv. *trifolii, phaseoli,* and *viciae* and at higher concentrations, to *R. meliloti* (Triplett & Sadowsky, 1992). Strains belonging to genera such as *Bradyrhizobium, Agrobacterium,* and

Pseudomonas are unaffected by trifolitoxin (Triplett & Sadowsky, 1992). When trifolitoxin-producing strain T24 is coinoculated with trifolitoxin-sensitive strains, nodulation by these sensitive strains is limited (Triplett & Barta, 1987). Also, transposon Tn5-induced mutants of strain T24 which were unable to produce trifolitoxin were no longer able to limit nodulation by trifolitoxin sensitive strains (Triplett & Barta, 1987). In fact, it would appear that T24 would be the ideal solution to the so-called competition problem for clover. However, nodules induced by strain T24 are unable to fix nitrogen, although this was unrelated to the ability to produce trifolitoxin (Triplett & Barta, 1987).

The region encoding the production of and resistance to trifolitoxin was introduced through marker exchange to the genome of *R. leguminosarum* bv *trifolii* TA1, a symbiotically viable strain (Triplett, 1990). When this strain was coinoculated with a trifolitoxin sensitive strain, nodule occupancy of the bacteriocin producing strain approached 100%. Although these tests were not performed under agricultural conditions, the preliminary results suggest that a bacteriocin such as trifolitoxin could play a significant role in the competitive ability of *Rhizobium*.

2.2.2 Cell Surface Characteristics

Acidic exopolysaccharides (EPS's) are complex carbohydrates produced and secreted in large quantities by most *Rhizobium* species (Milner *et al.*, 1992). *R. meliloti* EPS's have been the most extensively studied, and it has been proposed that EPS may act as a specific signal that promotes growth of the infection thread in the plant (Leigh & Walker, 1994). 22 exopolysaccharide synthesis genes have been identified in *R. meliloti*, each having a role in construction of

the major EPS succinoglycan (Leigh & Walker, 1994). Succinoglycan is required for nodule invasion; mutants deficient in succinoglycan are able to initiate nodule formation but, nodules remain uncolonised (Leigh & Walker, 1994). Indeed, EPS is required at some stage of nodule development for all of the *Rhizobium*-legume interactions that form indeterminate (that is, continually elongating) nodules (Leigh & Walker, 1994). Examples of this include *Rhizobium meliloti* on alfalfa (Finan *et al.*, 1985, Hynes *et al.*, 1986, Muller *et al*, 1988), and *Rhizobium leguminosarum* bv *viciae* on peas (Ivashina *et al.*, 1994). Other genes playing a role in EPS production in *R. leguminosarum* bv. *phaseoli* have also been isolated, and their roles in effective nodulation examined. For instance, a mutation in *psi* (polysaccharide inhibition) a plasmid-linked *Rhizobium leguminosarum* bv. *phaseoli* gene which inhibits EPS production also was shown to abolish symbiotic nitrogen fixation (Borthakur *et al.*, 1985). It was proposed that *psi* is a regulatory gene that acts to repress in the bacteroid the expression of gene(s) involved in EPS production (Borthakur *et al.*, 1985). In addition, a mutant strain of *R. leguminosarum* bv *phaseoli* which was unable to synthesise EPS was able to induce functional nodules on *Phaseolus* beans, the normal host of this species (Borthakur *et al.*, 1986). However, when the gene responsible for this phenotype (*pss* - for polysaccharide synthesis) was mutated and introduced into *R. leguminosarum* bv *viciae*, nodulation of peas by this species of *Rhizobium* was prevented (Borthakur *et al.*, 1986). These results fit the proposal that EPS is not required for successful nodulation in systems involving determinate (round) nodules (Leigh & Walker, 1994) as seen in *Rhizobium fredii* on soybean, *R. loti* on lotus, and *R. leguminosarum* bv. *phaseoli* on common bean (Milner *et al.*,

1992; Borthakur *et al.*, 1985, 1986). The reason for these phenomena is poorly understood (Leigh & Walker, 1994; Borthakur *et al.*, 1986).

Acidic exopolysaccharides may play a role in competitive ability of strains of *Rhizobium* and *Bradyrhizobium* which do not require EPS to induce nodule formation; however, experiments performed have demonstrated conflicting results. For instance, Tn*5* mutagenesis was carried out on strain USDA110 of *Bradyrhizobium japonicum,* and mutants were screened first for altered extracellular polysaccharide production, and then for decreased competitive ability (Bhagwat *et al.*, 1991). One Tn*5* mutant was deficient in both acidic polysaccharide and lipopolysaccharide production, and also showed reduced competitive ability (Bhagwat *et al.*, 1991). However, several EPS mutants isolated were equally competitive as the wild type strain (Bhagwat *et al.*, 1991). 12 of 13 Tn5 expolysaccharide-deficient mutants of *R. tropici* were decreased in competitive ability (Milner *et al.*, 1992), but, one EPS mutant was equal in competitive ability to that of the wild type strain, indicating that competitive ability may depend upon the nature of the defect in EPS production, as well as the quantity of EPS's produced, and that the relationship of EPS to competitive ability is poorly understood (Milner *et al.*, 1992).

2.2.3 Motility

Motility, like cell surface characteristics may or may not play a role in competitive ability of Rhizobia. A slow-migrating mutant of *B. japonicum* was unable to occupy as many nodules as the wild-type strain when the two were coinoculated together even though both had the same generation time in the bacterial growth medium (Hunter & Fahring, 1980). The authors concluded that, as many of the slow-migrating mutant cell population were non-motile, they were unable to be chemotactic; therefore, these organisms would be unable to respond to chemotactic attractants released by the legume root (Hunter & Fahring, 1980).

More recent studies have drawn different conclusions. A Tn*7* non-motile mutant of *B. japonicum* was used in competetive studies in non-sterile soil. Although the non motile Tn*7* mutant was about one-fourth to one-third less competitive than the wild type, the group concluded that this small difference may not have been due to motility at all, but rather to the lag in growth initiation associated with the Tn*7* mutant (Lui *et al.*, 1989). Lui *et al.*, (1989) speculated that motility only becomes significant when the plant root encounters the rhizobia as it grows through the soil. At this point, water films on a smooth patch of root surface may allow motility to play a role (Lui *et al.*, 1989). Normally, bacteria are only able to move if the soil they are in contains large pores with interconnecting water bridges, and if the soil is at near-saturation or saturation point (Lui *et al.*, 1989).

2.2.4 Speed of Nodulation

As with motility, speed of nodulation may or may not be significant in competitive ability, although it would seem that there would be a positive correlation between the two given that early rhizobial infection of legume roots induces an autoregulatory response in the plant preventing infection by subsequent inoculations (Stephens & Cooper, 1988). Kosslak *et al.*, (1983) tested the effects of preexposure of soybean (*Glycine max* L. Merrill) roots to *B. japonicum* strains and subsequent establishment of other strains in the nodules. They looked at delayed inoculation with combinations of effectively nodulating strains (USDA 110 and 138) and effective-ineffective strains (USDA 110 & SM-5) (Kosslak *et al.*, 1983). Delaying introduction of USDA 110, the superior competitor, into the root zone resulted in an enhancement of nodule occupancy by either USDA 138 or SM-5 (Kosslak *et al.*, 1983). However, when either of the two less competitive strains (USDA 138 or SM-5) was introduced 48 hours or longer before USDA 110, it formed the majority of the nodules (Kosslak *et al.*, 1983). Thus, strains which are more effective at nodulation and that are able to initiate nodulation before other strains will stand a greater chance of occupying more nodules. Similarily, Sargent *et al.*, (1987) using Tn*5* mutants of *R. trifolii* slightly impaired in their ability to nodulate plants effectively coinoculated onto a "split root" system with the parent strains, found that the mutant strain was almost completely inhibited in nodulation ability, indicating that highly competitive strains are faster at competing for the highly infectible root hair cells, and so rapidly infect them, thus inducing a systemic response in the plant, inhibiting nodulation by other strains.

Stephens & Cooper (1988) agreed with the principle of speed of nodulation and its positive correlation with competitive ability in *R. trifolii*; however, they proposed a different explanation. They found that although the region of the primary root which bore no root hairs (NRH zone) was the site of the first nodules formed for all strains examined, strains differed in the speed at which they nodulated this zone. Ten days after inoculation, the highest percentage of plants possessing nodules in the NRH zone were associated with those strains which formed nodules most rapidly in the NRH zone (Stephens & Cooper, 1988). When strains were coinoculated, the more competitive strain was that which had nodulated the NRH zone of the root more rapidly (Stephens & Cooper, 1988).

Finally, examination of strains of *B. japonicum* produced conflicting data, showing that in sterile soil, a more competitive strain was actually not the organism forming the majority of early nodulations (Zdor & Pueppke, 1988). Unfortunately, it is difficult to draw any conclusions from all of these experiements, as not only are there conflicting reports of the possible relationship between speed and competitive ability, but also, in each case, unrelated strains were used rather than genetically defined isogenic ones.

2.2.5 *nifA* Dependent Nodulation Efficiency Genes

In *R. meliloti*, a 5 kb region located on a non - symbiotic plasmid was found to play a role in competitive ability (Sanjuan & Olivares, 1991). Mutation in this region delays nodule formation and reduces nodulation competitiveness; as a result, this new symbiotic region is termed *nfe* or Nodule Formation Efficiency (Sanjuan & Olivares, 1991). Expression of *nfe* genes is dependent on the *nifA-ntrA* regulatory system that positively controls transcription of *nif* and some *fix*

genes, the genes directly involved in the nitrogen fixation process (Sanjuan & Olivares, 1991). Hybridisation experiments demonstrated that the *nfe* region was not present in other wild type strains of *R. meliloti*; however, when *nfe* DNA was transferred to each of 4 other wild type strains, a significant increase in competitive ability of each of these strains was observed (Sanjuan & Olivares, 1991). In addition, 128 base-pairs downstream of the *nfe2* gene, Soto *et al.*, (1994) discovered orfC, which shows amino acid similarity with ornithine cyclodeaminase of *Agrobacterium tumefaciens* which converts ornithine into proline. When this region was mutated and the strain tested on alfalfa plants, it also exhibited a less efficient nodulation phenotype to that of the wild type strain (Soto *et al.*, 1994). A similar region to the *nfe* region of *R. meliloti* is chromosomally located in *B. japonicum*, although it was found to be under a different regulatory system than that in *R. meliloti* (Chun & Stacey, 1994).

2.2.6 Role of Host Plant

The host plant may play a role in competitive ability by 'selecting' only *Rhizobium* strains which possess certain genes, thus excluding all other strains from nodulating. For example, the pea cultivar Afghanistan is not nodulated by European or North American strains of *R. leguminosarum* bv. *viciae*, yet is nodulated by the strain TOM, isolated from Turkey, which also can nodulate commercial peas (Davis *et al.*, 1988). Resistance to nodulation is determined by a recessive gene in the plant, *sym-2* (Lie, 1984). Strain TOM was found to contain an extra gene, termed *nodX* which is transcriptionally activated by root exudates from Afghanistan and which enables strain TOM to overcome this resistance to nodulation (Davis *et al.*, 1988). Further examination revealed that NodX is involved

in a novel modification of the nodulation factor made by *R. leguminosarum* bv. *viciae* strain TOM. Specifically, *nodX* codes for an enzyme involved in producing novel *O*-acetylation of the C-6 of the reducing sugar of the N-acetylglucosamine backbone of the *R. leguminosarum* bv. *viciae* nodulation factor Nod*Rlv*-V(Ac,$C_{18:4}$) (Figure 3)(Firmin *et al.*, 1993). Firmin et al., (1993) postulated that cv. Afghanistan may have a modified 'receptor' which positively recognizes the TOM Nod factor, and this modified receptor would now be much less efficient at recognizing 'normal' Nod factors made by strains lacking *nodX*. Alternatively, NodX-mediated acetylation may increase the resistance of the Nod factor to degradation, so that strains carrying NodX make a more persistent factor, thereby allowing nodulation to proceed (Firmin *et al.*, 1993). Yet another model suggests that those Nod factors not acetylated at the reducing end are normally acetylated by the plant and the 'resistance' to nodulation in cv. Afghanistan is a loss of an enzyme that normally acetylates the Nod factor (Firmin *et al*, 1993). Attempts have been made to exploit the relationship between *sym-2* and *nodX* to improve a strain's competitive ability by constructing a Tn5 derivative containing *nodX* (Fobert *et al.*, 1991) When a *nodX* containing strain was coinoculated with the parent strain onto a desireable pea cultivar (one containing the *sym-2* locus), the *nodX* containing strain could successfully outcompete the parent strain under greenhouse conditions (Fobert *et al.*, 1991). This example illustrates that it is not simply a phenotype present in the bacterial cell which determines nodulation competitiveness; rather, the host plant may also play a role by choosing for the presence of certain genes, perhaps even plasmid encoded genes.

Recent studies do suggest a role for plasmid encoded genes in competitive ability. Hynes & O'Connell (1990) looked

at the effect of different genera and species of host plants within the same cross inoculation group, on interstrain competition. *R. leguminosarum* bv. *viciae* nodulates plants from 4 genera - *Pisum, Vicia, Lens*, and *Lathyrus. Lens culinaris*, *Vicia faba* and *Pisum sativum* were grown up in soil containing a commercial multistrain *Rhizobium* inoculant, or in soil from native pasture (Hynes & O'Connell, 1990). Nodules were then surface sterilised, crushed, and plated, and plasmid profiles examined using Eckhardt gels which are modified agarose gels allowing the visualisation of intact plasmids. Eight different plasmid profiles were found; however, the majority of nodules formed on peas in both soils were from a plasmid group not found in bean nodules, and the same results were found with faba beans. Lentils were found to be less fastidious (Hynes & O'Connell, 1990). Some plasmid group representatives were never found in nodules of a particular host plant, although all strains were determined to be able to nodulate all 3 hosts. When plasmid profiles of isolates were compared with those from the commercial mix, the commercial mix profiles showed up only infrequently (Hynes & O'Connell, 1990), thus reinforcing Triplett's (1992) definition of the *Rhizobium* competition problem. It was proposed that strains carrying specific sets of plasmids might be better suited to one host plant than another (Hynes & O'Connell, 1990). In terms of competitiveness, this suggests that a strain may be more competitive due to the presence of certain plasmids. In fact, this might provide a possible role for *Rhizobium* plasmids which are found in the majority of *Rhizobium* species. More specifically, plasmids could possible aid in competitive ability due to the presence of plasmid encoded catabolic genes, which would provide a strain with a

competitive advantage if it could catabolise a substrate which was produced, for example, by a specific host plant.

2.3 Plasmid Encoded Catabolic Genes

As previously stated, many species of *Rhizobium* contain one or more large stable plasmids which contain a large proportion of the genome (Hynes *et al.*, 1988). Apart from containing nitrogen fixation genes, the role of the majority of these plasmids is largely unknown, and the plasmids are generally referred to as 'cryptic' (Baldani *et al.*, 1992). Within the past several years, attempts have been made to characterise these cryptic plasmids. Hynes *et al* (1986), not only demonstrated the presence of a second megaplasmid in *R. meliloti*, but also discovered that this plasmid carried genes involved in extracellular polysaccharide production, which is in fact required in this species for the production of functional nitrogen fixing nodules. This second megaplasmid was also found to contain 2 loci required for thiamine biosynthesis (Finan *et al.*, 1986), and a region which is required for C_4 dicarboxylate transport (Watson *et al.*, 1988). In addition, the second megaplasmid also contains the gene for the inducible form of ß-galactosidase (Charles *et al.*, 1990). One of the six plasmids of *R. leguminosarum* bv. *viciae* strain VF39 was found to contain genes for melanin production although it is as yet unknown what role melanin plays (Hynes *et al.*, 1988). Work with a *Rhizobium* species isolated from *Lablab purpureus* revealed that the ability to use catechol, a central metabolite in phenolic catabolism, is plasmid borne (Gajendiran & Mahadevan, 1990). Some of the most interesting data reveal that there are many instances of catabolic genes being plasmid encoded in several strains of *Rhizbium*.

