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MANIPULATIONS OF Rhizobium meliloti
FACILITATING MUTAGENESIS, GENE TRANSFER AND
GENOME REARRANGEMENTS

by

Gopalan Selvaraj, M.Sc.

A thesis submitted to the Faculty of Graduate
studies in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy.

Department of Biology,
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To my parents, with love

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ABSTRACT

Rhizobium meliloti is a Gram-negative bacterium that fixes atmospheric nitrogen in symbiotic association with alfalfa, a major forage crop of significant economic importance. The purpose of this project was to develop methods for the genetic analysis and manipulation of R. meliloti, with the intent of eventually applying these methods to study and manipulate the bacterial component of the R. meliloti-alfalfa symbiosis.

Plasmid DNA-mediated genetic transformation is one of the commonly used methods in introducing native, and engineered plasmids into bacterial cells. Such a method was developed for a number of R. meliloti isolates of diverse origin. Insertion mutants of R. meliloti are valuable tools in the genetic analysis and manipulations of an organism. A procedure for deriving transposon insertion mutants of R. meliloti was devised. This involved the construction of a set of self-transmissible plasmids carrying one of the transposons, Tn1, Tn5, Tn9. These plasmids were conjugative in matings with R. meliloti, but were incapable of replication and maintenance in this host (suicide plasmids). As a result, they could be used as delivery vehicles for transposons in insertion mutagenesis of R. meliloti.

While using the suicide vehicles bearing Tn5 in generating insertion mutants of R. meliloti strains, it was noticed that all of the putative mutants acquired streptomycin-resistance (Sm^r) phenotype. The basis of

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this observation was studied further and it was shown that Tn5 carries a gene that specifies Sm-resistance in Rhizobium, but not in Escherichia coli.

Using the suicide vehicles constructed as a part of this study, a large number of insertion mutants of R. meliloti was isolated and the phenotypes of these mutants were identified. This collection of mutants includes auxotrophs, UV-sensitive mutants, and mutants defective in symbiosis (Sym^-). Among the Sym^- mutants, those that were defective in nodulation (Nod^-), and in nitrogen-fixation (Fix^-) were identified. The Fix^- mutants were further classified based on the number of nodules, and the morphology of the nodules induced by them. These mutants should be useful in the genetic analysis of R. meliloti.

Derivatives of transposons Tn1 and Tn5 carrying the "mob (oriT)" region of a P-group conjugative plasmid were constructed. The "mob (oriT)" region confers upon a genetic element carrying it, the ability to be mobilized by a P-group conjugative plasmid. Suicide vehicles similar to the ones described above, but carrying these engineered transposons were constructed. These should be useful in further manipulations of R. meliloti.

J.

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List of Abbreviations

(The genetic abbreviations used conform to the proposals of Demerec et al. (1966) and Novick et al. (1976) that have been widely accepted)

Ade	Adenosine
<u>am</u>	Amber mutation
Ap	Ampicillin
Arg	Arginine
Asp	Aspartic acid
Asn	Asparagine
dATP	Deoxyadenosine triphosphate
Bio	Biotin
C	Celsius
Cb	Carbenicillin
dCTP	Deoxycytosine triphosphate
<u>che</u>	Chemotaxis
Cm	Chloramphenicol
cpm	Counts per minute
Cys	Cysteine
<u>fix</u>	Nitrogen fixation
<u>fla</u>	Flagella
<u>flu</u>	5-fluorouracil
Gln	Glutamine
Gly	Glycine
Gm	Gentamycin
dGTP	Deoxyguanosine triphosphate

h	Hours
Hac	Root hair curling
His	Histidine
Ile	Isoleucine
Ilv	Isoleucine valine
Inc	Incompatibility
IS	Insertion Sequence
Kb	Kilo basepairs
Km	Kanamycin
Lac	Lactose
Leu	Leucine
Lys	Lysine
Mdal	Megadaltons
Met.	Methionine
min	Minutes
<u>mob</u>	Mobilization
Nm	Neomycin
Nod	Nodulation
<u>nif</u>	Nitrogen-fixation gene
<u>oriV</u>	Origin of replication
<u>oriT</u>	Origin of transfer
Pan	Pantothenate
Phe	Phenylalanine
Pro	Proline
Pur	Purine
Pyr	Pyrimidine
R	Resistance
r m	DNA-restriction and modification

rep	Replication
Rif	Rifampicin
s	Sensitive
Ser	Serine
Sm	Streptomycin
<u>str</u>	Streptomycin-resistance gene
Sp	Spectinomycin
Sym	Symbiosis
Tc	Tetracycline
Thi	Thiamine
Thr	Threonine
Thy	Thymine
Tra	Conjugal transfer
Trp	Tryptophan
Tyr	Tyrosine
uCi	Microcurie
ug	Microgram
ul	Microlitre
Ura	Uracil
UV	Ultraviolet radiation

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INTRODUCTION

Rhizobium meliloti is a Gram-negative soil bacterium which interacts physically and functionally with alfalfa, a forage crop of significant global economic importance. It belongs to a physiological group of Rhizobium strains known as "fast growers" which includes R. leguminosarum, R. phaseoli and R. trifolii that nodulate peas, beans and clover respectively (see Table 1). These Rhizobium strains have relatively shorter generation times than certain other members of this genus (Martinez-de Dreuys and Arias, 1972; see Table 1). After infecting a host plant, the bacterial cells induce the formation of specialized plant organs called root nodules, and within these structures the bacterial cells undergo morphogenetic changes to become the nitrogen-fixing forms, the bacteroids. The host plant supports bacterial metabolism, and in turn the plant derives its supply of fixed nitrogen through the symbiotic association. Apart from these symbiotic nitrogen-fixers, certain other prokaryotes, but none of the eukaryotes that have been examined also fix atmospheric nitrogen in associative relationship with other organisms or in a free-living state.

However, symbiotic associations of legumes and Rhizobium strains are the prime source of nitrogen fixation in agriculture (Vincent, 1974; Newton and Burgess, 1983). A study of the Rhizobium-legume symbiosis is thus both of fundamental and applied interest.

In our laboratory we have been interested in

Table 1

Some commonly used species of Rhizobium and their hosts¹

<u>Rhizobium</u>	Host plant(s)	Comments (if any)
<u>R. meliloti</u>	alfalfa, sweet clover	"Fast-growing"
<u>R. leguminosarum</u> ²	Pea, sweet pea, broad bean	"Fast-growing"
<u>R. trifolii</u> ²	Red clover, white clover	"Fast-growing"
<u>R. phaseoli</u> ²	Bean	"Fast-growing"
<u>R. japonicum</u> ³	Soybean	"Slow-growing"
<u>R. lupini</u>	lupine	"Slow-growing"
<u>Rhizobium</u> sp. ⁴ "cowpea miscellany"	A variety of legumes	"Slow-growing"

¹The different species of Rhizobium are defined by their host-specificity with respect to nodulation and nitrogen-fixation.

²The species are closely related to each other (see Johnston et al., 1976; Benyon et al., 1980; Beringer et al., 1980; Hooykaas et al., 1981).

³Fast-growing isolates of R. japonicum have been isolated from soybean varieties, indigenous to mainland China (Keyser et al., 1982)

⁴These Rhizobium strains are promiscuous; they nodulate a variety of legumes that includes peanuts, cowpea etc.

studying the bacterial endosymbiont of the alfalfa-R. meliloti symbiosis. In general one might ask the following questions about the endosymbiont:

a) How does a Rhizobium cell recognize its specific host?

b) What are the genetic and biochemical factors that induce and govern the morphogenetic changes in host tissue resulting in the formation of a root nodule?

c) How do the bacteria fix nitrogen within these nodules?; is there an induction of specific host factors by the bacteria?

d) Can this process be improved to the benefit of the host plant?

f) Can the genes concerned with nitrogen fixation be installed in other plants so as to extend the range of nitrogen-fixers?

These questions can be addressed systematically by a combination of genetic and biochemical analyses. This thesis is primarily concerned with the genetics of the endosymbiont, R. meliloti.

The bulk of the innovations and development of concepts in bacterial genetics has been due to research on the enteric bacterium, Escherichia coli and closely related bacteria. In more recent years, a variety of novel methods of manipulating this bacterium and its genome have become available. In a large measure, this is due to the discovery and study of its extrachromosomal

elements and movable genetic elements (insertion elements and transposons); and through the development of methods for the in vitro manipulation of its genome. This has resulted in answering or making accessible a variety of questions at the molecular level. These developments have paved the way for studying other bacterial species. It was the purpose of this project to develop such methods for the genetic analysis of R. meliloti with the intent of eventually applying them to the study of symbiosis. In the following chapters the development of these methods is described and discussed, after reviewing pertinent literature on R. meliloti. In this review the developments made in other laboratories subsequent to the initiation of my thesis project (Jan. 1980) are described in a separate section (pp 21) so as to place this thesis in perspective.

The development of a method for introducing plasmid DNA into R. meliloti cells is described in Chapter I. The construction of a set of plasmid vehicles, suitable for insertion mutagenesis in Rhizobium and an extensive assesment of their use in deriving transposon insertion mutants of R. meliloti constitute Chapter II. While using one of the plasmids carrying transposon Tn5 to derive Tn5-insertion mutants of R. meliloti, it was noticed that all of the insertion derivatives had also acquired streptomycin-resistance. Since Tn5 does not specify Sm-resistance in E. coli, a host in which it has been

intensively studied, the basis of this observation was studied further. It was concluded that Tn5 carries a gene that specifies Sm-resistance in Rhizobium strains, but not in E. coli. These results are presented in Chapter III. Chapter IV contains the description of a large number of insertion mutants of R. meliloti strains. This collection includes auxotrophs, mutants defective in symbiosis, and UV-sensitive mutants.