Upon infection of a plant by *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, the plant is instructed to produce specific amino acid derivatives, called opines, which are then catabolised by *Agrobacterium* strains present in the rhizosphere (Wilson *et al*, 1995). It has been proposed that these opines act as nutritional substrates, providing a competitive advantage to these *Agrobacterium* strains present in the rhizosphere (as cited in Wilson *et al.*, 1995). Definitive evidence for such a relationship was provided by Wilson et al. (1995). This group used 2 strains of *Pseudomonas syringae*; one containing plasmid pYDH208 conferring the ability to catabolise the mannityl opines mannopine and agropine and the other, a near-isogenic non opine catabolising strain. When each strain was inoculated onto transgenic mannityl opine-producing tobacco plants, it was found that the population size of the strain containing pYDH208 was significantly greater than that of the wild type strain on similar leaves (Wilson *et al.*, 1995).

The opine concept has been expanded to include *Rhizobium*. In 1987, P. Murphy *et al.* found that in alfalfa nodules induced by strain L5-30 of *R. meliloti*, a compound termed L-3-*O*-methyl-*scyllo*-inosamine (3-*O*-M*S*I) is synthesized . What is significant is that this compound is also a specific growth substrate for strain L5-30 (Murphy *et al.*, 1987). As this compound is produced in the nodule and can be used as sole N- and C-source by the free-living bacterium, it has been termed a "Rhizopine", due to the similarity in biological properties between 3-*O*-M*S*I and opines produced by *Agrobacterium* (Murphy *et al.*, 1987).

The production of rhizopines in *R. meliloti* L5-30 is closely tied to the symbiotic process. Catabolic and synthetic genes for this compound are closely linked and on the Sym

plasmid of strain L5-30 (Murphy *et al.*, 1987; Murphy *et al.*, 1995). In fact, the promoter of the synthetic locus *mos*, is regulated by the symbiotic nitrogen-fixation regulatory gene *nifA* (Murphy *et al.*, 1988), and *mos* genes are only expressed in the terminally differentiated nitrogen-fixing bacteroids (Murphy *et al.*, 1988). The *mos* locus has a symbiotic promoter, which is highly homologous to the *nifH* promoter, and has both *nifA* and *rpoN* (which produces a factor that acts in concert with the product of the *nifA* gene) regulatory regions (Murphy *et al.*, 1988). It is this duplicated copy of the symbiotic promoter which enables genes for rhizopine production to be expressed in the bacteroid and to be coordinately regulated with nitrogen fixing genes (Murphy *et al.*, 1988). Murphy *et al.*, (1988), and Rossbach *et al.*, (1994, 1995) propose that the production of rhizopines enhances survival of the bacterial partner in symbiosis, in that certain free-living organisms possessing the catabolic gene(s) for 3-*O*-M*S*I will be able to survive in the rhizosphere, whereas other strains of *Rhizobium* will be less competitive as they are unable to utilise this particular compound.

Both the synthetic and catabolic regions of 3-O-MSI in *R. meliloti* strain L5-30 have been further characterised. The synthetic or *mos* locus is comprised of four open reading frames termed ORF1, *mosA*, *-B*, and *-C*. Work with immunogold labelled antibodies prepared to the three translated gene products, MosA, B, and C has shown that these proteins are located in the cytoplasm or membranes of bacteroids (Murphy *et al.*, 1995). The 5' region of ORF1 was found to be the region containing a high degree of homology to the *nifH* 5' region. As no protein presence could be detected, it was suggested that *mos*ORF1 may be the consequence of the fusion of a duplicated *nifH* gene (Murphy *et al.*, 1995), and that this duplicated copy

of the symbiotic promoter enables these genes to be expressed in to bacteroid and be coordinately regulated with nitrogen fixation genes (Murphy *et al.*, 1993; Murphy *et al.*, 1995). Interestingly, the MosA protein was found to show extensive homology over its entire length with the *dapA* gene products (dihydrodipicolinate synthetase)from *E. coli, Corynebacterium*, and wheat, which condenses pyruvate and aspartic semialdehyde to 2,3-dihydrodipicolinic acid (the first step in lysine biosynthesis). MosA also shares 29% identity with NanA from *E. coli*, which reversibly cleaves N-acetylneuraminic acid to pyruvate and N-acetyl-D-mannosamine (Murphy *et al.*, 1993). With discovery of a different rhizopine, *scyllo*-inosamine which lacks *mosA*, Murphy *et al.*, (1993) proposed that the purpose of the MosA gene product is to add a methyl group to *scyllo*-inosamine in the production of 3-*O*-M*S*I.

The central domain of MosB shares homology with a range of proteins including DegT from *E. coli*, and DnrJ from *S. typhimurium*. Both of these proteins have been shown to be regulatory. Perhaps, MosB could be involved in controlling housekeeping genes within the nodule which are involved in the biosynthesis of the rhizopine backbone (Murphy *et al.*, 1993). MosC does not show significant homology to any proteins in data bases; however, it is a very hydrophobic protein with 12 putative membrane-spanning regions characteristic of proteins involved in sugar transport across membranes (Murphy *et al.*, 1993). It is proposed that MosC could transport a precursor for rhizopine biosynthesis into bacteroids, where the rhizopine is synthesized, or even export the finished rhizopine from the cell (Murphy *et al.*, 1993).

The region encoding catabolic (*moc*) genes for 3-*O*-M*S*I has also been examined (Rossbach *et al.*, 1994). The amino acid sequence for MocA shows the highest degree of homology with

StrI product of *Streptomyces griseus*, a protein of unknown function encoded by a gene located in the antibiotic streptomycin biosynthesis operon (Rossbach *et al.*, 1994). It was suggested that MocA catalyzes an NADH-dependent dehydrogenase reaction, involved in the degradation of the rhizopine (Rossbach *et al.*, 1994). MocB exhibits the characteristic features of standard signal peptides, enabling proteins to be inserted into or translocated across cytoplasmic membranes, thus it was postulated that MocB functions as a rhizopine sensing and binding protein, interacting in the periplasmic space with a transport system to translocate the rhizopine across the membrane into the bacterial cytoplasm (Rossbach *et al.*, 1994). Interestingly, MocR shows homology with the GntR regulator class of bacterial proteins, in addition to similarity to pyridoxal phosphate-binding enzymes (Rossbach *et al.*, 1994). Although this does not suggest a role for MocR, Rossbach *et al.*, (1994) suggests that this gene product has a regulatory function. In addition, MocR may also have enzymatic activity, or its regulatory role may involve binding of a pyridoxal phosphate. From the DNA sequence, a role for MocC could not be determined. It is also unknown what function ORF334 and ORF293 have, although it appears highly likely that they encode proteins (Rossbach *et al.*, 1994). With the characterisation of the rhizopine catabolism (*mosABRC*) genes of *R. meliloti* L5-30, Rossbach *et al.*, (1994) have even proposed to design a minimal *moc* gene cassette consisting of the *mocABC* and *mocR* genes, and transfer and express these genes in different soil bacteria, for beneficial effects.

It should be noted that the presence of rhizopines is not as universal as are the opines produced by *Agrobacterium,* although inositol compounds like 3-*O*-M*S*I have been found in

nodules induced by *R. leguminosarum*, and unrelated compounds have been found in nodules induced by *R. loti* (Murphy *et al.*, 1987). A similar rhizopine to 3-*O*-M*S*I - *scyllo* inosamine (*s*Ia) has been found to be produced by another strain of *R. meliloti,* strain Rm220-3 (Saint *et al.*, 1993). Again, the *mos* and *moc* loci are located on the pSym plasmid, and are in close proximity to each other (Saint *et al.*, 1993). The *mos* genes are also NifA/RpoN regulated suggesting that the *mos* genes are regulated by a symbiotic promoter (Saint *et al.*, 1993). The *mos* loci in strain Rm220-3 demonstrate a large degree of similarity to that of L5-30; however, as previously stated, *mosA* is missing, and presumed to play a role in methylation of the rhizopine in L5-30 (Saint *et al.*, 1993). In terms of catabolism, strain Rm220-3 can catabolise both rhizopines, 3-*O*-M*S*I, and *s*Ia, and percent DNA sequence divergence between the *moc* regions of both strains was found to be 1.5% only (Saint et al., 1993). Recently, 50 different electrophoretic type strains of *R. meliloti* from a worldwide collection were screened using PCR and *moc* specific primers, and 3 additional strains were isolated which can synthesize and catabolise rhizopines (Rossbach et al., 1995). The strains were isolated from different geographic locations; however, all came from nodules from *Medicago sativa* (Rossbach et al., 1995). In addition, P. Murphy has identified 7 additional rhizopine synthesizing and catabolising strains of *R. meliloti,* as well as 10 *R. leguminosarum* bv. *viciae* strains and 2 bv. *phaseoli* strains (Rossbach et al., 1995).

There are several examples of *Rhizobium* species having the ability to catabolise substances which are present in the rhizosphere and some of these catabolic genes are plasmid located. Substances termed calystegins are produced in abundance by *Calystegia sepium, Convolvulus arvensis* and

Atropa belladonna primarily by organs in contact with the rhizosphere (Tepfer *et al.*, 1988). One strain of *R. meliloti* was able to catabolise calystegins and use them as a sole source of carbon and nitrogen (Tepfer *et al.*, 1988). In fact, the calystegin catabolism gene(s) in this strain is located on a self-transmissible plasmid, which is not essential to nitrogen-fixing symbiosis with legumes (Tepfer *et al.*, 1988). Tepfer *et al.*, (1988) suggests that other cryptic plasmids could carry catabolic genes necessary to the saprophytic competence of the bacterium; the ability to catabolise substances prevalent in the rhizosphere would provide one strain with a competitive advantage over another.

Within the literature are more specific examples of compounds produced by leguminous plants, which can be utilised by *Rhizobium* species. For instance, when nodules were isolated from nodules of leguminous trees, some of the *Rhizobium* present had the ability to catabolise mimosine, a toxin found not only in the roots, but also in the seeds and foliage of plants of the genera *Leucaena* and *Mimosa*, although this ability was found not to be plasmid encoded (Soedarjo *et al.*, 1994). Examination of pea seedlings discovered that during the formation of lateral roots, approximately 70% of the released amino acid compounds were found to consist of homoserine (van Egeraat, 1975a & c), and indeed, lateral roots of pea seedlings growing in sterile agar upon inoculation with *Rhizobium leguminosarum* gave a zone of bacterial growth (van Egeraat, 1975b). Further studies demonstrated that *in vitro*, homoserine favours the growth of those *Rhizobium* strains which are capable of producing nodules with peas (van Egeraat, 1975c). This selective stimulation of *R. leguminosarum* by homoserine suggests that under natural conditions where considerable amounts of homoserine are released, a selective

stimulation of *R. leguminosarum* over other cross-inoculation groups would occur (van Egeraat, 1975c).

Trigonelline (nicotinic acid N-methyl-betaine) is a substance present in high quantities in the seeds of legumes (Goldmann *et al.*, 1991), and which is present in root exudates of legumes (Boivin *et al.*, 1990; 1991). In strain RCR2011, trigonelline is able to serve as the sole carbon and nitrogen source, and genes for the catabolism of trigonelline (*trc*) are carried by the symbiotic plasmid, in close proximity to two clusters of symbiotic genes *nifKDH* and *nok/fixVI'*, suggesting a close association with the symbiotic process (Boivin *et al.*, 1990; 1991; Goldmann *et al.*, 1991). In fact, *trc* genes were shown to be induced during all the symbiotic steps, suggesting that trigonelline is a nutrient source throughout the *Rhizobium*-legume association (Boivin, *et al.*, 1990). However, none of the general or symbiotic regulatory genes such as *rpoN*, or *nodD* seemed to be involved in trigonelline catabolism (Boivin *et al.*, 1990). Other betaines such as stachydrine (proline betaine) and carnitine are also produced by alfalfa; these catabolic genes were also found to be plasmid encoded, although these genes were located to the right of the *trc* genes (Goldmann *et al.*, 1991). Some information necessary for the catabolism of other betaines choline, and glycine betaine is also plasmid encoded, to the right of stachydrine catabolic gene(s) (Goldmann *et al.*, 1991). Especially in the case of trigonelline, as legumes produce these compounds, it is advantagous for the symbiotic partner to be able to catabolise these compounds.

Recent Tn5 mutagenesis experiments demonstrated the presence of a chromosomally located gene encoding a proline dehydrogenase (ProDH) in *R. meliloti* strain GRM8 (Jimenez-Zurdo *et al.*, 1995). When mutated in this gene, the *R. meliloti*

strain was impaired in nodulation efficiency and competiveness on alfalfa roots, suggesting, again, that the ability to utilise certain carbon substrates which would be present in the rhizosphere gives one strain a competitive advantage over another (Jimenez-Zurdo *et al.*, 1995). Work examining the non-symbiotic megaplasmid of *R. meliloti* also revealed the presence of loci necessary for the catabolism of compounds such as dulcitol, melibiose, raffinose, ß-hydroxybutyrate, acetoacetate, protocatechuate and quinate (Charles & Finan, 1991). As well, an inducible form of ß-galactosidase required for growth on lactose was found also to be located on the same megaplasmid (Charles *et al.*, 1990). Additional plasmid encoded catabolic genes were revealed when Baldani et al., (1992) examined plasmid cured derivatives of *R. leguminosarum* bv. *trifolii.* The loss of certain plasmids was found to alter the strain's ability to utilize specific carbon compounds, for instance, rhamnose, sorbitol, adonitol, lactose, and catechol (Table 1)(Baldani *et al.*, 1992). As well, unpublished observations in M. Hynes' laboratory made with *R. leguminosarum* bv. *viciae* strain VF39 suggests that the ability to use some of the same carbon sources is also plasmid encoded in VF39. As plants produced many different compounds, it is advantageous for a strain of *Rhizobium* to be able to catabolise these compounds; this provides one strain with a competitive advantage over another.