GENETICS OF Rhizobium meliloti

Isolation of mutants

The first step in the genetic analysis or manipulation of an organism is the isolation and assembly of mutants. This provides one with a handle in all subsequent experimentation. Prior to the use of transposons as mutagens in R. meliloti (Casadesus et al. 1980; Ruvkun et al., 1980), mutants of R. meliloti were isolated by screening for spontaneous ones, mostly antibiotic-resistant types, and by mutagenesis with nitrosoguanidine (NTG), ethylmethane sulphonate (EMS) or UV-irradiation. Different laboratories around the world were using natural isolates (of diverse origin) or their antibiotic-resistant derivatives as the subjects of genetic studies. These are listed in Table 2. The use of different strains offers certain advantages. For instance a particular strain may be recalcitrant to some crucial manipulations, in which case a suitable alternative strain would be useful. Another advantage is that the studies on different strains would enable a comparison of the genetics of diverse strains.

Mutagenesis of the different strains of R. meliloti with chemicals or irradiation led to the assembly of a large number of mutants. These mutants, their derivation and characteristics are listed in Table 3 (Appendix 1). In compiling such a list, insertion mutants derived very recently (upto July 1983) in different laboratories but excluding mutants derived in this study are included.

Footnotes to Table 2

¹Brockwell and Hely (1966)

²Casse et al. (1979)

³Johnston et al. (1978); It is not clear if the strain 2011 (6646) held in the Rothamsted Culture Collection is the same as Rm2011(SU47)

⁴Meade and Signer (1977); Rm2011str-3 is a Sm^r mutant of SU47. Some authors refer to this mutant as "Rm2011", while Rm2011 is an alternate designation for SU47.

⁵Ludwig and Johansen (1980)

⁶Spitzbarth et al. (1979); probably a different strain, since the plasmid profiles reported in Simon et al. (1982) for Rm20-1 and in Rosenberg et al. (1981) for SU47 are different

⁷Szende and Ordogh (1960)

⁸Banfaävi et al. (1983)

⁹Casadesus and Olivares (1979)

¹⁰Ditta et al. (1980)

¹¹Kowalski (1970)

¹²This laboratory; Selvaraj and Iyer (1981). JJ1C10 was derived by I.K. Hooper (Adachi et al., 1983)

¹³Meade et al (1982)

Table 2

A list of R. meliloti strains studied intensively in different laboratories

Strain	Alternate designation (if any)	Origin	Comments (if any)
SU47 ¹	RCR2011 ² 6646 ³ Rm2011 ⁴ Rm2 ⁵ Rm20-1 ⁶	Australia	Rm 1021 ¹³ is a Sm ^r -derivative of Rm2011, extensively used in genetic studies; has one megaplasmid (300M. dal)
Rm41 ⁷	-	Hungary	AK 631 ⁸ is a variant with a compact colony morphology; has two plasmids (300 Mdal; 140 Mdal)
GR4 ⁹	-	? (Spain?)	-
102F34 ¹⁰	-	? (U.S.A.?)	-
L5-30 ¹¹	-	Poland	has two plasmids (300 Mdal; 90. Mdal)
IZ450 ¹²	-	Brazil	has two plasmids (300 Mdal; 140 Mdal) JJ1 is a Rif ^r derivative
JJ1C10 ¹²	KN1001	-	Derivative of JJ1; devoid of the smaller plasmid

Also included in this Table are auxotrophs, behavioural (chemotaxis-related) and symbiotic mutants. As shown in Table 3, the majority of the auxotrophic mutants were derived by mutagenesis with NTG, EMS or nitrous acid. These mutants served as markers in the construction of preliminary chromosome maps of different R. meliloti (see Kondorosi et al. 1977a; Meade and Signer, 1977; Casadesus and Olivares, 1979a).

An alternative to the conventional mutagenesis procedures is the use of transposons as mutagens. Denarie et al., (1977) attempted to use the mutator phage Mu in R. meliloti strain 2011. Intracellularly, the linear DNA of bacteriophage Mu has a capacity to insert at several sites in the E. coli genome. Mutations caused by insertions of Mu are very stable (for reviews, see Howe and Bade, 1975; Bukhari, 1976). As the host range of Mu does not extend to R. meliloti, Boucher et al., (1977) and Denarie et al. (1977) constructed a cointegrate of RP4, an IncP1-group wide host-range conjugative plasmid and Mu. This plasmid RP4::Mu was as conjugation-proficient as the parental plasmid in E. coli X E. coli matings. However, in matings of E. coli donors with R. meliloti recipients, there was a 10^5 -fold reduction in its transfer frequency. Further analysis showed that the majority of the transconjugants harboured RP4-derivatives with a defective prophage of Mu. The basis of these results is still not well understood. For these reasons, the use of Mu as a mutagen was not

pursued. However, a consequence of these observations on the instability of RP4::Mu in Rhizobium was the development of such Mu-cointegrates as suicidal delivery vehicles for introducing transposons into members of the Rhizobiaceae family. A prototype suicide vehicle was developed by van Vliet et al. (1978). They constructed a derivative of RP4::Mu carrying transposon Tn7. Tn7 specifies resistance to trimethoprim and streptomycin (Barth et al. 1976). Upon mating an E. coli strain carrying this suicide vehicle with Agrobacterium tumefaciens, (a member of the Rhizobiaceae that is closely related to R. meliloti), Tn7-insertions could be induced in the recipient (van Vliet et al. 1978). Modelled after this plasmid, Beringer et al. (1978) constructed another P-group plasmid carrying Mu and transposon Tn5. Tn5 encodes resistance to neomycin/kanamycin in E. coli, a host in which it has been intensively studied (Berg et al. 1975). It also specifies streptomycin-resistance in Rhizobium as described in Chapter III. The suicide plasmid pJB4JI carrying Tn5 induced Tn5-insertion mutants in R. leguminosarum, R. trifolii and R. phaseoli, some of which were auxotrophic mutants (Beringer et al. 1978). Its use in R. meliloti was described later by Ruvkun et al., (1980) Meade et al. (1982) and Forrai et al. (1983) and these studies are discussed in a latter section.

Casadesus et al. (1980) derived Tn1-insertions in R. meliloti strain GR4 by selecting for ampicillin-resistant colonies following a mating with E. coli carrying RP4::Mu. RP4 carries transposon Tn1 that specifies resistance to ampicillin/carbenicillin (Heffron et al. 1977). A selection for Ap-resistant transconjugants of R. meliloti yielded Tn1-insertion derivatives of which some had Tn1 at a site close to the his31 or cys4 allele. Auxotrophs were not found among the insertion derivatives. In the remainder, the Ap-resistance was "curable" by growth at an elevated temperature of 40° C (the optimum growth temperature is 30° C). Based on these results, Casadesus et al. (1980) concluded that Tn1 has hot spots for insertion in R. meliloti. Another interesting observation was the concomitant loss of nodulation-proficiency in the Ap^S clones, suggesting that the insertion of Tn1 in the Ap^r derivatives could have been on a plasmid that specified a nodulation function.

There are certain shortcomings associated with the use of bacteriophage Mu-carrying vehicles. These shortcomings and the construction of a different type of transposon-delivery vehicle are described in Chapter II.

Gene transfer

When a set of markers (mutated sites) become available in R. meliloti, further direction in the genetic analysis is the mapping of these mutations. The reasoning

behind gene mapping in R. meliloti is that the genes concerned with symbiosis could be localized on this map. Conjugation, chromosomal DNA mediated transformation and transduction are the three natural gene transfer systems in bacteria, and they have been extensively used in constructing genome maps in different bacteria. In addition, these methods have also been adapted for genetic manipulations.

Transformation

Historically, chromosomal DNA-mediated transformation is the first gene transfer system explored in R. meliloti. In fact, R. meliloti is one of the first Gram-negative bacterium to be examined for the existence of a transformation system. This work was done by the late Dr. R. Balassa, and it has been subsequently reviewed by others (G. Balassa, 1963; Gabor-Hotchkiss, 1972). The major findings of Dr. R. Balassa are as follows. There is an optimal physiological state for the uptake of DNA (competence) in R. meliloti. Auxotrophic traits such as Cys⁻ could be corrected to Cys⁺ at a frequency of 0.05 (per 100 recipient cells); streptomycin-resistant (Sm^r) phenotype could be transmitted to R. meliloti with DNA from Sm-resistant strains of R. meliloti, R. lupini and R. japonicum. The heterologous donors are not closely related to R. meliloti (Vincent, 1974), and yet in these experiments Sm-resistant transformants of R. meliloti

could be obtained. Another interesting observation concerns symbiotic traits in R. meliloti. Strains of R. japonicum and R. lupini that nodulate soybean and lupine respectively, could be transformed to nodulate alfalfa with DNA from R. meliloti. Such transformants were selected directly by assay of DNA-treated recipients on alfalfa plants. The derivatives were not tested for the ability to nodulate their original hosts. Unfortunately none of those experiments has been extensively followed up by other investigators. The Rhizobium strains used in these studies were natural isolates of Hungarian origin, and it is not known whether they are in current use elsewhere. The transformation system described above has not been exploited to generate a genome map.

Conjugation

Bacterial conjugation is a mode of gene transfer that involves participation of two cells of which the donor carries a self-transmissible plasmid either as an autonomous entity or in a state in which it is inserted into the chromosome. Bechet and Guillaume (1978) provided the first physical evidence for the presence of plasmid DNA in R. meliloti. Based on sedimentation analysis of alkaline lysates, they concluded that four out of seven isolates of R. meliloti had a plasmid. Subsequently, Casse et al., (1979), Spitzbarth et al. (1979) and Adachi and Iyer (1980) demonstrated, using procedures developed

for the isolation of large plasmids, that most of the many R. meliloti isolates examined had one or more plasmids. The size of these plasmids ranged from 90-260 megadaltons (Mdal). The physical comparison of some of these plasmids and recent studies that have shown the presence of at least one larger plasmid species (> 300 Mdal) in all of the R. meliloti isolates that have been examined will be described in a latter section.