Table 1
Plasmid-Encoded phenotypes in 3 *R. leguminosarum* bv. *trifolii* strains (Baldani et al., 1992; Hynes, unpublished observations)

Plasmid	Strain W14-2
a	LPS; *fixN*; growth on malate, lactose
b	growth on adonitol
c	growth on rhamnose, sorbitol
d	*nod, nif, fix*; catechol, SOD
	Strain W11-9
a	*nod, nif, fix*
b	growth on inositol, arabinose, nitrate; SOD, hexokinase, carbamate kinase
	Strain W8-7
b	growth on malate, glycerol; LPS, *fixN*
c	growth on adonitol, *nod, nif, fix*
e	SOD, growth on nitrate, malate

2.4 Plasmids and Relation to Competitive Ability

Studies have been performed examining the contribution of different plasmids to competitive abilities of *Rhizobium* strains. For instance, when 4 different symbiotic plasmids from *R. leguminosarum* were introduced into 3 different recipient strains, lacking plasmid-linked symbiotic determinants, it was found that the sym plasmid had no effect on competitiveness in the rhizosphere, that is on the ability of one strain to grow faster than another on the surface of legume roots (Brewin *et al.*, 1983). More recently, a study was performed using what were then classified as two strains of *R. leguminosarum bv. phaseoli.* Type I strains (which are now *R. etli*, Martinez-Romero, 1994) have multiple copies of the nitrogenase structural genes, and they produce melanin, whereas type II strains (which are now *R. tropici*, Martinez-Romero, 1994) have an extended host range (Martinez-Romero & Rosenblueth, 1990). *R. etli* are also more competitive than *R. tropici*; however, introduction of a non-Sym, 225 kilobase plasmid, b, from a *R. tropici* strain into a *R. etli* strain improves nodulation competitiveness of *R. etli* (Martinez-Romero & Rosenblueth, 1990). The mechanism operating in this situation is unknown (Martinez-Romero & Rosenblueth, 1990). In another strain of *R. leguminosarum* bv. *phaseoli*, effects on competitive ability were examined upon curing the strain of each of its 6 plasmids (pa to pf)(Brom *et al.*, 1992). Without pc, pf, pe, or pb(required for nodulation), no diminished effect on nodulation or nitrogen fixation was seen, but the resultant strains were significantly reduced in their ability to compete with the wild type organism (Brom *et al*, 1992). It should be noted that this study was performed under sterile conditions (Brom *et al.*, 1992).

More recent work with strain W14-2 has demonstrated that interactions between plasmids also contribute to the saprophytic competence of the strain (Moenne-Loccoz & Weaver, 1995). This group looked at strains with combinations of 3 out of 4 plasmids, or with only one plasmid out of 4 when introduced into the rhizosphere of the host plant (Moenne-Loccoz & Weaver, 1995). Strains lacking one plasmid were comparable to the wild type in terms of growth (Moenne-Loccoz & Weaver, 1995). However, strains only possessing one plasmid showed a prolonged lag phase or declined, and the strain lacking plasmids simply declined (Moenne-Loccoz & Weaver, 1995). Interestingly, when strains containing single plasmids were coinoculated into the rhizosphere with the wild type strain, the strains lacking plasmids were shown to be less competitive than the wild type strain (Moenne-Loccoz & Weaver, 1995). As well, this group carried out experiments to determine saprophytic competence of these cured derivatives of strain W14-2 in sterile soil. When only plasmids a or d were present in cells, the derivative was unable to grow in soil, although when plasmids b or c were present, growth was delayed leading to a lower population in 10 days than the wild type strain (Moenne-Loccoz & Weaver, 1995). Coinoculation into soil of the wild type and the derivatives cured of plasmids a, c or d resulted in a wild type population 10 times larger than that of the derivatives' (Moenne-Loccoz & Weaver, 1995). Again, these results all suggest that plasmids contribute to the competitive ability of *Rhizobium* strains, as well as to the saprophytic competence of the strain in both the rhizosphere and in sterile soil.

Recent work from M. Hynes and B. Tovell examines *R. leguminosarum* bv. *viciae* strain VF39, and the competitive

ability of strains cured of each of the 4 largest plasmids. 3 of these plasmids are known to carry genes involved in symbiosis (pRleVF39c, pd, and pe) (Table 2); in addition, these plasmids were found to play a role in competitive ability (Hynes & Tovell). The fourth plasmid, pRleVF39f, when cured had no effect on nodulation and nitrogen fixation ability; however, the loss of the plasmid leads to a substantial decrease in the competitive ability of the organism, especially in non sterile soil (Hynes & Tovell). This plasmid encodes genes necessary for the utilisation of ornithine, and arginine as carbon sources, as well as nitrate as a nitrogen source, although it is unknown what component of the plasmid is significant in competitive ability (Hynes & Tovell). It was suggested that the loss of competitiveness may be due to an inability to assimilate and/or catabolise nutrients in root exudates, thus decreasing saprophytic competence in the rhizosphere (Hynes & Tovell).

2.5 Hypothesis and Objectives

As *Rhizobium* plasmids represent a large proportion of the genome of the organism and are very stable, it is conceivable that they contain genes which contribute to the saprophytic competence of the organism. It is our hypothesis that genes necessary for the catabolism of certain carbon sources, present on plasmids of *R. leguminosarum* bv. *viciae* strain VF39 and bv. *trifolii* strain W14-2, make significant contributions to the competitive ability of these two strains. The overall objective of this study was to isolate plasmid-encoded catabolic genes from these two strains, and to perform studies examining the effects on competitive ability, and on nitrogen fixation ability when these genes are mutated.

Table 2
Plasmid-Encoded Phenotypes in *R. leguminosarum* bv. *viciae* Strain VF39 (Hynes, unpublished observations)

Plasmid	Phenotypes
pRleVF39a	melanin production; plant inducible locus
pRleVF39b	restriction system; reiterated sequence/transposase
pRleVF39c	LPS genes; growth on glycerol; growth on lactose; *fixG*, *HI*; *fixN* copies; *fix LJK*; plant inducible locus
pRleVF39d	*nod*; *nif*; *fix*; growth on trigonelline and adonitol
pRleVF39e	Fix minus phenotype; auxotrophic and poor growth phenotypes; growth on sorbitol and rhamnose
pRleVF39f	growth on nitrate; growth on ornithine, arginine, glutamate; competition

3.0 MATERIALS AND METHODS

3.1 Bacterial Strains and Plasmids

Bacterial strains used in this study are listed in Table 3. Bacterial strains were maintained in 30% (v/v) glycerol at -80 °C. Plasmids used in this study are listed in Table 4. Purified plasmid DNA was stored at 4 °C.

3.2 Growth Media and Culture Conditions

Growth of *E. coli* for genetic manipulations was performed in Luria Broth (L-B) (10.0 g/L tryptone, 10.0 g/L sodium chloride, and 5.0 g/L yeast extract). Solid media were made by adding agar (BDH) to 1.25% (w/v).

Growth of *R. leguminosarum* strains for genetic manipulations was performed in TY medium (Beringer, 1974), containing 5.0 g/L tryptone, 3g/L yeast extract, 0.5 g/L $CaCl_2$, 0.1g/L $MgSO_4$; or PH medium (Hynes et al., 1985) containing 4 g/L peptone, 1 g/L tryptone, 1 g/L yeast extract, 0.2 g/L $CaCl_2$, 0.2 g/L $MgSO_4$. In both cases, solid media were made by adding agar (BDH) to 1.25%.

For examination of growth of *R. leguminosarum* strains on specific carbon sources, minimal medium (VMM) was that described by Vincent (1970). This included Solution A - 1.0 g/L-K_2HPO_4, 1.0 g/L KH_2PO_4, and 0.6 g/L KNO_3. Upon autoclaving, Solution B (0.1 g/L $FeCL_3$, 2.5 g/L $MgSO_4$, 1 g/L $CaCl_2$) to a final concentration of 10% (v/v), and 100x Solution C (0.01 g/L biotin, 0.01 g/L thiamine, and 0.01 g/L calcium pantathenate) to a final concentration of 1x were added. Carbon sources were filter sterilized prior to addition to the medium at a final concentration of 0.4% (w/v).

Table 3
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
A) *Escherichia coli*		
DH10B	F-, *mcr*A, Δ(*mrr-hsd*RMS *mcr*BC), Ø80d*lac*ZΔM15, Δ*lac*X74,*end*A1, *rec*A1, *deo*RΔ(*ara*, *leu*)7697, *ara*D139,*gal*U, *gal*K, *nup*G, *rps*L	Lorow-Murray & Jessee, (1992)
HB101	F-, *mcr*B, *mrr*, *hsd*S20, (r_B,m_B), *rec*A13, *leu*B6,*ara*-14, *pro*A2, *lac*Y1, *gal*K2, *xyl*-5*mtl*-1, *rps*L20(Sm^r), *sup*E44 I-	Roulland-Boyer & Dussoix (1969)
S17-1	*pro*, *hsd*R, *rec*A{RP4-2 (Tc::Mu)(Km::Tn*7*)}; RP4 integrated into its chromosome	Simon *et al.*, (1983)
B) *Rhizobium leguminosarum*		
W14-2	bv. *trifolii*, Sm^r, 4 plasmids, poor growth on ribose, gluconate, fucose, dulcitol	Baldani *et al.*, (1992)

Table 3 Continued
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
W14-2 b	Sm^r, lacks sym plasmid, unable to utilise rhamnose,sorbitol, malate, lactose, catechol	Baldani *et al.*, (1992)
W14-2 c	Sm^r, lacks sym plasmid, unable to utilise adonitol,malate, lactose, catechol	Baldani *et al.*, (1992)
VF39SM	bv. *viciae*, Sm^r, 6 plasmids, fixes dinitrogen	Priefer *et al.*, (1989)
LRS 39301	derivative of VF39SM missing c plasmid	Hynes & McGregor (1990)
LRS 393401	derivative of VF39SM missing c and d plasmid	Hynes, unpublished
LRS 39501	derivative of VF39SM lacking e plasmid	Hynes & McGregor (1990)

Table 3 Continued
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
LRS 39601	derivative of VF39SM missing f plasmid	Hynes & McGregor, (1990)
248	wild-type strain of *R. leguminosarum*	Hirsch (1979)
306	wild-type strain of *R. leguminosarum*	Hirsch (1979)
309	wild-type strain of *R. leguminosarum*	Hirsch (1979)
336	wild-type strain of *R. leguminosarum*	Hirsch (1979)
3841	derivative of *R. leguminosarum* bv. *viciae*300, Sm^r	Poole *et al.*, (1994)
6015	derivative of 3841 with a deletion in its pSym, Sm^r, Rif^r	Hirsch (1979)

Table 3 Continued
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
8002	wild-type *R. leguminosarum* bv. *phaseoli*	Lamb *et al.*, (1982)
8401	derivative of 8002 cured of its pSym, Sm^r	Lamb *et al.*, (1982)
3855	wild-type strain of *R. leguminosarum*, Sm^r	Brewin *et al.*, (1983)
B151	derivative of 3855 cured of its pSym, Sm^r	Brewin *et al.*, (1983)
Y10	wild-type strain of *R. leguminosarum* bv. *phaseoli*	Moenne-Loccoz *et al.*, (1994)

Table 3 Continued
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
1-1	W14-2::Tn*5*B20, Km/Nmr, Sm^r, unable to utilise adonitol and arabinose	This Study
1-2	W14-2::Tn*5*B20, Sm^r, Nm/Km^r unable to utilise mannitol	This Study
17-3	W14-2::Tn*5*B20, Km/Nm^r, Sm^r, unable to utilise adonitol	This Study
22-1	W14-2::Tn*5*B20, Km/Nmr, Sm^r, unable to utilise glycerol	This Study
32-2	W14-2::Tn5B20, Km/Nmr, Sm^r, unable to utilise arabinose	This Study

Table 3 Continued
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
W14-2pLFWR1	Sm^r, Tc^r, contains pLFWR1, b plasmid	This Study
W14-2pLFWR2	Sm^r, Tc^r, contains pLFWR2, b plasmid	This Study
W14-2pLFWS	Sm^r, Tc^r, contains pLFWS, b plasmid	This Study
W14-2pLFWA	Sm^r, Tc^r, contains pLFWA, c plasmid	This Study
W14-2pLFVFA	Sm^r, Tc^r, contains pLFVFA, c plasmid	This Study
22-1pLFVFG1	Sm^r, Tc^r, Km/Nm^r, contains Tn*5*B20, pLFVFG1	This Study
22-1pLFVFG2	Sm^r, Tc^r, Km/Nm^r, contains Tn*5*B20, pLFVFG2	This Study

Table 3 Continued
Bacterial Strains

Bacterial Strain	Genotype/Phenotype	Source/ Reference
W14-2A1	derivative of W14-2 containing Tn*5*B20 insertion in ribitol dehydrogenase, Sm^r, Km/Nm^r	This Study
W14-2A2	derivative of W14-2 containing Tn*5*B20 insertion in ribitol dehydrogenase, Sm^r, Km/Nm^r	This Study
W14-2A3	derivative of W14-2 containing Tn*5*B20 insertion in ribitol dehydrogenase, Sm^r, Km/Nm^r	This Study

Table 4
List of Plasmids

Plasmid	Characteristic	Source/ Reference
pUC19	Amp^r, mcs of M13mp19, α peptide of *lacZ*, 2.69Kb, high copy number	Yanisch-Perron, *et al.*, (1985)
pBluescript SK+	Amp^r, polylinker derived from pUC19, αpeptide of lacZ, 2.96Kb, high copy number	Stratagene Cloning Systems, (1995)
pJQ200 SK+	Gm^r, mcs of pBluescriptIISK, α peptide of *lacZ*, 4.9Kb, contains *mob* region of RP4, contains *sacB* from *Bacillus subtilis*	Quandt & Hynes, (1993)
pRK2013	RK2*tra*, Km^r, ColE1	Figurski & Helinski, (1979)

Table 4 Continued
List of Plasmids

Plasmid	Characteristic	Source/ Reference
pRK7813	Tc^r, mcs of pUC9, α peptide of *lacZ*, 11.5Kb, contains *mob* region of RP4, derived from pRK404, contains λ *cos* site	Jones & Gutterson, (1987)
pSUP101::Tn*5*B20	Cm^r, Km/Nm^r contains *mob* region of RP4, derivative of pACYC184, transposon carrier replicon	Simon *et al.*, (1983)
pLFWA	derivative of pRK7813 with 40 Kb of Sau3A digested W14-2 DNA allowing for growth on adonitol, Tc^r	This Study
pLFVFA	derivative of pRK7813 with 40 Kb of Sau3A digested VF39 DNA allowing for growth on adonitol, Tc^r	This Study

Table 4 Continued
List of Plasmids

Plasmid	Characteristic	Source/ Reference
pLFWR1	derivative of pRK7813 with 40 Kb of Sau3A digested W14-2 DNA allowing for growth on rhamnose, Tc^r	This Study
pLFWR2	derivative of pRK7813 with 40 Kb of Sau3A digested W14-2 DNA allowing for growth on rhamnose, Tc^r	This Study
pLFWR3	derivative of pRK7813 with 40 Kb of Sau3A digested W14-2 DNA allowing for growth on rhamnose, Tc^r	This Study
pLFWS	derivative of pRK7813 with 40 Kb of Sau3A digested W14-2 DNA allowing for growth on sorbitol, Tc^r	This Study

Table 4 Continued
List of Plasmids

Plasmid	Characteristic	Source/ Reference
pLFVFG1	derivative of pRK7813 with 40 Kb of Sau3A digested VF39 DNA allowing for growth on glycerol, Tc^r	This Study
pLFVFG2	derivative of pRK7813 with 40 Kb of Sau3A digested VF39 DNA allowing for growth on glycerol, Tc^r	This Study
pLFWA5	derivative of pLFWA containing Tn*5*B20, Tc^r, Km/Nm^r	This Study
pLFWA66	derivative of pLFWA containing Tn5B20, Tc^r, Km/Nm^r	This Study
pLFWA5-19	SalI fragment of pLFWA5 in pUC19 Amp^r, Km/Nm^r	This Study
pLFWA66-19	SalI fragment of pLFWA66 in pUC19 Amp^r, Km/Nm^r	This Study

Table 4 Continued
List of Plasmids

Plasmid	Characteristic	Source/ Reference
pLFWA5-JQ	SalI fragment of pLFWA5 in pJQ200 SK+, Gm^r, Km/Nm^r	This Study
pLFWA66-JQ	SalI fragment of pLFWA66 in pJQ200 SK+ Gm^r, Km/Nm^r	This Study

When appropriate, antibiotics were added at the following concentrations to solid and liquid media to culture *E. coli*: ampicillin (Ap), 100 µg/mL; tetracycline (Tc), 10 µg/mL; kanamycin (Km), 50 µg/mL; gentamycin (Gm), 15 µg/mL Antibiotics were added at the following concentrations to solid or to liquid media to culture *R. leguminosarum*: tetracycline, 5 µg/mL; neomycin (Nm), 100 µg/mL; streptomycin (Sm), 600 µg/mL; gentamycin (Gm), 30 µg/mL.