Bedmar and Olivares (1980) discovered the presence of a conjugative plasmid in R. meliloti strain Rm4. This plasmid, pEZ1 (60 Mdal) confers sensitivity to phage ALI, and the trait was used as a marker in these studies. Although pEZ1 was transmissible at a high frequency (0.01 per donor cell), its chromosome mobilizing ability [see Holloway (1979) for a review on this phenomenon] was not assessed. pEZ1 was also transmissible between R. meliloti X E. coli matings, suggesting a wide host-range of the plasmid. Recently Denarie et al. (1981) have identified a conjugative plasmid in strain Rm41. The transfer efficiency of this plasmid (pRm41a) was not reported. A more recent study in our laboratory has shown that the strain IZ450 carries a 140 Mdal plasmid that is transmissible at a frequency of 10^{-3} (Adachi et al., 1983). The chromosome mobilizing ability of this plasmid is under investigation (I.K. Hooper, personal communication).

In another approach, Meade and Signer (1977) and Kondorosi et al. (1977a) introduced P-group plasmids into R. meliloti strains and screened for plasmid-mediated transfer of the chromosome by using various auxotrophic and antibiotic markers. Certain P group plasmids mediate transfer of chromosomal markers by a mechanism that is not well-understood [see a review by Holloway (1979) on chromosome mobilization]. The efficiency of chromosome-transfer by RP4 in strain Rm2011 was 10^{-6} to 10^{-5} (Meade and Signer, 1977), but the efficiency was much lower in strain Rm41 (Kondorosi and E. Vinze, unpublished results; see Kondorosi and Johnston, 1980). These observations indicate differences between the two strains with respect to this phenomenon. Strain-specific differences have been documented for other bacterial species as well (see Holloway, 1979). Kondorosi et al. (1977a) made use of R68.45, a derivative of another P-group plasmid, that was originally selected as a "high frequency mobilizing plasmid" in Pseudomonas aeruginosa (Hass and Holloway, 1976). R68.45 mobilized different genes of strain Rm41 at a frequency of 10^{-5} to 10^{-3} .

A general feature of chromosome mobilization in R. meliloti was the formation of haploid recombinants. Relatively large segments of chromosomes were transferred from several dispersed points of origin. This allowed one to study the inheritance of unselected markers by the recombinants. Using this strategy, Meade and Signer

(1977), Kondorosi et al. (1977a) and Casadesus and Olivares (1979a) constructed preliminary genome map of strains Rm2011, Rm41 and GR4 respectively. In all cases it was found to be a single circular map. None of the symbiotic functions were mapped at this time. Ludwig and Johansen (1980) reported the isolation of an R68.45-derivative that mediated chromosome-transfer in Rm2011 at a greater (70 to 170 -fold) efficiency. This derivative was isolated as a mutant that restored temperature resistance in a conditional DNA replication mutant (dnaGts) of E. coli. It is not clear why such a mutant plasmid possesses an enhanced chromosome-mobilizing ability in R. meliloti. This feature of R68.45 has not been exploited in subsequent manipulations of R. meliloti.

Julliot and Boistard (1979) devised another method to promote chromosome transfer in R. meliloti. These authors cloned parts of the total DNA of R. meliloti into a non-essential site of plasmid RP4. The resulting recombinant plasmids, referred to as "in vitro-generated R-primes" promoted polarized transfer of chromosomal markers in R. meliloti strain 2011, presumably via formation of "Hfr"-like strains by general recombination in the donors. Many auxotrophic markers were transferable by this method, but symbiotic functions were not examined in this study.

Kiss et al. (1980) isolated an R68.45-prime factor in vivo. This plasmid pGY1 was isolated following serial

transfer of the wild-type allele of cys46 to Cys⁻ mutants in R.68.45-promoted chromosome-transfer experiments. This method resulted the selection of a strain that transferred the Cys⁺ phenotype at a higher frequency, and such a strain was shown to carry a 17.8 Mdal insert in R68.45. In addition to carrying the wild-type allele of cys46, the insert also carried the attachment site for a Rhizophage, 16-3. The plasmid pGY1 also promoted polarized gene transfer of chromosomal markers anticlockwise to the cys46 allele.

While attempting to carry out R68.45-promoted inter-species gene transfer between R. meliloti and R. leguminosarum, Johnston et al. (1978a) had constructed R68.45-primers that carried different chromosomal segments of R. meliloti strain 2011. These R-primers carrying inserts ranging from 3-43 Mdal were also shown to promote polarized gene transfer in R. meliloti strain 41 (Johnston et al. 1978b; Kondrosi et al. 1980). An additional outcome of these studies is the indication that the chromosomes of R. meliloti strains Rm41 and 2011 are highly homologous. It was also concluded that the order of different gene in these strains and in R. leguminosarum strain 300 is very similar.

Transduction

Generalized transduction is a useful technique to study fine linkage of different markers that are

previously mapped by conjugational analysis. Kowalski (1967) reported the occurrence of generalized transduction in R. meliloti strain L5-30. The frequencies were 10^{-7} to 10^{-5} , and the markers examined were antibiotic-resistance or auxotrophic mutations (see Kondorosi and Johnston, 1980). Although co-transduction of some marker pairs was observed, these data did not contribute to the establishment of a gene map.

Another generalized transducing phage that has been productively used is DF3. This phage was used in conjunction with R68.45-promoted gene transfer in constructing a chromosome map of strain GR4 (Casadesus and Olivares, 1979a). This phage transduced chromosomal and R68.45-associated markers at a frequency of about 10^{-6} . A temperature-sensitive mutant of DF3 has been isolated that does not kill potential transductants at 34 C or above (Casadesus and Olivares, 1979b).

Sik et al. (1980) have also isolated a generalized transducing phage for another well-studied strain, Rm41. This temperate phage does not appear to mediate co-transduction at appreciable frequencies, and therefore its use in fine linkage mapping is limited. Svab et al. (1978) have described a specialized transducing phage that transduces cys46 (and its wild-type allele) at a frequency of 10^{-2} . Secondary sites of transduction have not been

reported. This phage should prove to be very useful, if any symbiotic genes are located in the vicinity of cys46 allele.

In vitro manipulations

Prior to 1981 there are few reports on in vitro manipulations of R. meliloti. The experiments of Julliot and Boistard (1979) on the in vitro construction of R-primes has been discussed earlier. These experiments were performed with E. coli as an intermediate host in all in vitro manipulations. Much of the delay in this area has been due to the lack of an efficient plasmid DNA-mediated transformation system in R. meliloti. Until the development of such a method, molecular cloning could not be performed directly in R. meliloti. I have developed a procedure for doing this and it is presented in Chapter I. Recently Kiss and Kalman (1982) have also demonstrated plasmid-mediated transformation of R. meliloti strain Rm41 by a different method. Their results are discussed in Chapter I. A steppingstone in the study of R. meliloti genetics is the demonstration of DNA sequence homology between the structural genes of the nitrogenase of Klebsiella pneumoniae and restriction fragments of R. meliloti DNA (Ruvkun and Ausubel, 1980). This corroborated the structural and functional homology of the nitrogenase isolated from different organisms. Ruvkun and Ausubel also cloned EcoRI-fragments of the

total DNA of strain Rm2011 in E. coli using the vector pBR322 and identified a clone that was homologous to the nitrogenase structural genes (nifHDK) of K. pneumoniae. These sequences will be referred to as "Kp nifHDK-like" in this review.

The limitations imposed by the narrow host-range vectors such as pBR322 was circumvented by Ditta et al. (1980) who constructed a cloning vector from the wide host-range P-group plasmid, RK2. This vector pRK290 is also mobilizable into different bacterial species by a conjugative helper plasmid. The latter is a recombinant plasmid of a P-group plasmid whose origin of replication (oriV) has been substituted with a ColE1 replicon. As a result, the helper plasmid is of narrow host-range with respect to replication and maintenance. Using pRK290 as a cloning vector, Ditta et al. constructed a gene bank (BglIII-generated fragments) of R. meliloti strain 102F34 in an E. coli host. A 26 kilobasepairs (kb) insert showing homology to nifHDK was also identified in these studies. More recent developments in the manipulation of R. meliloti are discussed below.

Recent developments in R. meliloti genetics

This section examines the progress made in R. meliloti genetics from 1980-81 to present (August 1983).

Location of nif and nod genes

The term nif and nod are defined as follows: a gene concerned with the formation of nodules is referred to as nod; nif genes are those that are responsible for the fixation of nitrogen. 'fix' is sometimes used as a synonym for nif. The term sym encompasses all genes concerned with symbiotic functions.

The involvement of a bacterial plasmid is well-established in the case of crown gall disease in dicotyledonous plants (see a review by Holsters et al. 1982). Subsequent studies on different strains of R. leguminosarum and the closely related species, R. trifolii has also convincingly shown that a plasmid (about 150 Mdal) encodes symbiotic functions in these bacteria. These studies provided the impetus for the examination of the plasmid composition of R. meliloti. The presence of moderately large plasmids (90-260 Mdal) in R. meliloti has been discussed earlier. However most of these plasmids, with the exception of a few (described below); did not show any homology to the nifHDK sequences (Szeto et al. 1981; Puhler et al. 1981; Jouanin et al. 1981; Adachi et al., 1983). Denarie et al. (1981) however showed that all R. meliloti isolates harbour, in addition to the medium-sized plasmids, a very large plasmid (>300Mdal), referred to as "megaplasmid". The method used in this study was a milder analytical procedure in which R. meliloti cells were lysed gently in agarose gel slots and

the lysate was directly electrophoresed (Eckhardt, 1978). Using this method Banfalvi et al. (1981), Rosenberg et al. (1981) and Puhler et al. (1982) analysed some 50 R. meliloti isolates of independent origin, and concluded that all these strains carried a megaplasmid. It should be noted that size estimations are based on extrapolations of linear relationship between the logarithm of the molecular weight and the relative electrophoretic mobility of smaller reference plasmids. Such calculations are largely underestimates (Casse, et al. 1979; Hanson and Olsen, 1978). A further comment is that the molecular masses of the megaplasmids have not been verified by an independent technique.