3.3 Enzymes and Chemicals

Restriction endonucleases and accompanying buffers, T4 DNA ligase and accompanying buffers, Ribonuclease IA, phenol, Calf Intestinal Alkaline Phosphatase and accompanying buffers, lambda molecular weight markers, and agarose were purchasesd from GIBCO-BRL, Burlington, ON. Lysozyme and antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. The Prep - A - Gene kit was purchased from Bio-Rad Laboratories. Boehringer Mannheim supplied the DIG DNA Labeling and Detection System, in addition to isopropyl--D-thiogalactopyranoside (IPTG). Proteinase K was supplied by Pharmacia, Uppsala, Sweden. All other chemicals and media components were supplied by BDH.

3.4 Isolation of Total Genomic DNA for *Rhizobium*

Chromosomal DNA was isolated by the method of J. Quandt, (personal communication) which is modified from the method of Meade *et al.*, (1982). Cells were grown in 50 mL volumes in TY to late logarithmic phase, and harvested by centrifugation at 10 000 rpm. Pellets were then resuspended in 33.3 mL 1M NaCl and left 1 hour on ice. Cells were then pelleted again by centrifugation at 10 000 rpm, and the supernatant decanted. Cells were then vortexed in 8.3 mL 20%

sucrose in TE buffer (10 mM Tris, pH 8.0, 10 mM EDTA), and 8.3 mL lysozyme (5 mg/mL)/ RNase (1mg/mL) in TE was added. The suspension was then vortexed and incubated for 30 minutes at 37 °C. 3.0 mL of 5% sarcosyl/proteinase K (5 mg/mL) in TE were added, where proteinase was added fresh to the solution. The suspension was then vortexed and left overnight at 37 °C. The next day, 2.2 mL of 3M sodium acetate pH 5.4, 6.7 mL phenol and 6.7 mL phenol/chloroform (1:1 ratio) were added, and the suspension was mixed gently without vortexing for 2 - 5 minutes. After centrifuging at 10 000 rpm for 5 minutes, the upper phase containing the DNA was removed into a sterile tube. 6.7 mL of phenol/chloroform was then added, the suspension mixed gently, centrifuged as before, and the upper phase transferred to a new tube. 23.3 mL of isopropanol was added to the DNA, the tube inverted several times, and the tube stored at -80 °C for 1 hour. The mixture was then centrifuged for 15 minutes at 10 000 rpm, and the DNA pellet washed 2 times with 70% ethanol. The pellet was then dried in a vacuum oven for 5 minutes at 65 °C, then 500 µL of sterile water were added, and the solution heated at 65 °C for 30 minutes. DNA was then stored at 4 °C.

3.5 Plasmid DNA Isolation

3.5.1 Small Scale Plasmid Isolation

The method used was a modified alkaline lysis procedure (Sambrook *et al.*, 1989). Cells were grown in 5.0 mL L-B broth overnight at 28 °C, and harvested by centrifugation at 13 000 rpm for 5 minutes. The cell pellet was resuspended in 100 µL Solution I (50 mM glucose, 10 mM EDTA , 25 mM Tris-HCl, pH 8.0), and lysed with 200 µL Solution II - 0.2 M NaOH, 1% (w/v) SDS. After storing on ice for 5 minutes, cell debris was

precipitated by the addition of 150 µLSolution III (3.0 M potassium, 5.0 M acetate), and storing on ice for another 5 minutes. Debris was pelleted by centrifuging at 13 000 rpm for 10 minutes. The supernatant was then removed to a fresh tube, and an equal volume of phenol/chloroform (1:1 ratio) was added. Plasmid DNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol, and centrifugation at 13 000 rpm for 10 minutes. The pellet was then washed with 70 % ethanol, and vacuum dried, finally being resuspended in 40 µL of distilled, deionised water.

3.5.2 Large Scale Plasmid Isolation

Large scale plasmid isolation was performed by the unpublished method of R. Treisman from Sambrook *et al.*, (1989). Cultures were grown in 500 mL L-B broth overnight with shaking at 37 °C, and cells were harvested by centrifugation at 7 000 rpm for 10 minutes and resuspended in 18 mL Solution I (see previous section). 2.0 mL lysozyme to a final concentration of 10 mg/mL in 10 mM Tris-HCL, pH 8.0 was added, and 40 mL Solution II was added prior to the cells being stored at room temperature for 10 minutes. 20 mL ice cold Solution III was added to precipitate cell debris, and the cells were centrifuged at 7 000 rpm for 10 minutes. The supernatant was decanted and extracted once with an equal volume of phenol/chloroform (1:1). The DNA was precipitated from the aqueous phase by adding 0.6 volumes of isopropanol, centrifuged at 7000 rpm for 10 minutes at 4 °C and dried. Plasmid DNA was then resuspended in 3.0 mL of TE (10 mM Tris, pH 8.0, 1.0 mM EDTA), and an equal volume of ice-cold 5.0 M LiCl was added to the solution. The sample was then centrifuged at 13 000 rpm at 4 °C, and the supernatant transferred to a fresh tube. An equal volume of isopropanol

was added, and the DNA was precipitated by centrifugation at 13 000 rpm for 10 minutes. Upon washing the pellet with 70 % ethanol and allowing the residual ethanol to evaporate, the pellet was resuspended in 500 mL TE (pH 8.0), and 500 µL of 1.6 M NaCL with 13% PEG_{8000} (w/v) was added. Upon thorough mixing, the DNA was precipitated by centrifugation at 13 000 rpm at 4 °C, and resuspended in 400 µL TE. The solution was then extracted once with an equal volume of phenol, once with an equal volume of phenol/chloroform (1:1), and once with an equal volume of chloroform. 10 µL of 100 mM ammonium acetate was added to the aqueous phase, as was 2.5 volumes of 95% ethanol. Upon thorough mixing, DNA was pelleted yet again by centrifuging at 13 000 rpm for 10 minutes at 4 °C, washed with 70% ethanol, and dried in a vacuum oven, before resuspending in 500 µL TE, and storing at - 20 °C.

3.6 Recombinant DNA techniques

3.6.1 Restriction Endonuclease Digestion

All restriction endonuclease digestions were performed according to the manufacturers recommendations to obtain optimal enzyme activity and to avoid non-specific DNA cleavage. DNA obtained from small scale plasmid isolation procedures was digested for 3 to 18 hours. As DNA obtained from large scale plasmid isolation contained little contaminating cellular debris, plasmid DNA was digested for 3 hours. Chromosomal DNA was digested for 3 to 18 hours.

3.6.2 Ligation of DNA Fragments into Plasmids

Vector and insert DNA were mixed at a ratio of 1:3. Ligation reactions were carried out in a final volume of 10 µL DNA Ligase Buffer with 1.0 unit of T4 DNA Ligase. Reactions

were carried out for 18 hours at 18 °C, or for 4 hours at 18 °C, or for 2 hours at 37 °C.

3.6.3 Dephosphorylation of Digested DNA

Dephosphorylation of digested vector DNA was performed according to the method of Sambrook *et al.*, (1989). After completion of digestion, the enzyme was removed with extraction with phenol/chloroform (1:1) w/v, and precipitated down. Upon resuspension of the DNA in 90 µL of distilled, dionised water, the supplied 10x CIP dephosphorylation buffer was added at a final concentration of 1x, and 1 unit of Calf Intestinal Alkaline Phosphatase per 100 pmoles of DNA was added, and the mixture incubated for 30 minutes at 37 °C. The Calf Intestinal Alkaline Phosphatase was then inactivated at 75 °C for 10 minutes, and then the suspension purified by extraction with phenol chloroform (1:1). 0.1 volume of sodium acetate (pH 7.0) was added, as was 2.5 volumes of 95% ethanol, and the DNA precipitated down, washed, dried, and resuspended.

3.7 Gel Electrophoresis

3.7.1 Agarose Gel Electrophoresis

DNA electrophoresis was performed using agarose gels ranging in concentration from 0.8 to 1.0% in Tris-borate buffer (90 mM Tris-base, 2 mM EDTA, 90 mM boric acid). Samples were loaded in agarose sample buffer dye (0.1% (w/v) bromophenol blue, 33 % (v/v) glycerol). Electrophoresis was carried out at a constant voltage of 80 V for 1 to 3 hours. DNA was stained with ethidium bromide (0.002 mg/mL) and viewed with ultraviolet light. Low melting point agarose was used at a concentration of 0.6% in Tris-borate, and electrophoresis was carried out at a constant voltage of 20 V for 15 - 18 hours. DNA was stained and viewed in the same manner.

3.7.2 Modified Eckhardt Gel Electrophoresis

The technique used for resolving and visualising plasmids was that of Eckhardt, (1978) as modified by Hynes & McGregor, (1990). Gels were prepared in tris borate buffer with SDS at a final concentration of 1.0% Gel concentrations of 0.5 - 1.0 % agarose were used depending on the size of the plasmids worked with. The cells were grown up in PH. At an OD_{600} of 0.3, 0.1 mL of culture was added to an Eppendorf tube on ice. 0.5 mL cold 0.3% sarkosyl (w/v in Tris borate buffer) was added to the culture, the tube mixed by inversion, and the cells spun down for 5 minutes at 13 000 rpm. After careful removal of the supernatant, the pellet was resuspended in 20 µL of Lysis Solution (10% w/v sucrose, 10 µg/mL RNase, all prepared in tris-borate buffer; with lysozyme added to a final concentration of 100 mg/mL). This amount was then loaded into the well of the gel, and the gel was run at 5 V for 15 - 30 minutes until cell lysis (clearing of the well) occurred. The

gel was then run at 80 - 120 V depending upon gel size for 2 to 6 hours.

3.8 Isolation of DNA fragments from Agarose Gels

Two different methods were used to isolate fragments from agarose gels. DNA was first digested with restriction endonucleases according to the manufacturers' recommendations and electrophoresed on 1 % agarose gels using Tris-borate buffer. The DNA band of interest was then excised from the gel and purified using the Prep - A - Gene kit, according to the manufacturers instructions.

If DNA was electrophoresed on low melting point agarose, the band(s) of interest were excised, and the slices melted at 68 ^{o}C. The solution was then extracted with a 0.5 volume of phenol, and the aqueous phase removed to another tube. This procedure was repeated 3 more times, and then 0.1 volumes of 3.0 M sodium acetate pH 5.4 was added, as was 2.5 volumes of 95% ethanol, to precipitate down the DNA. The DNA was then washed with 70% ethanol, and dried, and then resuspended in ligase buffer for ligations.

3.9 Transformation of *Escherichia coli*

E. coli DH10B or S17-1 cells were made competent by the $CaCl_2$ method of Sambrook *et al.*, (1989). Cultures were grown in 500 mL of L-B broth at 30 ^{o}C to an OD_{600} of 0.5 to 0.6, centrifuged at 6000 rpm for 10 minutes, then resuspended in 40 mL 0.1M $MgCl_2$. The cells were again pelleted in the same manner, the supernatant discarded, and the cells resuspended in 40 mL $CaCl_2$ and incubated on ice for 30 minutes. Once again, the cells were pelleted and the supernatant discarded, and the cells were resuspended in 6 mL of $CaCl_2$/glycerol mix (4.3 mL 0.1M $CaCl_2$, 0.7 mL glycerol) This mixture was then

frozen at -80 °C. DNA was transformed into competent cells by incubating the DNA mixture with the cells on ice for 35 minutes. Cells were then heat shocked at 42 °C for 2 minutes, 1 mL L-B was added and incubation at 37 °C occurred for 1 hour. Transformed cells were then plated on the appropriate selective medium.

3.10 Matings

300 µL of a fresh overnight culture of *E. coli* was mixed with 1.0 µL of a fresh overnight culture of *Rhizobium* (mutants may have taken 2 days to grow), and the cells were centrifuged at 13 000 rpm. The cell pellet was resuspended in 40 µL of medium, and this amount was spotted onto a TY plate lacking antibiotics. After incubation overnight at 28 °C, all of the cells were scraped up and resuspended in 1.0 mL of distilled deionized water. Cells were then centrifuged again at 13 000 rpm, resuspended in 100 µL of distilled deionized water and plated onto the appropriate selective medium.

3.11 Preparation of DNA Probes

For probes, DNA was labelled with digoxygenin - labelled d-UTP according to the manufacturer's protocol from the DIG-DNA Hybridization and Detection Kit from Boehringer Mannheim

3.12 Southern Hybridisation

0.8 - 1.0% (w/v) gels were prepared, DNA was digested and electrophoresed, and, upon depurination in 0.25 M HCl, the DNA was transferred to a Bio-Rad Zeta-Probe membrane using alkaline blotting as outlined in the manufacturer's protocol. After transfer, again, manufacturer's specifications from the DIG - DNA Hybridization and Detection Kit from Boehringer

Mannheim were followed for washing, prehybridizing, hybridizing, and developing the finished blot.

3.13 Creation of Cosmid Libraries

3.13.1 Preparation of DNA

Total DNA from *Rhizobium leguminosarum* strains W14-2 and VF39 was prepared, and partially digested using *Sau*3A. DNA fragments were then separated by low melting point agarose gel electrophoresis, and fragments approximately 40 kb in size were removed from the gel by melting the gel fragments and extracting with phenol. The vector pRK7813 (Jones & Gutterson, 1987) was digested with *Bam*HI, dephosphorylated with calf alkaline phosphatase, and ligated to DNA of each of the two strains of *Rhizobium* in an approximate ratio of 1 (vector): 2 (insert). Packaging was accomplished using a commercially prepared packaging mix from Promega following the manufacturer's instructions.

3.13.2 Plating of Cosmid Libraries

The protocol used was taken from Sambrook *et al.*, (1989). *E. coli* cells were grown up overnight in the presence of 10 mM $MgCl_2$ and 0.2% (w/v) maltose. 200 µL of cells were mixed with 100 µL of Phage Dilution Buffer (10 mM Tris HCL pH 7.4, 10 mM $MgSO_4$, 0.01% w/v gelatin), and 1 or 10 µL of phage lysate were added (made up to 100 µL in Phage Dilution Buffer). The bacteriophage particles were allowed to adsorb by incubation at 37 ^{o}C for 20 minutes, then 1 mL of L-B medium was added, and incubation continuted for another hour. The culture was then spread onto the appropriate selective medium.

3.14 Screening of Cosmid Libraries

The cosmid libraries were screened for the presence of catabolic genes by complementation. Pools of cosmids in *E. coli* strain S17-1 were mated into the appropriate strain of *Rhizobium* known to be missing a particular plasmid, and thereby being unable to utilise a particular carbon substrate. These matings were then plated on VMM with the appropriate carbon source and antibiotics, and examined to see if the ability to use that particular carbon source was regained.

3.15 Transposon Mutagenesis

3.15.1 Using λ as the Delivery Vehicle

The methods followed were those proposed by Simon *et al.*, (1989). The lambda mutant λ221cI857*P*am80 loaded with the Tn*5* derivative Tn*5*B20 was allowed to infect *E. coli* strain S17-1 (containing the plasmid to be mutated) by the same protocol used for plating of cosmid libraries. Upon completion of infection, cells were plated on the appropriate selective medium.

3.15.2 Using pSUP102::Tn*5*

The methods followed were those proposed by Simon *et al.*, (1983). Derivative Tn*5*B20 was incorporated into a mobilisable vector, mobilising *E. coli* strain S17-1 was used to introduce pSUP102::Tn*5* into either *R. leguminosarum* strain. Spontaneous Tn*5* transposition occurred from the introduced vector onto the host DNA, and the mating mixtures were selected on a medium containing Sm (to select against the donor cells) and Nm (to select for stable Tn*5* insertion). It should be noted that the vector can not be stably maintained in the recipient cell. Resulting colonies were then replica-plated

onto minimal medium with mannitol as sole carbon source where growth was ensured, as well as several different carbon sources to determine if the *Rhizobium* strain had lost the ability to grow on that particular carbon source.

3.16 ß-Galactosidase Assays

The method provided by Sambrook *et al.*, (1989) was modified for use with *Rhizobium.* The mutant was grown overnight in TY broth then inoculated into tubes of VMM containing one of 3 different conditions: 1) 0.4% glycerol (control); 2) 0.4% glycerol + 0.4% carbon source to be tested; 3) 0.2% glycerol + 0.2% carbon source to be tested. The mutants were then grown overnight at 28 °C and 0.1 mL of the cell culture was mixed with 0.9 mL Z Buffer (0.06 M $Na_2HPO_4/7H_2O$, 0.04 M NaH_2PO_4/H_2O, 0.01 M KCl, 0.001 M $MgSO_4/7H_2O$, 0.05 M ß-mercaptoethanol), 2 drops of chloroform were added and 1 drop of 0.1% w/v SDS was added. The tubes were then vortexed and equilibrated to 28 °C. 0.2 mL of 4 mg/mL ONPG was added to the lysed culture, and when a yellow colour developed, the reaction was stopped by addition of 0.5 mL of a 1.0 M Na_2CO_3 solution. The time at which colour development occurred was recorded. The absorbance at 600 nm of the original cultures was read, as was the absorbance at 550 nm and 420 nm. Miller units of ß-galactosidase activity were calculated by the formula:

$$\text{Units of Activity} = 1000 \times \frac{A_{420} - (1.75 \times A_{550})}{\text{Time (min)} \times 0.1 \times A_{600}}$$

3.17 Plant Tests

Plant tests were carried out using the methods of Hynes *et al.*, (1986). Seeds were surface sterilised by 5 minutes of

70% ethanol treatment followed by 10 minutes in a 1/6 dilution of hypochlorite. Seeds were then rinsed in 4 changes of sterile distilled deionised water. Mutants and wild-type strains were coinoculated in a 1:1 ratio onto the appropriate host plant seeds and plants were grown in Petri dishes on Plant Medium which contains1 mL/L of each of Solution A: (294 g $CaCl_2$/L); Solution B: (136 g KHPO/L); Solution C: (6.7 g Fe-citrate); and Solution D: (123 g/L $MgSO_4/7H_2O$; 87 g/L KSO; 0.338 g/L $MnSO_4/H_2O$; 0.247 g/L HBO; 0.288 g/L $ZnSO_4/7H_2O$; 0.1 g/L $CuSO_4/7H_2O$; 0.056 g/L $CoSO_4/7H_2O$; 0.048 g/L $NaMo/2H_2O$) made solid with the addition of 1.2% agar. After 4 weeks in a growth chamber, nodules formed were surface sterilised, crushed and plated as outlined in Hynes and O'Connell, (1990). The identity of strains in nodules was confirmed by antibiotic resistance and the ability to grow/or not on the carbon source the mutant strain was unable to utilise.

3.18 Sequencing

A 24 base primer 5' - TAGGAGGTCACATGGAAGTCAGAT - 3' was constructed from Tn*5*-IS50R by Gibco-BRL and used in automated sequencing by the University Core DNA Services using the ABI system. This primer corresponds to a unique sequence on Tn*5*B20 as this construct contains the promotorless *lacZ* gene at the opposite end of the construct. To obtain DNA for sequencing, *Sal*I fragments of digests of pLFWA2 and pLFWA66 cosmids containing the Tn*5*B20 insert were ligated into pUC19. Plasmid DNA was prepared by modified alkaline lysis (Sambrook *et al.*, 1982) with the addition of a PEG8000 purification step. 1 µg ds DNA was then used in the sequencing reactions.

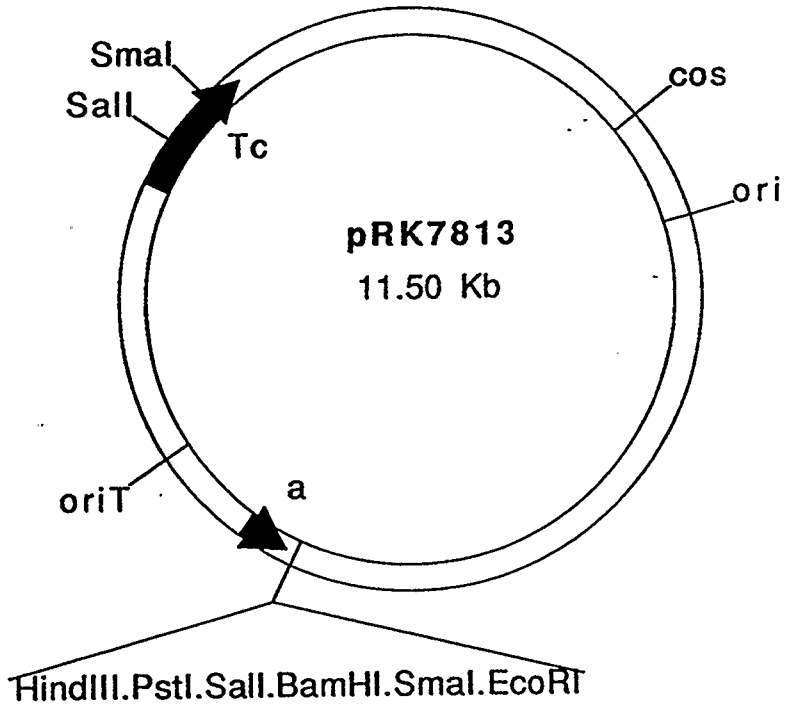
4.0 RESULTS AND DISCUSSION

4.1 Isolation of Catabolic Genes from Strains W14-2 and VF39

Two cosmid libraries were created in vector pRK7813 (Jones & Gutterson, 1987), one for *R. leguminosarum* bv. *viciae* strain VF39, and one for *R. leguminosarum* bv. *trifolii* strain W14-2. Although the regions of interest were plasmid-located, and separate plasmid libraries could have been constructed, a cosmid library provided two important advantages. First of all, the plasmids are themselves very large and constructing a separate library from each one would have in itself taken a considerable amount of time. More importantly, though, a cosmid library of the entire genome of the organism allowed us to screen not only for any plasmid-encoded catabolic genes, but also will allow for screening of regulatory determinants which may not all be plasmid-encoded. In addition, cosmid libraries of these two strains have many applications beyond the scope of this study.

The vector pRK7813 (Jones & Gutterson, 1987) (Figure 4) was chosen as a cosmid vector for a number of reasons. First of all, it is RK2 based and consequently contains the *oriT* region thus allowing mobilisation by a transfer-proficient plasmid to a wide range of Gram negative genera (Jones & Gutterson, 1987; Stanley et al., 1987). Second, pRK7813 replicates in both *Rhizobium* and *E. coli* (Stanley et al., 1987). In addition, this vector can accommodate a large insert of DNA (approximately 10 Kb more than pLAFR1 for example) (Jones & Gutterson, 1987). It is smaller than many other cosmid vectors, which means that there is less background of vector DNA in the cosmid library (Jones & Gutterson, 1987). Finally,

Figure 4. Cosmid vector pRK7813.
Jones & Gutterson, (1987)
oriT, RK2 origin of transfer; cos, the λ *cos* site; α, the *lacZα* gene; *ori*, origin of replication.


SmaI
SalI
Tc
cos
ori
pRK7813
11.50 Kb
oriT
a
HindIII.PstI.SalI.BamHI.SmaI.EcoRI

it can be used for cloning target DNA which has been partially digested with *Sau*3A into the *Bam*HI site (Jones & Gutterson).

Approximately 800 cosmids were obtained for strain VF39 plated on strain S17-1, which showed relatively high efficiency as a plating strain; 10 μL of λ lysate at a titre of 10^8 colony forming units/mL when used to infect a fresh overnight culture of S17-1 produced approximately 10 colonies. 700 cosmids were obtained for W14-2, packaged in HB101. Plating on strain S17-1 was less efficient for strain W14-2, 10 μL of lysate did not produce any colonies, consequently, strain HB101 was used, with a similar plating efficiency as VF39 on S17-1. In both cases, it was unclear as to why the host strain of *E. coli* chosen made a difference in plating efficiency.

In order to determine if the colonies obtained from packaging and plating were cosmids, two or three colonies per plate were selected, and modified alkaline lysis experiments were then carried out to examine the plasmid DNA. Once the DNA had been isolated, double digests with *EcoR*1 and *Hind*III were carried out, as this combination of enzymes would digest on either side of the cloning site, releasing the vector. Upon visualisation of the digested DNA, the approximate size was noted, which then allowed verification of the cosmids. This also allowed verification that no polycosmids or series of vectors joined head to tail had been formed, and in fact, no polycosmids were evident.

In order to determine if the entire genome of each *Rhizobium* strain was represented, the Poisson Distribution Formula was used:

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

where N=number of recombinants to screen
f=fraction of genome/recombinant
p=desired probability of detection

A genome size of 6 000 Kb was used as an estimate based on the size of the genome of *R. meliloti* strain 1021 (Honeycutt *et al.*, 1993) as the genome size of each strain of *R. leguminosarum* used is unknown. Using this genome size of 6 000 Kb, an approximate cosmid insert size of 35 Kb, and a p value of 0.99, it was calculated that 787.2 cosmids would need to be screened in order to represent 99% of the genome. Thus it was assumed that both cosmid libraries were likely to represent the majority of the genome of each strain of *Rhizobium* from which they were obtained.

Initially, complementation experiments were performed with both cosmid libraries using carbon sources for the catabolic genes which had been shown to be plasmid located (Baldani *et al.*, 1992; Hynes, unpublished observations). Cosmids were mobilised from donor *E. coli* strain S17-1 into a recipient *Rhizobium* strain, lacks the ability to grow on a particular carbon source. Several cosmids which complemented for different carbon sources were isolated in this manner (Table 5). Through complementation with W14-2c, 2 cosmids, one isolated from W14-2, and one from VF39 were obtained which enabled the growth of the mutant strain on adonitol. A cosmid allowing growth on lactose from W14-2 was isolated in a similar manner. From complementation experiments using W14-2b, 1 cosmid allowing for growth on sorbitol was isolated from the W14-2 cosmid library, and 2 cosmids allowing for growth on rhamnose were isolated, also from W14-2. A mutant strain of W14-2, 22-1 which contained a Tn*5*B20 insertion and thus was unable to grow on glycerol was also used in complementation experiments, and gave rise to 2 cosmids from VF39 which enabled growth on glycerol. Since several different catabolic genes were isolated from each strain of *Rhizobium*, this served to verify results of the

Table 5
Cosmids, Strain Origin, Strain Used for Complementation, and Corresponding Carbon Source On Which Growth Is Able to be Complemented

Cosmid	Strain Cosmid From	Strain Used to Isolate Cosmid	Carbon Source On Which Cosmid Complements for Growth
pLFVFA	VF39	W14-2c	Adonitol
pLFWA	W14-2	W14-2c	Adonitol
pLFVG1	VF39	22-1	Glycerol
pLFVG2	VF39	22-1	Glycerol
pLFWL1	W14-2	W14-2c	Lactose
pLFWR1	W14-2	W14-2b	Rhamnose
pLFWR2	W14-2	W14-2b	Rhamnose
pLFWS1	W14-2	W14-2b	Sorbitol

Poisson Distribution calculations indicating that a significant portion of the genome for each strain was represented.

Other genera of bacteria, for example, *E. coli* were also used in attempts to obtain catabolic genes from the 2 cosmid libraries created. Specifically, *E. coli* strain HB101, due to the fact that cosmids were already contained within this strain was also used for complementation studies. In addition, examples exist in the literature of complementation experiments using HB101; for instance, McDermott and Kahn, (1992) successfully used an *E. coli* isocitrate dehydrogenase mutant in complementation experiments with *R. meliloti*. As HB101 is a *lacY* mutant, the lactose cosmid isolated from W14-2 was mobilised into HB101, and several colonies were subsequently purified. Unfortunately, when colonies growing on lactose were induced to lose this cosmid through repeated streaking on tetracycline minus medium, the colonies still retained the ability to grow on lactose. Apparently, the problem of reversion was not unique to this study as demonstrated by a group working with *Agrobacterium radiobacter* (Williams *et al.*, 1992). A similar type of complementation experiment was carried out in HB101, with similar results, leading the research group to conclude that the *lac*$^+$ phenotype was probably due to the reversion of the host organism to *lac*$^+$ mediated via the low-affinity lactose-H+symport system (Williams *et al.*, 1992), which may have occurred during this study. Similarly, HB101 cells which appeared to have gained the ability to grow on arabinose, galactose, or mannitol were found to have done so by mutation rather than by complementation with a cosmid. The rate of reversion of these characteristics was higher than the rate of complementation, hindering isolation of catabolic genes from strains VF39 and W14-2.

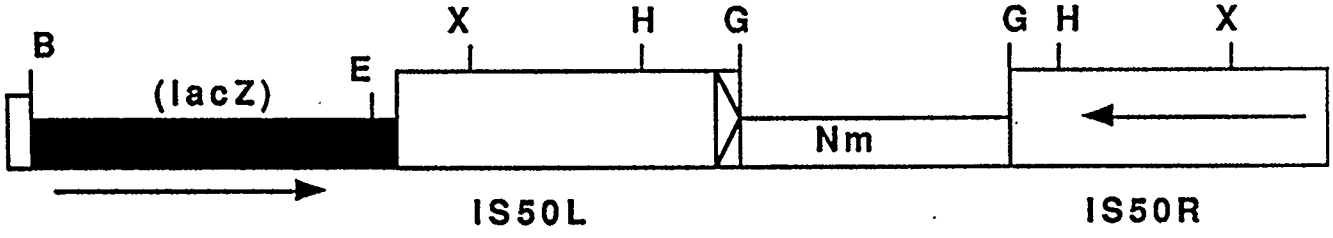
Once isolated by complementation, cosmids were verified by first mobilising them back into *E. coli* strain S17-1 Plasmid preparations were then performed, and the DNA was subsequently digested with various restriction enzymes such as *EcoR*I and *Hind*III which would digest on either side of the insert, and electrophoresed.