Megaplasmids have been shown to carry DNA sequences homologous to Kp nifHDK, thus providing physical evidence for the presence of nif genes on them (Banfalvi et al., 1981; Rosenberg et al., 1981, 1982; Puhler et al., 1982). Puhler et al. (1982) also found that smaller plasmids in some of the natural isolates of German origin also showed homology to Kp nifHDK. In one of the strains, Rm20-1, a 260 Mdal plasmid and the megaplasmid showed Kp nifHDK homology (Simon et al., 1982). By a combination of site-directed mutagenesis (described on page 25), cloning and heteroduplex analysis, Simon et al., (1982) were able to demonstrate that the Kp nifH-like sequence of the megaplasmid of Rm2011 and Kp nif-like sequence of the 260 Mdal plasmid in strain Rm20-1 are homologous. Sequences

surrounding nifH were, however, heterologous. It is not known if Rm20-1 carrying a Tn5 insertion in the nifH sequence of the 260 Mdal plasmid is phenotypically Fix⁻. Therefore any speculations regarding pseudogenes or nif gene duplications in R. meliloti must await further studies.

An independent approach to identify the sym genes is the isolation of random Sym⁻ mutants of R. meliloti by insertion mutagenesis (Meade et al., 1982; Forrai et al., 1983; Chapter II and IV). Meade et al. and Forrai et al. isolated Tn5-insertion mutants of R. meliloti strains 1021 and Rm41 respectively with the aid of pJB4JI, a Mu-based suicide vehicle (Beringer et al., 1978). Forrai et al. (1983) identified the location of Tn5 in some of the Nod⁻ and Fix⁻ mutants. While none of Nod⁻ mutants had the Tn5 on their chromosomes, 5 of the 11 Fix⁻ mutants had Tn5 at various chromosomal locations. The remainder carried Tn5 on their megaplasmids. Thus, it seems that the fix genes are dispersed.

Genetic and physical analysis of the nod function

Long et al. (1982) exploited the ability of plants to select Nod⁺ bacteria from a mixed inoculum of Nod⁺ and Nod⁻ cells to identify a recombinant clone carrying the nod gene(s). Earlier, Friedman et al. (1982) had constructed a mobilizable wide host-range cosmid cloning vector, pLAFR1. These authors also constructed a gene

bank (EcoRI-fragments) of R. meliloti strain Rml021 in an E. coli host. These E. coli derivatives were mated en masse with the Nod⁻ mutants (Meade et al., 1982) derived from a pJB4JI-facilitated mutagenesis, and the transconjugants were inoculated on alfalfa seedlings. This operation resulted in the isolation of Nod⁺ transconjugants (Nod⁻/pLAFR1::nod). By further DNA hybridization studies, Long et al. (1982) were able to show that an 8.7 kb EcoRI fragment of the megaplasmid carried the insertions in the original Nod⁻ mutants, and that this segment was about 20 kb away from the Kp nifHDK-like sequences (see Fig.1)

Genetic and physical analysis of the fix function

Much of the progress made in this area can be attributed to the development of a technique for site-directed mutagenesis in vivo (Ruvkun and Ausubel, 1981). Basically this technique involves cloning of a DNA sequence of interest in an E. coli host, mutagenesis of the passenger DNA with transposons, identification of the location of the insertions, and re-transfer of the plasmids carrying insertions to the original "wild-type" strain followed by the elimination of them by an incompatible plasmid while maintaining selection for the transposon-associated marker. This operation predominantly results in the transfer of the transposon from the recombinant plasmid to the "wild-type" sequences

by general recombination between the "wild-type" sequence and the corresponding sequences that bracket the transposon insertion in the plasmid. The end-product is a strain mutated with a transposon, in which the precise location of the transposon is known. Using overlapping clones from a gene library, one can sequentially mutate and analyze any given region of a genome.

Using this technique in conjunction with the isolation of Kp nifHDK-like sequences of R. meliloti strains, Ruvkun and Ausubel (1981), Simon et al. (1982), Ruvkun et al. (1982), and Corbin et al. (1982, 1983) analyzed the fix region of different R. meliloti. Their results are summarized below, and are also shown in Fig. 1 to facilitate a comparison of different strains:

- 1) Some of the fix genes are dispersed in clusters within a segment of a megaplasmid. The approximate size of this region is 14 Kb.

- 2) There are two clusters of Tn5 insertions in Fix⁻ mutants of Rml021; one 6.3 kb and the other 5 kb, separated by a 1.6 kb region (Ruvkun et al., 1982). Insertions into the 1.6 kb region do not affect the Fix phenotype. There are three transcriptional units as identified by complementation studies involving subclones of the 14 kb segment. The Kp nifHDK-like sequences are present in the 6.3 kb segment that is transcribed as a unit. Based on partial DNA sequencing of the Kp nifHD-like segment, Ruvkun et al. also concluded that the

Legend to Fig. 1

1a. Organization of some of the megaplasmid-associated nod and fix regions in R. meliloti strain 1021. The information from Ruvkun et al. (1982) and Long et al. (1982) is presented here. The scale in the original figures has been changed to facilitate a comparison. nif, D, and K, homologous sequence to the nifHDK of K. pneumoniae; the boundaries of K are not known. No. 1, 2, 3, transcriptional units as determined by Tn5-insertions, and complementation analysis. B, BamHI; Bg, BglII; E, EcoRI; and H, HindIII. The sites for BamHI, BglII, and HindIII in the nod region have not been determined.

1b. Organization of fix regions in R. meliloti strain 102F34. The information from Corbin et al. (1983) is presented here, with proportionate changes in the scale of the Figure to facilitate a comparison to Rm1021. The nif region is arbitrarily aligned to the corresponding region in Fig. 1a. D, and n, homologous sequence to the nifHDK of K. pneumoniae. a, b, c, and d, transcriptional units as identified by Tn5-insertions. P₁ and P₂ are the promoter regions at which the bacteroid-specific RNA's are initiated as indicated in thick bars. The dotted line denote uncertainty about termination.

(Rm 1021)

3

2

1 Kb

(102 F 34)

nifHDK genes of R. meliloti are transcribed starting from nifH. The nifH gene of another strain, Rm41 has been sequenced (Torok and Kondorosi, 1981), and the partial sequence of the nifH of Rm1021 matches exactly with the corresponding sequence in Rm41 (Ruvkun et al., 1982).

Since the nifHDK genes of Rm41 are contiguous (Banfalvi et al., 1981), the functional organization of nifHDK is also probably similar in both strains.

3) In strain 102F34, a 35 kb region encompassing the Kp nifHDK-like sequences was analyzed with 70 independent Tn5 insertions (Corbin et al., 1982; 1983). Furthermore, bacteroid-specific RNA transcripts were mapped using the technique of Berk and Sharp (1977). These studies have revealed four clusters of fix gene(s) in a 14 kb region flanked by 10 kb regions of symbiotically unimportant sequences. There are two major RNA transcripts, each transcribed in opposite directions. The RNA I covers the nifHDK region, and the direction of transcription is the same as in strain Rm1021. Thus the direction of transcription of the nifHDK cluster of R. meliloti is similar to that of K. pneumoniae. However, in K. pneumoniae all of the 17 nif genes are transcribed in the same direction, whereas in R. meliloti strain 102F34, at least one cluster is transcribed in the opposite direction.

Linkage of nod and fix genes

Banfalvi et al. (1981) and Rosenberg et al. (1981) reported that in spontaneous and heat-induced Nod⁻ mutants of different R. meliloti isolates the Kp nifHDK-like sequences were also lost. Banfalvi et al. (1981) estimated the deletion to be a minimum of 24 kb. Long et al., (1982) identified the location of Tn5 in two Nod⁻ mutants isolated by insertion mutagenesis and the insertions were about 20 kb away from the nifHDK cluster (see Fig. 1). It is not known if the Nod⁻ mutants isolated by Banfalvi et al. and Rosenberg et al. had lost sequences similar to the nod sequences identified by Long et al. (1982).

Rosenberg et al. (1982) have described a procedure for the preparative isolation of megaplasids which is a modification of the one described by Schwinghamer (1980). This procedure yields distinct chromosomal and plasmid DNA bands in cesium chloride-ethidium bromide gradients, but further manipulations result in the breakdown of the circular plasmid DNA. Similar observations have been made in our laboratory (I.K. Hooper, personal communication). However, the plasmid preparation obtained by this method should be useful for molecular cloning and DNA hybridization experiments. Such studies should provide more insights into the structural and functional organization of the megaplasmid-associated nod and fix genes.

Transfer of sym functions to other bacteria

The plasmids of R. leguminosarum that specify sym functions, referred to as Sym plasmids, have been transferred or mobilized into other Rhizobium strains, including R. meliloti and into A. tumefaciens, a bacterium closely related to R. meliloti. The nod functions have been expressed in these hosts (Banfalvi et al. 1981; Hooymaas et al. 1982). Similar experiments were done with R. meliloti strains (Kowalczyk et al., 1981; Kondorosi et al. 1982; Banfalvi et al. 1983). In the experiments of Kowalczyk et al., Nod⁺ phenotype was transferred from R. meliloti L5-30 carrying R68.45 to a Leu⁻Nod⁻ mutant of R. trifolii by bacterial mating. All of the carbenicillin-resistant (Cb^r; an R68.45-associated marker) transconjugants were also able to nodulate clover (a host of R. trifolii), but not alfalfa. The transconjugants carried an additional plasmid, designated as pUCS202. The size of this plasmid was 40 Mdal, in comparison to R68.45 which is 35 Mdal. Some Leu⁻ mutants of Rhizobium are also known to be defective in nodulation (Denarie et al. 1976). However, the Leu⁺ revertants of the Nod⁻ mutant of R. trifolii remained Nod⁻, suggesting that some other genetic determinant other than a suppressor of the Leu mutation must have been transferred from the R. meliloti donors. It has been reported that the presence of pUCS202 in the R. trifolii is responsible for the production of certain lipopolysaccharides by the bacterium

(Russa et al. 1982). Since further physical studies on pUCS202 were not done, it is difficult to explain the basis of these observations.

Kondorosi et al. (1982) and Banfalvi et al. (1983) provided convincing evidence to show that all or some of the sym functions of R. meliloti can be transferred to A. tumefaciens and to other Rhizobium. Two approaches were used in these studies. Kondorosi et al. cloned the oriT (mob) sequence of RP4, and a Km-resistance marker into the nif sequence of R. meliloti strain Rm41, cloned within a narrow host-range vector. Using this composite plasmid, the "mob-Km^r" sequences were transferred to the nifH region of the megaplasmid of Rm41 by site-directed mutagenesis. The resultant derivative of the megaplasmid was now rendered mobilizable by a P-group plasmid to Rhizobium Sp., A. tumefaciens and Nod⁻ mutants of Rm41. In Rm41 Nod⁻ hosts, the plasmid restored nodulation-proficiency. In the other hosts it conferred the ability to induce white Fix⁻ nodules on alfalfa.

In a different approach, Banfalvi et al. (1983) constructed R-primes of a P-group plasmid carrying some of the sym genes of Rm41. It is known that transposon Tn5, once established in R. meliloti does not re-transpose at frequencies exceeding 10^{-8} (Meade et al. 1982; Forrai et al. 1983; Selvaraj and Iyer, unpublished results). Therefore a selection for the transfer of Km-resistance (Tn5-associated marker) in a mating between sym::Tn5

derivative carrying a P-plasmid and an E. coli recipient yields R-primes carrying segments of R. meliloti DNA. Such R-primes carrying segments of the chromosomal DNA (from chromosomal Fix⁻ mutants) did not carry nod genes, whilst the ones carrying megaplasmid DNA carried all or some of the nod genes. The latter R-primes conferred upon A. tumefaciens the ability to induce small white nodules. The E. coli hosts carrying these plasmids did not promote nodulation in alfalfa. The isolation of these R-primes should expedite further analysis of sym genes.

Weber and Puhler (1982) and Puhler et al. (1983) studied the expression of cloned nifHDK sequences of R. meliloti (Rm nifHDK) in an E. coli minicell system. Since the insert DNA was present within an antibiotic-resistance gene, in one orientation it could be transcribed under the control of the antibiotic-resistance gene, provided there was no transcriptional stop signals preceding the nif HDK sequence. When the nifH was proximal to the extraneous promoter, a fusion protein comprising the nifH gene product and a part of the antibiotic-resistance gene product was detected in the minicell system. However, the distal genes were not expressed unless cloned separately downstream to an extraneous promoter. Thus it seems that the R. meliloti nif transcripts are not translated in E. coli unless they are fused to a translatable message. However, premature transcriptional termination might be another explanation. Another interesting observation is

the demonstration of the activation of *Rm nifH* promoter by the *Kp nifA* gene product in an *E. coli* host. Such activation was inhibited by the presence of the *Kp nifL* gene product. Similar observations have been made by Sundaresan *et al.* (1983). In *K. pneumoniae* the *nifHDK* genes are regulated as described above (Kennedy *et al.* 1981). Recently Ow *et al.* (1983) have shown that some of the *Kp nifA*-regulated promoters, including that of *Rm nifH* share a consensus sequence. It is not known if the *R. meliloti nif* genes can be phenotypically expressed in appropriate mutants of other nitrogen-fixing organisms. There are reports of such complementation with other *Rhizobium* species as the donors (Bishop *et al.* 1977; Maier *et al.* 1978; Page, 1978).

With reference to the expression of *nif* or *nod* genes from other bacteria in *R. meliloti*, there are different results. Johnston *et al.* (1979) reported that the *nod* functions of a Sym plasmid of *R. leguminosarum* are not expressed in *R. meliloti*. In contrast, the results of Hooykaas *et al.* (1982) suggest that some of the *nod* functions of *R. leguminosarum* are expressed in *R. meliloti*. These differences could be due to strain specific variations. Interestingly, Dixon *et al.* (1976) have detected *Kp nif*-specified nitrogenase components in *R. meliloti* by immunological methods. However, there was no phenotypic expression.

Genetic improvement of symbiotic nitrogen-fixation

Alfalfa is the most important forage crop in global agriculture (Bolton et al. 1974). Therefore the genetic improvement of R. meliloti-alfalfa symbiosis is of paramount importance. The efficiency of symbiotic nitrogen-fixation is influenced by a variety of factors that includes among others the competitive ability of a R. meliloti strain in soil, the genetic constitution of the host plants, the rate and magnitude of nitrogen-fixation by the bacteria, and the overall energetics of the process of nitrogen fixation.

The earlier literature on the genetic improvement of R. meliloti (and other Rhizobium strains) has been reviewed by Schwinghamer (1977). In these studies "better nitrogen-fixers" were sought among irradiated R. meliloti cells or among strains mutated to auxotrophy, resistance to antimetabolites etc. The effect of these mutations on symbiotic capacity has also been reviewed by Denarie et al. (1976) and Kuykendal (1980). Recent studies have included seeking chemotaxis-related mutants of R. meliloti, as an initial step towards studying the competitive ability of such mutants (Ames et al. 1980; Ames and Bergman, 1981). Plazinski (1981) reported the isolation of a Tn5-insertion mutant of R. meliloti strain Rm41 that showed increased nitrogen-fixation. His attempts to map the location of Tn5 on the chromosome were unsuccessful, leading to the suspicion that the Tn5 was

located on an indigenous plasmid. Further physical studies were not done. The basis of this observation remains obscure.

Recently there is an upsurge in the study of hydrogen oxidation in root nodules (see reviews by Evans and Barber, 1977; Dixon, 1978; Lim et al. 1980; Eisbrenner and Evans, 1983). It is well established that the energy supplied to biological nitrogen fixation is partitioned between nitrogen fixation and hydrogen evolution. Therefore hydrogen evolution is energetically a wasteful process. Accumulation of hydrogen also inhibits nitrogenase (see Robson and Postgate, 1980). Some isolates of R. japonicum and R. leguminosarum have evolved an enzymatic mechanism for the recycling of the hydrogen produced in the root nodules. This phenomenon referred to as hydrogen uptake seems to improve the yield of biomass and fixed nitrogen in Rhizobium-legume systems (see Eisbrenner and Evans, 1983). A search for Hup⁺ isolates of R. meliloti in different laboratories has shown that none of the R. meliloti isolates of diverse origin had a significant capacity for recycling hydrogen (Ruiz-Argueso et al. 1979; Miller and Sirois, 1982; R.M. Behki, personal communication). It is of considerable interest to transfer hup genes from Hup⁺ organisms to R. meliloti in an effort to study the expression of these genes in this host. Perhaps the phenotypic expression of the hup genes might enhance the nitrogen-fixing ability of

R. meliloti. In R. leguminosarum isolates it has been shown that the acquisition of a plasmid specifying hydrogen uptake results in an increased efficiency of nitrogen fixation (DeJong et al. 1980). We have explored the possibility of transferring hup genes from Alcaligenes eutrophus, a hydrogen-utilizing organism, to R. meliloti (Behki et al. 1983).

Genetic analysis of symbiotic nitrogen fixation by the other Rhizobia

This section summarizes recent progress made in the genetics of symbiosis involving Rhizobium species other than R. meliloti. There are reviews dealing with the genetics of Rhizobium in general (Kondorosi and Johnston, 1980), of R. lupini (Heumann, 1979), and of the molecular biology of Rhizobium-legume symbiosis (Ausubel, 1982, Beringer et al. 1980, Verma and Long, 1983). Among the different commonly studied species of Rhizobium (see Table 1, p), R. meliloti and the R. leguminosarum sub-group (R. leguminosarum, R. phaseoli and R. trifolii) are the intensively studied organisms. Following the identification of "fast-growing" isolates of R. japonicum (referred to as "PRC" strains), and a "fast-growing" Rhizobium that induces root and stem nodulation in a tropical legume there have been some reports on the

molecular genetics of these organisms as well. Among the "slow-growers", R. japonicum has been studied to some extent.

The R. leguminosarum subgroup

It is now widely accepted that the chromosomes of the members of the R. leguminosarum subgroup are very similar and that the individual species are defined by their Sym plasmids that confer the host-specificity (see Johnston et al. 1978c; Benyon et al., 1980; Beringer et al., 1980; Hooykass et al., 1981). Therefore, one can conclude that the chromosomal sym genes, if present, are also functionally identical in this Rhizobium group. The presence of large plasmids (130-220 Mdal) encoding sym functions has been well-documented for all the three members of this group, R. leguminosarum, R. phaseoli and R. trifolii (discussed below). The sym plasmids carry homologous regions that include the nifHD genes, suggesting a close relationship among Sym plasmids of these organisms (Prakash et al. 1981).

R. leguminosarum

In general only one Sym plasmid is found in a strain although a given strain may contain in addition cryptic plasmids or plasmids conferring other phenotypes such as bacteriocin-production. The approximate sizes of the Sym plasmid in different strains vary from 130-220 Mdal (Hombrecher et al. 1981). In addition to encoding nod,

fix and host-specificity functions, some of the Sym plasmids also specify other phenotypes such as bacteriocin-production, hydrogen-uptake and conjugal transfer (see Beringer et al. 1980). The Sym plasmid of some strains of the R. leguminosarum sub-group and the Ti (Tumor-inducing) plasmids of different phytopathogenic A. tumefaciens strains have some homologous regions indicating a structural relationship between them (Prakash et al. 1981)

Sym⁻ mutants have been isolated following mutagenesis with NTG, UV, transposon Tn5, and by growth at high temperatures (see Beringer et al. 1980). These treatments have resulted in deletion derivatives, insertion mutants and temperature-sensitive mutants affected in sym function. The genetic analysis of some of these mutants has revealed that a 45 kb segment of the Sym plasmid is involved in symbiosis; within this region, 12 Tn5-induced mutations conferring Sym⁻ phenotype define a nod region bracketed by two fix regions. One of the fix regions has DNA sequences homologous to the nifHD region of K. pneumoniae (Ma et al., 1982; Downie et al., 1983a,b). The other fix region has Kp nifA-like sequences, suggesting the some of the regulatory genes such as nifA might also be conserved in Rhizobium strains (Downie et al., 1983b).