As a result of screening cosmid libraries of 2 strains of *Rhizobium leguminosarum*, several different cosmids carrying putative catabolic genes were isolated. Although a greater number of cosmids was produced from strain VF39 than from strain W14-2, W14-2 produced a greater variety of catabolic genes when screened. Perhaps the VF39 library produced was less random than the W14-2 library. Or, as strains of W14-2 missing various plasmids were what cosmids were mobilised into in order to screen for catabolic genes; perhaps VF39 DNA was unable in many instances to complement the W14-2 strain for the use of several of the carbon sources examined. Work by Baldani *et al.* (1992) demonstrated that 3 strains of *R. leguminosarum* bv. *trifolii* were each able to utilise unique carbon sources, rather than all of the strains being able to utilise the same carbon sources. As mentioned, carbon sources were chosen for complementation studies based on work already done with strain W14-2. This suggests that VF39 can very likely utilise several different carbon sources which are, for the most part, distinct from those used by strain W14-2; however, the cosmid libraries were not screened using these carbon sources.

4.2 Creation of Transposon Tn*5* Mutants

Transposon Tn*5* derivative Tn5*B*20 (Figure 5) was used to conduct a random mutagenesis of *Rhizobium* strains W14-2 and

Figure 5. Transposon Tn*5* derivative Tn*5*B20. Open box with arrow indicates transposase. Open arrowhead indicates *npt*-promoter. lacZ= promoterless ß-galactosidase gene with short sequence at left end necessary for transposition Abbreviations: B, *BamH*I; H, *Hind*III; X, *Xho*I; G, *Bgl*II; E, *EcoR*I (Simon *et al.*, 1989).


B
(lacZ)
E
X
H
G
G
H
X
Nm
IS50L
IS50R

VF39. Transposon Tn*5* itself has many advantages for use in random mutagenesis (Simon *et al.*, 1983): 1) Tn*5* transposes with high frequencies in many different Gram negative bacterial species; 2) Tn*5* inserts with little target sequence specificity; 3) Tn*5* exhibits low probability of genome rearrangements upon transposition and a high stability once established in a genome; and 4) Tn*5* encodes an aminoglycoside 3' phosphotransferase (*nptII*) conferring resistance to neomycin and kanomycin, useful for selection purposes. Putative Tn*5* mutants were obtained which were unable to grow on adonitol, arabinose, adonitol and arabinose, glycerol, and mannitol (Table 6). As well, auxotrophs requiring adenosine, isoleucine, valine, uracil, and tryptophan were also obtained. Over 2000 colonies were screened, resulting in the discovery of numerous different mutants, suggesting that insertion of Tn*5*B20 into each strain occurred at random. Regulatory studies were then carried out on all of the sugar mutants as Tn*5*B20 contains a promoterless *lacZ* gene and these studies were replicated twice and average values calculated. These studies examined whether or not the presence of the carbon source the strain was unable to utilise induced the production of ß-galactosidase. Two assumptions were made: first of all that Tn*5*B20 had caused the mutation, and second, that Tn*5*B20 had inserted in the correct orientation with respect to the putative catabolic gene promoter. Results from those cases which appeared to fulfil these criteria are shown in Table 7. Strain 1-1 showed a 5-fold increase in activity upon addition of arabinose, and a similar increase on addition of adonitol. Upon increasing the concentration of arabinose, added, a 40-fold increase in activity was seen, whereas activity appeared to drop on addition of two times more adonitol. Addition of higher

Table 6
Carbon Source Catabolism Mutants of *R. leguminosarum* strain W14-2

Mutant Designation	Strain	Carbon Source Which Cannot be Utilised
1-1	W14-2	Adonitol and Arabinose
1-2	W14-2	Mannitol
17-3	W14-2	Adonitol
22-1	W14-2	Glycerol
32-2	W14-2	Arabinose

Table 7
Carbon Source Effect on β-Galactosidase Activity of Mutants of *R. leguminosarum* strains VF39 and W14-2

Mutant/ Carbon Source Added	Units of ß-Galactosidase Activity		
	Glycerol	Glycerol + Carbon Source Mutant Cannot Utilise	Fold Induction
1-1	112.0		
0.2% Arabinose		551.7	5x
0.2% Adonitol		411.0	4x
0.2% Both		308.3	3x
0.4% Arabinose		4320.4	40x
0.4% Adonitol		134.5	1x
0.4% Both		3440.0	30x
1-2	372.0		
0.2% Mannitol		370.0	1x
0.4% Mannitol		594.8	2x
17-3	780.6		
0.2% Adonitol		7324.4	10x
0.4% Adonitol		5069.1	7x
32-2	1142.8		
0.2% Arabinose		3598.7	3x
0.4% Arabinose		2755.9	2x

concentrations of both sugars showed approximately the same activity as the more concentrated arabinose alone. Strain W17-3 was also very significant, showing approximately a 10-fold increase in ß-galactosidase activity upon addition of adonitol althought this activity did not increase upon doubling of the adonitol concentration. Strain 32-2 showed approximately a 3-fold increase in ß-galactosidase activity upon addition of arabinose, again, not changing with increasing the increasing concentration of arabinose. 1-2 showed no increase in activity upon addition of 0.2% mannitol, but a slight increase in activity upon addition of 0.4% mannitol. As ß-galactosidase production depends upon the Tn*5* construct being introduced in the correct orientation with respect to a functional promoter, these results suggest that this occurred only for strains 1-1, 1-2, 17-3, and 32-2. Although all of these mutants were subsequently set aside, further studies could utilise these mutants for competition studies.

4.3 Subcloning of Catabolic Genes from Cosmids

4.3.1 Use of Adonitol Probe

As mutant 17-3 was unable to utilise adonitol, it was thought that DNA flanking the Tn5 insert would be useful in creating a probe with which to characterise further both adonitol cosmids. Initially, total DNA from 17-3 was digested with *Cla*I as it was believed that this enzyme would not digest within the Tn*5* construct. It was later discovered that there is a *Cla*I site present within the *lacZ* gene; however, at the time, this was not realised. It was discovered that the vector pSUP102 had also become incorporated into the *Rhizobium* genome along with the Tn*5* construct. This greatly simplified subcloning, as the digest was simply religated. At this point,

the subclone was digested with *ClaI/KpnI* and compared with Tn*5*B20 contained in the vector pSUP102 digested with *ClaI/KpnI*. Digestion of the subclone with these two enzymes resulted in 3 fragments which were not present in the Tn*5*B20 so they were presumed to contain *Rhizobium* DNA from the adonitol region. These 3 bands were subsequently isolated, one approximately 800 base pairs in size, and the other 2 forming a doublet approximately 500 base pairs in size. These fragments were precipitated down together and DIG labeled. Digests of pLFVFA DNA and pLFWA DNA, the 2 cosmids complementing for growth on adonitol, were performed, the gel was blotted, and Southern hybridisation was carried out. Bands which showed hybridisation were then isolated from the agarose gel, Prep - A - Gene treated, and each of the fragments subcloned into pRK7813, which was chosen due to the ease with which it could be mobilised into *Rhizobium*. Resulting subclones were then mobilised into wild-type strain W14-2c only and checked to see if the strain had regained the ability to grow on adonitol. Unfortunately, this method did not yield any subclones which were able to complement for adonitol use. This may in part be due to the fact that the probe may have contained a portion of the *lacZ* gene from pRK7813 resulting in the probe being complementary to more than just the adonitol region. In addition, the enzymes chosen to digest the cosmids may very well have cut the genes or operons in two. Consequently, several bands were probably isolated and used in subcloning which had no actual portion of the adonitol region in them.

As many Tn*5* mutants were available which could not utilise various carbon sources, it was believed that additional probes for carbon sources such as rhamnose and sorbitol could be created in a similar fashion. Unfortunately, what had

looked to be promising catabolic mutants which could have been used in the same way as 17-3 to manufacture probes, turned out not to be mutants at all and were not included in the results of this study. Even under the best circumstances though, it would be expected that not all putative mutants isolated would actually turn out to be legitimate. The Tn*5* construct may not have caused the mutation, and consequently revertants may have very readily occurred. Some mutants were found to have multiple Tn*5* inserts. As well, mutant phenotypes were also caused when plasmids were lost. Finally, unpublished observations (M. Hynes) have suggested that with the mode of delivery of Tn*5*B20 used, there is a high occurrance of vector integration, and again, many of these mutants would readily revert.

4.3.2 Other Attempts at Subcloning

In addition, random subcloning was attempted for each of the cosmids obtained. Enzymes *Hind*III, *Pst*I, *BamH*I, and *EcoR*I which cut vector pRK7813 at one location were used separately to digest each of the cosmids and these digests run on agarose gels. Resulting bands between 2 Kb and 12 Kb were isolated from agarose gels, ligated into vector pRK7813, and subsequently mobilised into the appropriate *Rhizobium* mutant. Also, *Hind*III digests and *BamH*1 digests of each of the cosmids were precipitated, resuspended in ligase buffer, and religated under conditions favouring recircularisation. Again, resulting subclones were mobilised into the appropriate *Rhizobium* strain; however, in both cases, no subclones having the ability to complement for growth on the desired carbon source were obtained. This may have been due to the actual size of the cosmids themselves as compared to the size of the gene(s). As there is such a significant amount of DNA which is

not part of the catabolic region, it is very likely that most subclones made would not even contain the desired region. Again, it is very likely that enzymes chosen would have cut within the gene or operon, leaving fragments which would only contain portions of the gene or operon, and this was confirmed upon construction of a restriction map of the adonitol region, as described in section 4.5.

4.4 Tn*5* Mutagenesis

As subcloning by the previous methods was not successful, Tn*5* saturation mutagenesis of the catabolic genes using construct Tn*5*B20 was carried out on the cosmids carrying putative catabolic genes. The lactose cosmids obtained were not used in this or in any further experiments, as the phenotype was not clear enough to provide reliable selection. Through Tn*5* mutagenesis, cosmids which lost the ability to complement for each carbon source were obtained. The cosmid DNA was then examined by agarose gel electrophoresis to verify that a Tn*5* insertion event had occurred. Wild-type cosmid DNA, and cosmid DNA assumed to contain a Tn*5* insert were each digested with *Sal*I, as this enzyme does not cut within Tn*5*B20. Digests were then electrophoresed and compared, and the patterns examined to see if a fragment from a mutated cosmid corresponding to one from the wild-type cosmid had disappeared, and a new fragment approximately the size of the missing fragment plus the Tn*5*B20 construct had appeared.

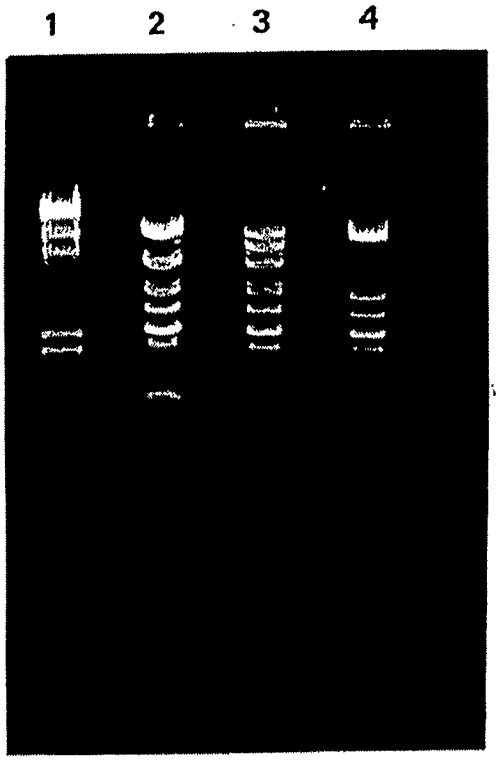
Glycerol, rhamnose and sorbitol cosmids which appeared to have Tn*5* insertions based on antibiotic resistance and the phenotype of the recipient *Rhizobium* strain upon mating, when examined on agarose gels seemed to have undergone some sort of deletion event, resulting in the loss of most of the cosmid.

Later work demonstrated that the *E. coli* strains which contained these cosmids after being mobilised into *E. coli* from *Rhizobium* were resistant to Km as well as Tc suggesting that the helper plasmid pRK2013, used in the mating of the cosmid into *E. coli* was also present in the cell. Consequently, pRK2013 may have been mobilised into the *Rhizobium* strain where Tn*5* insertion would not have been required to produce the Km resistant phenotype. This too would have resulted in the glycerol, rhamnose or sorbitol minus phenotype in the recipient *Rhizobium* strain as pRK2013 would be unable to complement for growth on any carbon source examined, and would have produced the banding pattern obtained on the agarose gels. As this was not realised until much later, these cosmids were subsequently set aside. However, using strains devoid of pRK2013, Tn*5* mutants in two rhamnose and 1 sorbitol cosmid have been obtained. No pLFVA:: Tn*5* mutants were obtained. pLFWA::Tn*5* mutants were obtained and 15 were selected for further study. *Sal*I digests of the wild type cosmid and the two different Tn*5*B20 insertions (pLFWA5 and pLFWA66) were performed to confirm the presence of the Tn*5* insert. *Sal*I digests of pLFWA5 and pLFWA66 were compared back to the *Sal*I digested wild type cosmid and examined to determine if a band present in the wild type cosmid was missing from the cosmid plus insert, and that a band equal in size to the size of the Tn*5* fragment plus the missing band had appeared (Figure 6).

4.5 Mapping of pLFWA Tn*5* Insertions

Three different approaches were used in combination to obtain restriction maps of the Tn*5* regions in pLFWA. The entire cosmids containing each of the two different inserts, and the cosmid lacking the Tn*5* insert were all digested with

Figure 6. *Sal*I digests of pLFWA5 and pLFWA66 showing location of Tn*5*B20 insertion.
Lane 1, λ*Hind*III Marker; Lane 2, pLFWA *Sal*I; Lane 3, pLFWA2 *Sal*I; Lane 4, pLFWA66 *Sal*I.
Lane 3 demonstrates that a fragment approximately 1 Kb in size is missing and a fragment approximately 9 Kb has appeared corresponding to the insertion of Tn*5*B20 (8.4 Kb) into the 1 Kb band.
Lane 4 demonstrates that a fragment approximately 5 Kb is missing and a fragment approximately 13 Kb is migrating very closely to the 12 Kb band present in the wild type cosmid pattern.
Arrows indicate the bands in the wild type cosmid DNA which contain a Tn*5*B20 insertion in pLFWA5 or in pLFWA66.