A 10 kb fragment spanning the nod region confers nodulation-proficiency and host-specificity to Sym⁻ mutants of R. leguminosarum and a strain of R. phaseoli suggesting that the genes that determine host-specificity and the nod genes are located within a short region of the Sym plasmid (Downie et al., 1983a).

R. trifolii

The Sym plasmids in different strains differ with respect to conjugative ability. The conjugative plasmids are transferable at a frequency of 10^{-6} to 10^{-4} , and the others are mobilizable (Hooykaas et al., 1981; Zurkowski, 1981; Scott and Ronson, 1981). One of the Sym plasmids has been transferred to A. tumefaciens, and the nod functions are expressed to some extent (Hooykaas et al., 1981). Random insertion mutants of R. trifolii strains SU329 and SU843 have been obtained with pJB4JI (carrying Mu and Tn5) and some Sym⁻ mutants have been identified in this collection. These include mutants defective in root-hair curling and nodule development (Rolfe et al., 1982; Scott et al., 1982). However, the organization of the sym genes in this strain is not clear. The sequences surrounding a fix mutation hybridize to a segment of the total DNA of A. tumefaciens (Scott et al., 1982). It is not known if these sequences of R. trifolii are important for symbiosis.

R. phaseoli

The Sym plasmids of R. phaseoli strains are about 190 Md in size and are self-transmissible at a frequency of about 10^{-7} ; these plasmids also specify production of melanin (Benyon et al., 1980; Hombrecher et al., 1981; Lamb et al., 1982). One of the Sym plasmids has been randomly mutagenized with Tn5, resulting in six independent mutants with Fix⁻ phenotype (Lamb et al., 1982). Among eight melanin⁻ mutants isolated following mutagenesis of the Sym plasmid one was Fix⁻, and three were Nod⁻. These Nod⁻Mel⁻ mutants had suffered a deletion in the Sym plasmid, suggesting linkage of nod and fix genes. The Fix⁻Mel⁻ mutants is suspected to be a double mutant (Lamb et al., 1982). Thus it seems that melanin-production, a distinct characteristic of R. phaseoli, is not essential for symbiosis. Raleigh and Signer (1982) have isolated Nod⁻ mutants by selecting for derivatives of R. phaseoli, resistant to certain bacteriophages. These mutants had undergone alterations in their cell surface. Interestingly, Nod⁺ "revertants" could be obtained by isolating derivatives of the Nod mutants that are now resistant to a different bacteriophage.

Recently Quinto et al. (1982) have provided evidence for the reiteration of plasmid-borne nifHDK-like sequences in R. phaseoli strains. It is not known whether such reiterated sequences are present in the chromosome.

R. japonicum

Nod⁻ and Fix⁻ mutants of the slow-growing strains of R. japonicum (for example, strains 61A76 and 311b110) have been isolated following mutagenesis with NTG or UV irradiation (Maier et al., 1978; Noel et al., 1982; Stacey et al., 1982). However, these mutations have not been mapped. In general, the lack of suitable methods has been the impediment in the genetic analysis of this organism. Haugland and Verma (1981) and Masterson et al. (1982) have demonstrated the presence of moderately large plasmids (100-200 Mdals) in different R. japonicum strains. However, none of these hybridized to the nifHDK probes of R. meliloti or K. pneumoniae. Recently Cantrell et al. (1982) have demonstrated the presence of larger plasmids (approximately 290 Mdal) in some Hup⁻ mutants of R. japonicum strain 311b110. Since the parental strain does not have a discernible plasmid (Cantrell et al., 1982; Haugland and Verma, 1981), it leaves open the possibility that a much larger plasmid encoding hup functions might be present in the Hup⁺ strain, and that the smaller plasmid in the Hup⁻ mutants is a deletion derivative of such a "megaplasmid". This putative plasmid might have been recalcitrant to the isolation procedures used in these studies. Recently Moshiri et al. (1983) have shown that a single point mutation in a R. japonicum strain (a derivative of strain USDA 122) affects hydrogen uptake, nitrogenase activity and heme biosynthesis,

suggesting genetic relationship among these phenotypes. Taken together with these observations, the results of Cantrell et al. (1982) leave open the possibility that the hup functions of R. japonicum are specified by a megaplasmid, and that the sym genes are also associated with this plasmid.

Hennecke (1981) and Fuhrmann and Hennecke (1982) have identified in a genomic library of strain 110, a recombinant plasmid carrying the structural genes for nitrogenase. The insert DNA in this plasmid shows homology to the nifD but not nifH and nifK probes of K. pneumoniae and R. meliloti (Hennecke, 1981; Fuhrmann and Hennecke, 1982; Kaluza et al., 1983). Unlike R. meliloti and the R. leguminosarum group, the nifH gene of R. japonicum is not linked to the nifDK cluster (Kaluza et al., 1983). Its location is not known. The nifD-containing fragment of R. japonicum has been examined for its coding properties under the control of extraneous plasmid-associated promoters (Fuhrmann and Hennecke, 1982). It encodes two polypeptides in an E. coli minicell system, and these have been assigned as the nifD and nifK gene products. The nifDK genes are transcribed from nifD in a single unit as in the case of other Rhizobium species (Fuhrmann and Hennecke, 1981). Recently "fast-growing" R. japonicum strains have been isolated (Keyser et al., 1982). The presence of plasmids has been demonstrated in five of the six strains examined. The large indigenous

plasmid (100-200 Mdal in size) present in each strain hybridized to the nifHDK probes (Masterson et al., 1982). However, the organization of the nif genes in these plasmids is not known. The method for insertion mutagenesis of R. meliloti, developed in my study (Chapter II) has also been useful with some "fast-growing" R. japonicum strains (D.P.S. Verma, communication of unpublished results). Therefore it is now feasible to assemble Sym^- mutants for further characterization.

Other Rhizobia

Natural isolates of Rhizobium that can nodulate some legumes as well as a non-leguminous plant, Parasponia urgosa have been isolated (Trinick, 1973). Cen et al. (1982) have isolated pJB4JI-induced insertion mutants of one of these promiscuous strains CP283. These include Nod^- , Fix^- , and auxotrophic mutants. Recently Mohapatra et al. (1983) have demonstrated an ex planta nitrogenase activity in the parental strain grown on agar and in liquid media, suggesting that it is possible to develop a simple plate assay for Fix^- mutants.

A "fast-growing" strain, NGR234, that nodulates a variety of legumes, including alfalfa, and Parasponia has been identified (Trinick, 1980). This strain harbours a Sym plasmid (approximate size, 200 Mdal) that is lost upon growth at elevated temperatures; the plasmid can be re-introduced via mobilization with a P-group plasmid

carrying a transposon (Morrison et al., 1983). The Sym⁻ derivative does not nodulate the variety of legumes and Parasponia that are nodulated by the parental strain. However, upon acquisition of Sym plasmids from R. trifolii or R. leguminosarum it gained the ability to form Fix⁻ nodules on clover and peas respectively (Morrison et al., 1983). It is not known if the loss of the Sym plasmid results in the inability of the strain to induce nodulation in alfalfa. Further studies on these novel Rhizobium that have overcome the barrier between legumes, and between legumes and non-legumes should provide additional insights into plant-bacteria interactions.

The Rhizobium strain ORS571 forms a symbiotic association with Sesbania rostrata, a tropical legume; such an association can result in nitrogen-fixing nodules on both stems and roots (Dreyfus and Domergues, 1981). Recently Elmerich et al. (1982) have demonstrated nitrogen fixation by this strain under free-living conditions. Using growth on N-free media as an assay, these investigators have isolated Fix⁻ mutants that do not fix nitrogen in plant assays as well. These mutants regain their Fix⁺ phenotype upon introduction of a recombinant plasmid carrying Kp nifHDK-like sequences (Elmerich et al., 1982). Perhaps this strain of Rhizobium has a closer resemblance to A. tumefaciens in that both strains can react with roots or shoots.

Concluding remarks

In this review I have focussed attention on R. meliloti, and I have compared the organization of sym genes in different Rhizobium species. Excellent reviews have been written on other Rhizobium species, and on symbiotic nitrogen-fixation by Rhizobium in general (Heumann, 1979; Ausubel, 1982; Beringer et al. 1980; Verma and Long, 1983). As one can see from these articles and the review presented here, significant progress in the molecular biology of symbiosis has been made in the past two to three years. A comparison of the pertinent features of different Rhizobium species indicate that, apart from the conservation of nitrogenase genes there is an overall similarity in the chromosomal organization of other genes in physiologically related species of Rhizobium (R. meliloti and R. leguminosarum; see Kondorosi and Johnston, 1980). However, there is very little chromosomal genetic exchange between the two species, suggesting an intrinsic barrier between these different groups of Rhizobium. The nifHDK genes of slow-growing species (e.g., R. japonicum) are not located on discernible plasmids, indicating a possible deviation in the location of sym genes in this group (Haughland and Verma, 1981; Masterson et al. 1982). Recently fast-growing strains of R. japonicum have been identified, and Kp nifHDK-like sequences are present on indigenous plasmids in these strains (Masterson et al. 1982). The

basis and significance of such differences with respect to the location of sym genes in different groups of Rhizobium is not known. In addition unlike in R. meliloti, the structural genes of nitrogenase are organized as two separate and dispersed operons, nif and nifDK (Kaluza et al. 1983).

The presence of insertion elements in R. lupini and R. meliloti (strain 1021) has been demonstrated (Preifer et al. 1980; Ruvkun et al. 1980, 1982). The IS elements found in R. meliloti insert preferentially into sym genes (Ruvkun et al. 1980, 1982). The evidence from studies on E. coli carrying transposons suggest that the presence of translocatable elements confer selective advantages (Biel and Hartl, 1983; Chao et al., 1983). It is not known if the indigenous IS elements confer an evolutionary advantage in Rhizobium. These will be some of the questions that might be addressed in the near future. Considering the rapid progress being made in R. meliloti genetics and the current activity in plant cell manipulations, it is tempting to propose that a reasonable understanding of the organization of sym genes will be achieved in the near future, and that efforts will be directed towards installing some of the genes in plant genomes.

APPENDIX IMUTANTS OF DIFFERENT R. meliloti STRAINS

A compilation of all mutants of different R. meliloti strains that have been described (excluding the ones isolated in this study), is presented in the following Tables. This list of mutants includes auxotrophs, chemotaxis-related mutants, Sym, mutants defective in carbohydrate-utilization, and mutants defective in recombination. Together with the description of the mutants isolated in this study (Chapter IV), this forms a comprehensive reference source for all R. meliloti mutants that have been described. It is envisaged that this list of mutants will be useful for the construction of strains, and also in a variety of genetic experiments.

Table 3a

Mutants of *R. meliloti* strain Ra2011 and its derivatives¹

Strain	Characteristics ²	Method of Isolation	Comments and Reference
Ra2011A, 8	Glutamate synthase	EMS	Sym ⁺ ; Alf et al. (1981)
Ra2620	As above	?	Sym ⁺ ; Osburne and Signer (1980)
Ra1001, 4002	Ammonium transport	UV	Mod ⁺ Fix ⁺ ; Osburne (1982)
Ra20-26 ⁴	Ade ^{Sm}	Nitrous acid	Sym ⁺ ; Spitzbarth et al. (1979)
Ra2011 Str ^r Gly ^{Sm}	Gly ^{Sm}	?	Boucher et al. (1977)
NR1000	fla-1, non-motile	EMS	Mod ⁺ ; Ames et al. (1980)
NR1001	fla-2, non-motile	EMS	As above
NR1002	fla-3, non-motile	Spontaneous	As above
NR2000	mot-1, non-motile	As above	As above
NR2001	mot-2, non-motile	As above	As above
NR3000	che-1, aberrant swarming	As above	As above
NR3001	che-2, as above	As above	As above
NR3359	trp-33 his-39 pyr-49 spc-1 rif-1 nov-59 str-3	EMS	Juliot and Bolstard (1979)
Ra3378	trp-33 his-39 leu-53 phe-78 spc-1 rif-1 nov-51 str-3	As above	As above
Ra3374	trp-33 his-39 spc-1 rif-1 str-3 pan-44	As above	As above
GM1 61 (m61)	met-61 str-3	?	Juliot and Bolstard (1979)
GM1 66 (m69)	pyr-66 str-3	?	See Juliot and Bolstard (1979)
GM1 58 (m61)	gly-58 str-3	?	As above
GM1 59 (m113)	cys-59 str-3	?	As above
GM1 68 (m64)	pur-68 str-3	?	As above
GM1 73	pur-713 ilv str-3	?	As above
Ra3300	str-3 trp-33 spc-1	EMS	Lufwig and Johansen (1982)
Miscellaneous (no strain numbers)	arg-76, arg-15, arg-51, cys-24, cys-50, cys-11, 11u-74, gly-12, his-39, 11v-7, 11v-13, 11v-77, 11v-48, 11v-53, leu-79, leu-26, met-56, nov-57, nov-59, pan-44, phe-54, phe-78, pur-31, pyr-19, rif-30, trp-33	EMS	Meade and Signer (1977) all of these are individual mutants.
Ra1826	Lac	UV	Ucker, and Signer (1978)
Miscellaneous (no strain number)	Arg ⁺ , Met ⁺ , and Sulphur ⁺	Insertion mutagenesis with pUB4J1 (Tn5)	Meade et al. (1982)
Ra1023	met1023::Tn5	As above	As above
Ra1033	gly-1033 (Tn5)	As above	As above; linkage of Tn5 to gly not known
Ra1107	arg-1102::Tn5	As above	Meade et al. (1982)

Table 3a
Mutants of *R. meliloti* strain Rm2011 and its derivatives¹

Strain	Characteristics ²	Method of isolation	Comments and Reference
Rm1126	nod-1126::Mu-Tn5, Hae	Insertion mutagenesis with pJB4J1 (Tn5)	Long et al. (1982) Hirsch et al. (1982)
Rm1029	fix-1029::Tn5	As above	Heade et al. (1982)
Rm1027	nod1027::[SRm], Hae	As above	Long et al. (1982) Hirsch et al. (1982)
Rm1491	nif::Tn5	As above	Ruvkun et al. (1982)
Rm1308	nifK::Tn5	site-directed mutagenesis	Ruvkun et al. (1982)
Rm1310	nifK::Tn5	As above	As above
1312	nifD::Tn5	As above	As above
Rm1318	nifD::Tn5	As above	As above
Rm1491	nifH::Tn5	As above	As above
Rm1471	nifH::Tn5	As above	As above
Rm1145	causes root swelling, no infection thread	pJB4J1-mutagenesis (Tn5)	Hirsch et al. (1982)
Rm1028	As above	As above	As above
Miscellaneous	several individual Tn5-insertions in fix clusters	site directed mutagenesis	Ruvkun et al. (1982)

¹ Some of the mutants are derivatives of Rm1021, a streptomycin-resistant derivative of Rm2011.

² See page Xi for a list of abbreviations.

Table 3b

Mutants of *R. meliloti* strain Rm1 and its derivatives¹

Strain	Characteristics ^{2,3}	Method of isolation	Comments and Reference
ZB 121	Mod ⁺ Km ^S	Heat treatment of a Tn5-carrying strain (strain ZB 72; location of Tn5 ^W unknown)	Banfalvi et al. (1981)
ZB 125	Mod ⁺	As above (from ZB 73)	As above
ZB 126	Mod ⁺	As above from ZB 75	As above
ZB 135	Mod ⁺ Km ^S	As above from ZB 78 (Tn5 ^W present on the megaplasmid)	As above
ZB 138	Mod ⁺ Km ^S	As above	As above
ZB 147	Mod ⁺ Km ^S	As above from ZB 84	As above
JA 7	fix-4	pJ84J1-mutagenesis (Tn5)	As above; Fograt et al. (1983)
JA 10	Fix ⁺ , fixes more nitrogen	As above	Placzinsky (1981)
ZB 157, 158, 159	Mod ⁺ Km ^S	Heat treatment of a strain carrying Tn5 at an unknown location (strain AG 31::Tn5)	As above
ZB 160	Fix ⁺	Heat treatment of a strain carrying Tn5 at an unknown location (strain ZB 74::Tn5)	Placzinsky (1981)
ZB 161	Fix ⁺	As above from strain ZB 83	As above
Gy 889	fix-5	MTG	Forrat et al. (1983)
Gy 890	fix-8	MTG	As above
Gy 891	fix-9	MTG	As above
Gy 900	fix-7	MTG	As above
TF 178	fix-2	pJ84J1-mutagenesis (Tn5)	As above
JA 5	fix-3	As above	As above
ZB 273	fix-10	As above	As above
ZB 274	fix-11	As above	As above
ZB 277	fix-11	As above	As above
ZB 278	fix-13	As above	As above
ZB 313	fix-14	As above	As above
ZB 306	nod-2	As above	As above
AK 1282	nifH::Tn5	site-directed mutagenesis	As above

Table 3b
Mutants of *R. melioli* strain Bm1 and its derivatives¹

Strain	Characteristics ^{2,3}	Method of Isolation	Comments and Reference
JMP19-1	pur-19	NTG	Silk et al. (1980)
JML 44	leu-46	NTG	As above
JML 44-14	leu-44 pur-14	NTG	As above
JMP-1	met-1	NTG	As above
Bm F46 (-AK200)	cys-46	NTG	Kondorosi et al. (1977a)
AK 185	str-1 cys-46 gly-1 ade-4	NTG	Silk et al. (1980)
AK 194	str-1 cys-46 tyr-1 gly-1 ade-4	NTG	Silk et al. (1980)
AK 178	cys-46 met-5 str-3	NTG	Kondorosi et al. (1977a)
AK 180	cys-46 ade-3 str-3	NTG	As above
MG 10	cys-10	NTG	Svab et al. (1978)
K 7	arg-7	NTG	As above
K 3	trp-3	NTG	As above
A 1 (-AK74)	his-1 Sm ^r	NTG	Kondorosi et al. (1977a)
AK 104	cys-46 gly-1 leu-4 str-1	NTG	As above
-AK 193	cys-46 gly-1 ade-4 met-2 str-3	NTG	As above
AK 171	met-33 ade-1 str-1	NTG	As above
AK 173	ura-29 ade-1 str-1	NTG	As above
GY 133	cys-46 leu-4 str-1	Gly ⁺	Kiss et al. (1980)
revertant of			
N 69	NR ⁻ , ineffective modules	?	Kondorosi et al. (1973)
N 77	As above	?	As above
GY 39	narA-168 met-168	NTG	Kiss et al. (1979)
GY 43	narB-15 phe-15	NTG	As above
GY 44	narB-15 ade-15	NTG	As above
GY 45	narB-15 trp-15	NTG	As above
GY 152	narA-168	NTG	As above
GY 154	narB-15	NTG	As above
GY 156	narC-191	NTG	As above
GY 248	narD-240	NTG	As above
GY 62	narB-15 trp-15 str-15	NTG	As above
AK 1212	Mod ⁻ , nifHDK region deleted	Spontaneous	Banfalvi et al. (1981)
AK 627	Mod ⁻ , nifHDK region not deleted	Heat treatment	As above
AK 635	Mod ⁻ , nifHDK region deleted	As above	As above
AK 641	Mod ⁻ , nifHDK region deleted	NTG	As above
GY 1823	Mod ⁻	Freezing and thawing	As above
GY 561	Mod ⁻ , nifHDK deleted	?	Kondorosi et al. (1982)
AK 1168	cys-46 rif ^r 5fu ^r		