1
2
3
4

*BamH*I, *EcoR*I, or *Hind*III, and double digests with combinations of these enzymes, and these digest patterns compared (Figure 7). In addition, cosmid DNA containing each of the two different Tn*5* inserts was obtained by a small scale plasmid prep, digested with *Sal*I, and ligated into the *Sal*I site of pUC19 to create pLFWA5-19 and pLFWA66-19. From pLFWA66-19, a 3.5 Kb *BamH*I fragment containing part of the adonitol region was isolated (*Sal*I fragment indicated by arrowheads on Figure 9), labeled with DIG and used as a probe to hybridise with pLFWA cosmid DNA digested with *Sal*I, *EcoR*I, *Hind*III, *Pst*I, or *BamH*I (Figure 8). As well, 2 digests, *Sal*I or *EcoR*I, of pLFVFA DNA were included in order to determine if homologous regions were present in strain VF39. Interestingly though, the digests of pLFVFA contains bands which hybridised with the probe constructed from pLFWA5-19 although these bands are different sizes from those obtained from hybridisation with pLFWA DNA. Finally, ß-galactosidase activities of each Tn*5* containing derivative of pLFWA introduced into strain W14-2 were also carried out (Table 8). All of these results were used in construction of a restriction map of the two Tn*5* inserts (Figure 9) where orientation of inserts was determined by comparison of size of fragments produced by digestion of pLFWA, pLFWA5 and pLFWA66 with *BamH*I, *EcoR*I, *Hind*III, or pair combinations of these enzymes. Direction of transcription of the adonitol dehydrogenase gene is indicated in Figure 9 by an arrow; however, it is not known how large the

Figure 7. *BamH*I, *EcoR*I and *Hind*III digests of pLFWA5, and pLFWA66 compared with *BamH*I, *EcoR*I and *Hind*III digests of pLFWA.

Lane 1, λ*Hind*III; Lane 2, pLFWA *BamH*I; Lane 3, pLFWA5 *BamH*I; Lane 4, pLFWA66 *BamH*I; Lane 5, pLFWA *EcoR*I; Lane 6, pLFWA5 *EcoR*I; Lane 7, pLFWA66 *EcoR*I; Lane 8, pLFWA *Hind*III; Lane 9, pLFWA5 *Hind*III; Lane 10, pLFWA66 *Hind*III; Lane 11, pLFWA *EcoR*I/*BamH*I; Lane 12, pLFWA5 *EcoR*I/*BamH*I; Lane 13, pLFWA66 *EcoR*I/*BamH*I; Lane 14, pLFWA *Hind*III/*BamH*I; Lane 15, pLFWA5 *Hind*III/*BamH*I; Lane 16, pLFWA66 *Hind*III/*BamH*I; Lane 17, pLFWA *EcoR*I/*Hind*III; Lane 18, pLFWA5 *EcoR*I/*Hind*III; Lane 19, pLFWA66 *EcoR*I/*Hind*III.

Note: This figure was taken from one agarose gel although creation of the figure involved cutting and pasting removed lanes containing no useful information.

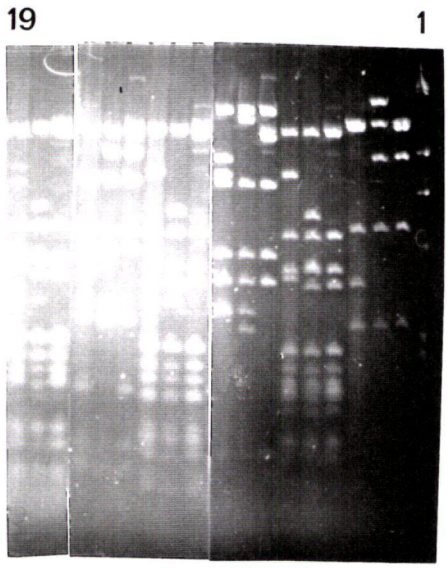

19
1

Figure 8. Southern hybridisation of digests of pLFWA and pLFVFA.

Lane 1, pLFWA *BamH*I; Lane 2, pLFWA *Pst*I; Lane 3, pLFWA *Hind*III; Lane 4, pLFWA *EcoR*I; Lane 5, pLFWA *Sal*I; Lane 6, pLFVFA *EcoR*I; Lane 7, pLFVFA *Sal*I. The probe was created from the central *Sal*I fragment indicated in Figure 9. This fragment was then digested with *BamH*I to produce a 3.5 Kb probe containing part of the adonitol region. Arrows indicate location of λ*Hind*III marker bands.

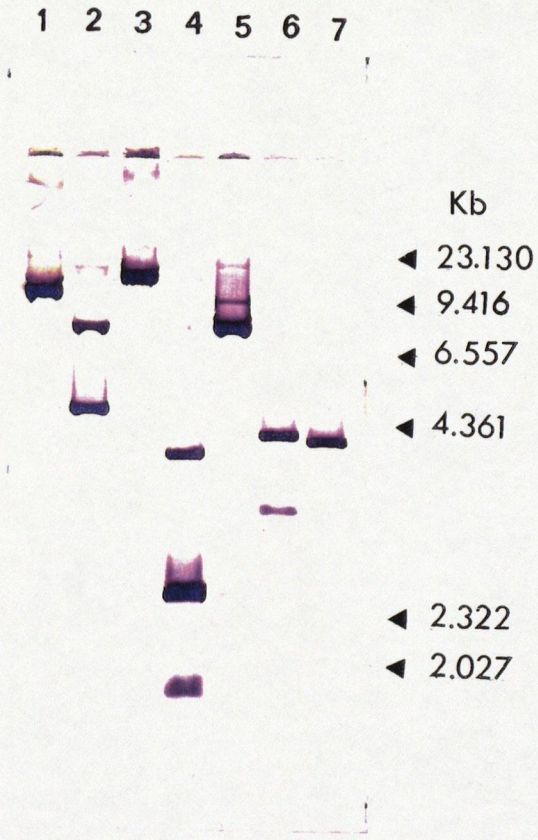
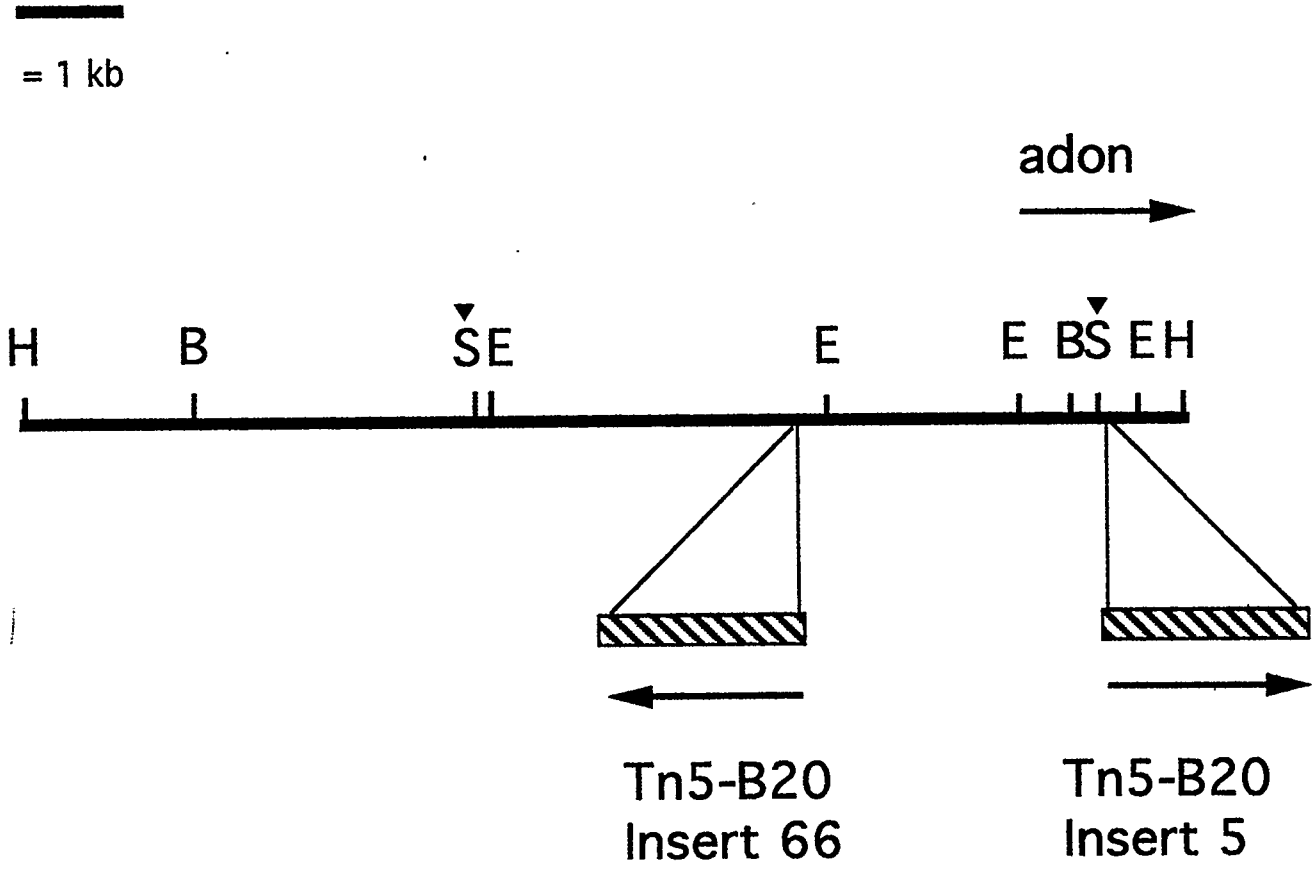

1 2 3 4 5 6 7
Kb
23.130
9.416
6.557
4.361
2.322
2.027

Table 8
Results of ß-Galactosidase Assays of pLFWA Cosmids carrying Transposon Tn*5*B20 in Strain W14-2 Interrupting the Adonitol Complementing Region

Cosmid+ Tn*5*B20/ Carbon Source Added	Units of ß-Galactosidase Activity Glycerol	Glycerol + Adonitol
pWLFWA5	150.0	
0.2%Adonitol		996.0
0.4%Adonitol		1667.2
pWLFWA66	0.0	
0.2%Adonitol		0.0
0.4%Adonitol		0.0

Figure 9. Restriction maps of pLFWA5 and pLFWA66 showing direction of transcription of ribitol dehydrogenase.

Abbreviations: B, *BamH*I; S, *Sal*I; E, *Eco*RI. Arrows show direction of Tn*5*B20 insertion. Arrowheads indicate region used in construction of probe for use in restriction mapping and identifying plasmid on which catabolic region is found.


= 1 kb
adon
H
B
S E
E
E B S E H
Tn5-B20
Insert 66
Tn5-B20
Insert 5

gene itself is, only that the arrow does coincide with the start of the coding region.

The same DIG-labeled *BamH*I fragment was then used to probe an Eckhardt gel of several different strains of *Rhizobium leguminosarum* belonging to 3 different biovars and from a range of geographical locations, where Figure 10a isthe Eckhardt gel (Figure 10a and b). A diagrammatic representation of the Eckhardt gel indicating the pSym plasmids in strains where known (Figure 10c) is included for clarification, as is a diagrammatic representation of the Eckhardt gel indicating the plasmids which demonstrated hybridisation in the Southern analysis (Figure 10d). All of these strains were found to possess the adonitol region on a plasmid. In strains VF39 and 3841 (lanes 17 and 11), the region was found to be located on the pSym based on the known gel mobility of the pSym in these strains (Hynes, personal communication). Strain 6015 (lane 10), a derivative of 3841 which lacks the *nod* region of the pSym still demonstrates hybridisation to the adonitol region suggesting that this region is not closely linked to the *nod* region. In all other strains, the adonitol region was found to be located on a plasmid other than the pSym, again based on previous studies showing size and location of the pSym plasmid in each strain (Hynes, personal communication).

4.6 Creation of Adonitol Mutants

In addition to *Sal*I subclones in pUC19, the *Sal*I fragment from pLFWA66 carrying the Tn*5* construct was also ligated into pJQ200 KS (Figure 11) creating pLFWA66-JQ. This construct was then mobilised into wild type *Rhizobium* strains W14-2, 3841, and VF39. Matings were then plated onto TY

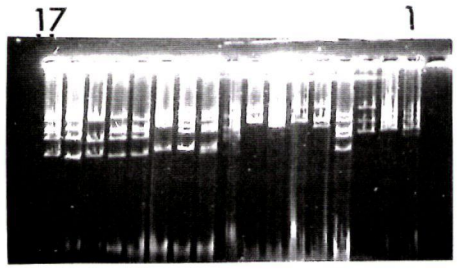
Figure 10a. Eckhardt gel showing plasmid profiles of several strains of *Rhizobium leguminosarum*.
Lane 1, 309; Lane 2, 306; Lane 3, Y10; Lane 4, 248; Lane 5, 3855; Lane 6, B151; Lane 7, 8002; Lane 8, 8401; Lane 9, W14-2; Lane 10, 6015; Lane 11, 3841; Lane 12, 336; Lane 13, LRS 39601; Lane 14, LRS 39501; Lane 15, LRS 393401; Lane 16, LRS 39301; Lane 17, VF39SM.

Figure 10b. Southern hybridisation of Eckhardt gel showing hybridisation patterns of several strains of *Rhizobium leguminosarum*.
Note: lane designations are as above.

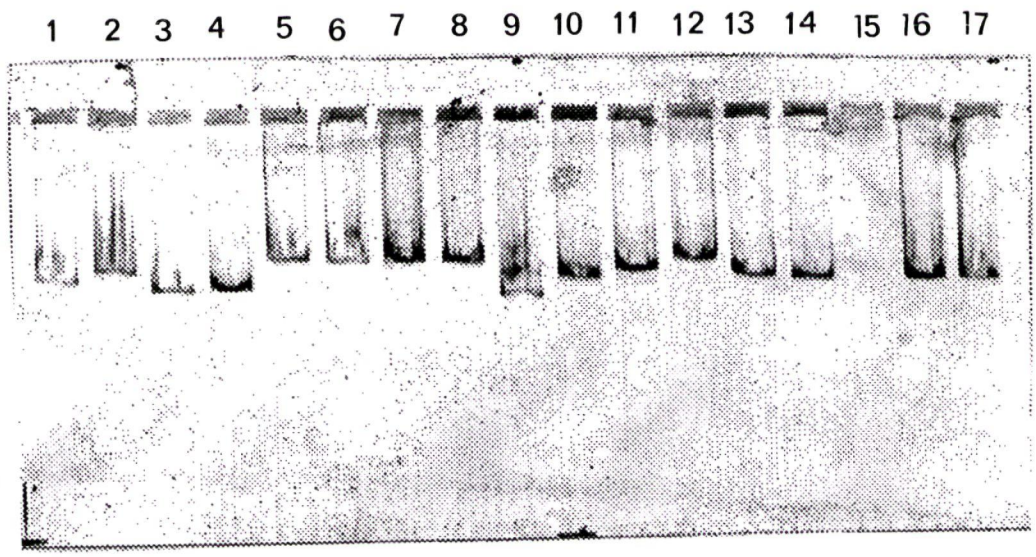
Figure 10c. Diagrammatic representation of Eckhardt gel from 10a showing plasmid profiles of several strains of *Rhizobium leguminosarum* indicating pSym plasmid where known.
Asterisks indicate pSym plasmid. Lane designations are as in 10a.

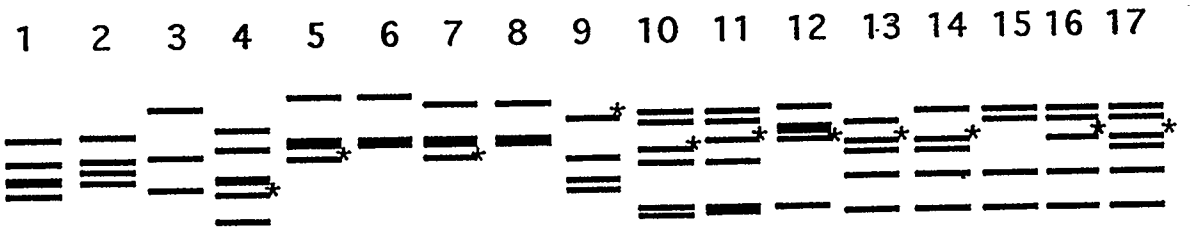
Figure 10d. Diagrammatic representation of Eckhardt gel from showing plasmid profiles of several strains of *Rhizobium leguminosarum* indicating hybridising band.
Arrowheads indicate hybridising band. Lane designations are as in 10a.

10a



10b




1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

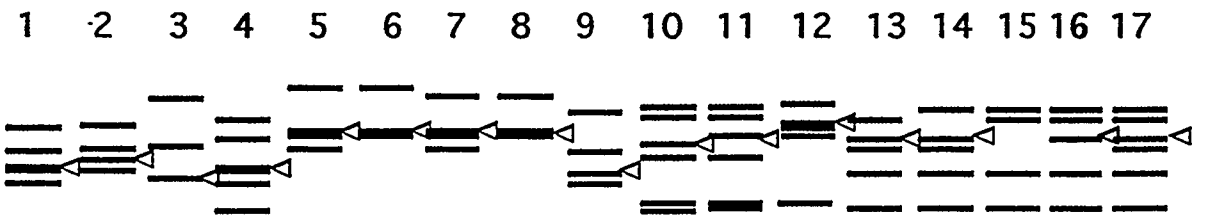
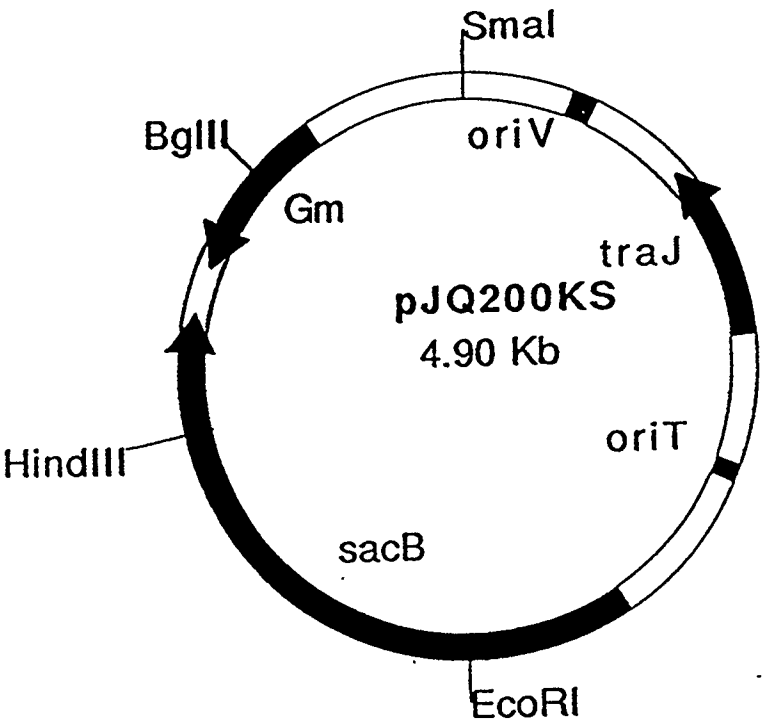

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 11. Suicide vector pJQ200 KS.
*Sma*I site = site of MCS insertion:
*Kpn*I *Apa*I *Xho*I, ***Sal***I, *Hind*III, *EcoR*V, *EcoR*I, *Pst*I,
*Sma*I, *BamH*I, *Spe*I, *Xba*I, *Not*I, *Bst*XI, *Sst*I.


SmaI
BglII
oriV
Gm
traJ
pJQ200KS
4.90 Kb
oriT
HindIII
sacB
EcoRI

medium containing 5% sucrose, Nm (for the transposon), and Sm (to select against *E. coli*). Colonies resulted from all matings; however, upon replica-plating onto VMM plates containing adonitol as sole carbon source, only W14-2 colonies were shown to have lost the ability to grow on adonitol as sole carbon source. Lack of colonies in 3841 and VF39; would suggest a transposition event rather than a recombination event. Replacement of the wild-type region with the Tn5 containing region in W14-2 was confirmed by Southern hybridisation (Figure 12) where *Sal*I digests of total DNA from W14-2 (Lane 6), and from 5 independent mutants W14-2A1-5 (Lanes 1-5), were run on an agarose gel, blotted, and hybridised with the DIG-labeled *BamH*I fragment described earlier, revealing an increase in hybridising fragment size confirming the presence of the Tn*5* insert.

4.7 Plant Tests

Three independent mutants of W14-2 were coinoculated in a 1:1 ration onto white clover (*Trifolium repens* cv. Ladino), red clover (*Trifolium pratense* cv. Ottawa) and red clover (cv. Norlac). Results obtained from white clover are shown in Table 9 as these were the plants which showed the best growth. Interestingly enough, in spite of a claim made by Baldani *et al.*, (1992) that W14-2 is fix$^-$ on crimson clover (*Trifolium incarnatum*), some fixation did occur on cv. Ottawa and a very small amount of fixation did occur on cv. Norlac. Unfortunately, recovery from nodules was limited, as many nodules were very small. The null hypothesis used was that there should be no significant difference between the ability of the adonitol mutants to occupy nodules as compared with

Figure 12. Southern hybridisation of total DNA digests demonstrating the presence of Tn*5*B20 insertion in the adonitol catabolic region.
Lane 1, W14-2A1; Lane 2, W14-2A2; Lane 3, W14-2A3; Lane 4, W14-2A4; Lane 5, W14-2A5; Lane 6, W14-2.
The probe was created from the central *Sal*I fragment indicated in Figure 9. This fragment containing Tn*5*B20 insert 66 was then digested with *BamH*I to produce a 3.5 Kb probe containing part of the adonitol region. Arrows indicate sizes of λ*Hind*III marker.

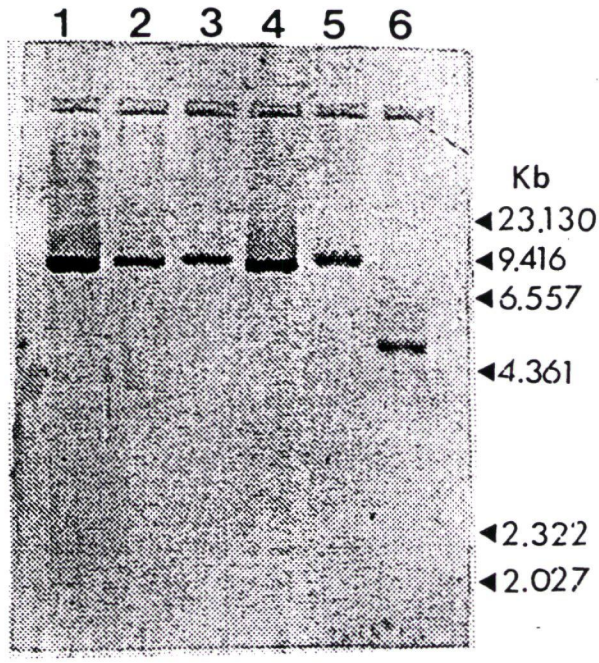

1
2
3
4
5
6
Kb
23.130
9.416
6.557
4.361
2.322
2.027

Table 9
Results of Competition Assays of W14-2A1,2, and 3 Coinoculated with W14-2 on *Trifolium repens* cv. Ladino

Strain Re-Isolated	Number of Nodules
W14-2	21
W14-2A1,2,3	12

the wild-type strain. A Chi square analysis performed on the numbers resulted in a probability value of $0.10 < p < 0.20$ suggesting that the null hypothesis should be accepted indicating no difference between ability to occupy nodules . Unfortunately, it is difficult to draw any conclusions from these results as the sample size was so small due to poor recovery from nodules. However, these plant tests are subsequently being repeated under more realistic conditions.

4.8 Sequencing Results

Results of the BLAST search of sequence data obtained are shown in Figure 13. 249 bases of sequence were obtained. For pLFWA5, significant homology was shown to ribitol dehydrogenase (*rbtD*) from *Klebsiella aerogenes* (Dothie *et al.*, 1985), confirming that the region isolated did contain a catabolic gene. Experiments analysing the ribitol dehydrogenase gene of *K. aerogenes* suggest that this gene is part of an operon including at least two enzymes, ribitol dehydrogenase and D-ribulokinase as well as a control region, *rbtC* (Charnetzky & Mortlock, 1974), and a repressor, *rbtR,* with a total size of approximately 3 Kb (Dothie *et al.*, 1985). In this study, difficulties were encountered when attempting to subclone the adonitol catabolic region. If this gene is indeed part of an operon in *Rhizobium* as it is in *K. aerogenes,* it would make it unlikely that the methods attempted for subcloning would have succeeded, explaining why so many difficulties were encountered at this particular stage of the study.

Figure 13. Results of BLAST analysis of sequence data generated with IS50 unique primer. Amino acid sequence obtained from pLFWA5-19 compared with sequence from *Klebsiella aerogenes* (indicated by asterisk). Top numbers indicate nucleotide position from sequence generated. *K. aerogenes* sequence numbers correspond to amino acid numbers from database. Middle sequence indicates identity between the 2 sequences.

```
72  VXRKTGDIILTSSVXGLVLVVWXPIYTASKXAVQAFVHTLRRQVAYHGXXXGA 230
    ++K+GDII TSS+ G+V V+W P+YTASK AVQAFVHT RRQVA +GVRVGA
135 IAQKSGDIIFTSSIAGVVPVIWEPVYTASKFAVQAFVHTTRRQVAQYGVRVGA 187
```

5.0 DISCUSSION

As previously stated, a characteristic of most *Rhizobium* species is that they contain large plasmids representing a significant portion of the genome of the organism (Hynes *et al.*, 1989). In addition, these large plasmids are very stable and resist curing by conventional methods (Hynes *et al.*, 1989). It can then be assumed that the genes contained on plasmids are important to the organism; however, it seems unusual that, if these genes are significant, they are not present on the chromosome. It is possible that plasmid encoded genes allow an organism a certain amount of flexibility. Although changes in the chromosome do occur over time, this is probably a much slower process. Eberhard, (1989) argues that some genes are always plasmid encoded because certain genes favour the plasmid mode of reproduction over the chromosomal one. Genes on plasmids are more likely to be transferred horizontally than those chromosomally located leading to a wider variety of genetic settings with which the plasmid can interact (Eberhard, 1989). Plasmid encoded genes are also able to be amplified more rapidly when circumstances require their expression (Eberhard, 1989). As well, plasmid encoded genes can evolve more rapidly than chromosomally located genes (Eberhard, 1989). Consequently, genes on plasmids are more likely to code for local adaptations.

It comes as no surprise that many catabolic genes in different strains of *Rhizobium* are plasmid located. Boivin *et al.*, (1990, 1991); Goldmann *et al.*, (1994); van Egeraat (1978); Tepfer *et al.*, (199) have all demonstrated that leguminous plants produce a variety of substances, and presumably, these substances are present in the rhizosphere surrounding the particular plant. It is interesting, though that various *Rhizobium leguminosarum* strains from different geographic

regions all demonstrated a plasmid-encoded adonitol region. This geographic diversity of the strains examined would suggest that adaptation to growth on adonitol is not a local adaptation; however, each different rhizosphere may be thought of as a local environment. Perhaps, as Eberhard, (1989) points out, the adonitol region may simply be plasmid located as the plasmid form of replication of that gene is preferred.

Results from this study as well as those from previous studies demonstrate that catabolic genes may be located on the symbiotic plasmid in a particular strain (Boivin *et al.*, 1990, 1991; Murphy *et al.*, 1987, 1993; Rossbach *et al.*, 1994, 1995). In the case of rhizopines, synthesis genes are under symbiotic regulation; rhizopines are produced by the bacteroid to be utilised by the free-living organisms in the rhizosphere. Similarly, *trc* genes for the catabolism of trigonelline are induced during all of the symbiotic steps, suggesting that trigonelline is a nutrient source throughout the *Rhizobium*-legume association (Boivin *et al.*, 1990). If catabolic genes are on the symbiotic plasmid, this suggests that they have evolved due to the presence of specific compounds which are produced by a particular host, although this does not necessarily mean that these catabolic genes are under symbiotic control. Perhaps in strains such as VF39 and 3841, a particular host plant produces adonitol, or some precursor. In which case, it would be advantageous for these two strains to be able to catabolise this particular compound. It may also be speculated that the adonitol genes may be part of a pathway degrading a more complex substrate; for example, an as of yet undiscovered rhizopine common to these strains. It could also mean that, as in the case of trigonelline, the plant derived compound is specifically used during a certain stage in the

symbiotic process, thus, control of the catabolic genes is under symbiotic control.

However, it has also been demonstrated in numerous cases that catabolic genes may also be found on non-symbiotic plasmids. Perhaps these genes are required for more ubiquitous substances or intermediates which commonly occur from the breakdown of a variety of substrates. Again, though, a plasmid may have been inherited from another strain, and the ability to utilise a variety of substrates proved useful for the recipient strain.

6.0 FUTURE DIRECTIONS

As Tn*5* inserts into the sorbitol and rhamnose regions on all 3 cosmids have now been obtained, similar experiments to insert these mutated regions back into the wild-type organisms will be carried out. In addition, it will then be possible to create double mutants of the regions already mentioned, to study the cumulative effect of several catabolic mutations on rhizosphere behavior and competitiveness. As well, more sequencing will be carried out to determine if the sorbitol and rhamnose regions are also genuine catabolic genes. Further competitive assays will then be carried out to obtain a more complete picture of the role of plasmid encoded catabolic genes in competition of *Rhizobium leguminosarum*. In using Tn*5*B20 to make these mutants, gene fusions were created, thus allowing studies of regulation of these loci to be carried out. For instance, the effect of the presence of roots on host and non-host plants on gene expression could be tested.

Population genetics studies will also be carried out to examine correlations between the presence of the adonitol region and incompatibility groups, allowing us to trace the evolutionary history of certain sets of plasmid encoded genes. The majority of studies carried out have focused on genes directly involved in symbiosis, such as *nod*, *nif*, and *fix* genes. Although these genes all are clustered together on a plasmid, the rest of the plasmid appears to be highly variable. Hynes (personal communication) has shown that different pSym plasmids of *R. leguminosarum* belong to at least 6 different incompatibility groups, and some sym plasmids are incompatible with non-pSym plasmids from other strains, or even plasmids in other species such as *Agrobacterium* (O'Connell *et al.*, 1987). This suggests that *nod*, *nif* and *fix*

genes are not reliable markers for the history of a given plasmid, as they may have exhibited considerable mobility in their evolutionary history. It can be speculated that catabolic genes may correlate more closely with the incompatibility group of a set of plasmids. In fact, preliminary evidence for this is suggested by the fact that the adonitol locus studied in this work is located on the pSym plasmid in two cases, 3841 and VF39 where it is known that the pSym plasmids do belong in the same incompatibility group (Hynes, personal communication). In strains such as 8002, 248 and 336 where the pSym plasmids belong to different incompatibility groups from those in VF39 and 3841, the adonitol locus is not located on the pSym plasmid. From using clones of other catabolic genes isolated in this study, it should be possible to answer questions about 1) the general plasmid location of these genes; and 2) whether sets of genes; for example, rhamnose and sorbitol, always stay together on the same basic plasmid replicon as determined by plasmid incompatibility.

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