Table 3b
Mutants of *R. meliloti* strain Rom1 and its derivatives¹

Strain	Characteristics ^{2,3}	Method of isolation	Comments and Reference
AK 495	<u>cys-46 gly-1 phe-15</u> NTG		Kondorosi et al. (1977a)
GY 493	<u>cys-46 phe-15 str-1</u> Gly ⁺	revertant of AK 495	Kiss et al. (1980)
AK 172	<u>pur-1 met-13 str-3</u> NTG		As above
ZS J	<u>pur-1 str-3</u> 7		Kondorosi et al. (1977a)
AK 175	<u>pur-1 cys-24 str-1</u> NTG		As above
AK 183	<u>cys-46 pur-3 his-2</u> NTG		Kiss et al. (1980)
AK 351	<u>narB-15 pur-15</u> NTG		Kiss et al. (1979)
AK 532	<u>cys-46 gly-1</u> NTG		Kiss et al. (1980)
AK 746	<u>cys-10 rif-10</u> NTG		As above
Unassigned numbers	<u>Arg, Cys, Gly, His, Leu, Met, Pur, Pyr, Trp, Tyr, Mod, Fix</u>		Forrai et al. (1983); all of these are individual mutants.
Unassigned numbers	<u>Arg, Cys, Lys, Met, Pur, Glu, Trp, Tyr, Mod, Fix</u> pJB4J1-mutagenesis (Tn5)		As above
NK 461	NR ⁻ , ineffective node	7	Kondorosi et al. (1973)
G 41	Glutamate synthase ⁻ Sym ⁺	NTG	Kondorosi et al. (1977b)
Tb 5	Glutamine ⁻ , Mod ⁺ Fix ⁻	NTG	As above

Table 3c

Mutants of R. meliloti strain GR4

Strain	Characteristics ^{1,2}	Method of isolation	Reference
GRC 18	<u>ala-1 ser-1</u>	MTG	Casadesus and Olivares (1979a)
GRC 30	<u>trp-3 his-39</u>	MTG	As above
GRC 50	<u>orn-1</u>	MTG	As above
GRC 51	<u>phe-15 arg-17</u>	MTG	As above
GRC 54	<u>cys-4 thi-1</u>	MTG	As above
GRC 55	<u>leu-5 his-31 lys-25</u>	MTG	As above
GRC 56	<u>ade-9 met-1 ura-2</u>	MTG	As above
GRC 59	<u>ade-3 arg-17</u>	MTG	As above
GRC 61	<u>leu-5 ade-1</u>	MTG	As above
GRC 38	<u>ala-1 ser-1 gly-10</u>	MTG	As above
GRC X1	<u>leu-5 gly-10</u>	MTG	As above
GRC X2	<u>ala-1 lys-25 str</u>	MTG	As above
GRC X6	<u>ura-2 ala-1 str</u>	MTG	As above
GRC 52	<u>leu-5</u>	?	Casadesus and Olivares (1979b)
TR 125, 139	Tn1 Inserted at	RP4::Mu	Casadesus et al. (1980)
143	unknown location; loss of Ap ^r results in the loss of Nod ⁺ phenotype	mutagenesis	

¹see page Xi for a list of abbreviations²The strains bearing multiple mutations were derived by sequential mutagenesis

Table 3d
Mutants of *R. melliloti* strain L5-30

Strain	Characteristics ¹	Method of Isolation	Comments and Reference
MD 2	Mannitol dehydrogenase	pJB4J1- mutagenesis	Tn5-linkage not shown; Duncan (1981)
MD 5	Ribose kinase	As above	As above
MD 3	Ara ⁻	As above	As above
MD 6	Xylose isomerase	As above	As above
MD 7	Fructose kinase	As above	As above
UR 6	Succinate dehydrogenase delayed modulation, Fix ⁺	MTG	Gardiol <i>et al.</i> (1982)
UR 1	Phosphoglucose isomerase, Fix ⁺	MTG	Arias <i>et al.</i> (1979)
UR 3	Phosphoglucose isomerase, fructokinase	Spontaneous	UR 1-derivative; Gardiol <i>et al.</i> (1980)
UR 4	fructokinase	Spontaneous	UR 3-derivative; as above
MD 1	alpha-ketoglutarate ⁺ Fix ⁺	MTG	Duncan and Frenkel (1979)
Unassigned	Ade ⁻ , Arg ⁻ , Cys ⁻ , Gly ⁻ ? Ile ⁻ , Ily-Leu ⁻ , Leu ⁻ Met ⁻ , Orn ⁻ , Phe ⁻ , Thi ⁻ , Trp ⁻ , Ura ⁻	?	Denarie <i>et al.</i> (1976); All of these are mutants
GM1 1801, 1802, 1812, 1817, 1823, 1824, 1825	Mod ⁻ derivatives	Spontaneous	Rosenberg <i>et al.</i> (1981)
UR 8	Defective in mannose- utilization	MTG	Arias <i>et al.</i> (1982)

Table 3e
Mutants of other R. meliloti strains

Strain	Parental strain	Characteristics of the mutant ²	Method isolation	Reference and Comments
AS 34	102F34	delta-ala ⁻	NTG	Leong et al. (1982); Mod ⁺ Fix ⁻
A 36	102F34	As above; leakier than A 34	NTG	Fix ⁺ with exogenous supply of delta-ala.
recA::Tn5	102F34	RecA ⁻	site-directed mutagenesis with Tn5	Better and Hillinski (1983)
Miscellaneous	102F34	Several individual Tn5- insertions in <u>fix</u> clusters	site-directed mutagenesis	see Corbin et al. (1983)
SaS 3W	Sa10	Mod ⁻	spontaneous	Rosenberg et al. (1981)
Lb1 1N, Lb1 3W	Lb1	Mod ⁻	spontaneous	Rosenberg et al. (1981)
WL113, 131	1u2F51	Mod ⁻ , do not bind to the roots	NTG	Paau et al. (1981)
WL 188	102F51	Mod ⁻ , binds to the roots	NTG	As above

² see page xi for a list of abbreviations.

EXPERIMENTAL PROCEDURES

Bacterial strains, Plasmids and Bacteriophages

The primary strains of E. coli and Rhizobium used in this study are listed in Table 4 (page 58). The derivatives of these, constructed in this study are described in the text.

The plasmids derived in this study and their parental plasmids are listed in Table 5 (page 60).

Bacteriophages PlCm carrying Tn9 (Rosner and Gottesman, 1977) is a gift of Dr. J.L. Rosner.

Bacteriophage λ b221 cI857 Pam Oam rex::Tn5 (Shanabruch and Walker, 1980), abbreviated as λ ::Tn5 was provided by Dr. G.C. Walker. λ c515 b519 int-am219 CI857, nin5::Tn1, referred to as λ ::Tn1 in this study, was from Drs. J. Way and N. Kleckner.

Media, Chemicals and antibiotics

For the growth of E. coli strains, TYS medium (Selvaraj and Iyer, 1980) that contained Tryptone (1%), Yeast extract (0.5%), Sodium chloride (0.5%), and 0.2% (v/v) of 1N NaOH was used. The growth temperature was 37°C unless otherwise stated.

Rhizobium strains were grown in TY medium (Beringer, 1974) containing Tryptone (0.5%), Yeast extract (0.3%) and CaCl₂ (10 ml of 6.6% CaCl₂ added to 1000 ml of TY medium after sterilization) was used. The growth temperature was 30°C. For solid media, agar (Difco) was used at a concentration of 1.5%.

Table 4a
E. coli strains^a.

Strain	Relevant Characteristics	Source and References
WAB03	Met ⁻ Thi ⁻ r _k ⁻ m _k ⁻	W. Arber, Universitat Basel
GS501	Spontaneous Rif ^r derivative of WAB03	This study
MM13	Spontaneous Tc ^r derivative of WAB03	M. Walkowiak
HB101	Pro ⁻ Leu ⁻ Thy ⁻ Thi ⁻ r _k ⁻ m _k ⁻ Sm ^r	H. Boyer, University of California (Bolivar <u>et al.</u> , 1977)
GS500	Spontaneous Rif ^r derivative of HB101	This study
CR34	Thr ⁻ Leu ⁻ Thy ⁻ Thi ⁻ , Pl-resistant	F. Bonhoeffer
C600	Thr ⁻ Leu ⁻ Thy ⁻ Thi ⁻	S. N. Cohen
PB3	His ⁻ Thi ⁻ , A-resistant	R.C. Portalier (Portalier <u>et al.</u> , 1974)
1830	NaI ^r ; carries pJB4JI encoding resistance to Gm and Nm (Tn5)	J.E. Beringer (Beringer <u>et al.</u> , 1978)

^aDerivatives of these strains have been described in the text.

Table 4b. *Rhizobium* strains

Strain	Derivation and Relevant Characteristics ^a	Source and Reference
<u><i>R. meliloti</i></u>		
I2450	Natural isolate; Brazil	E.A. Peterson, Agriculture Canada, Ottawa
JJ1 ^b	Spontaneous Rif ^r derivative of I2450	Selvaraj and Iyer (1981)
Rhodesia 304	Natural isolate; Zimbabwe	E.A. Peterson
GS1002 ^c	Spontaneous Rif ^r derivative of Rhodesia 304	This study
2043	Natural isolate, England	R.W. Miller and G. Kristjannsen, Agriculture Canada, Ottawa
GS1003	Spontaneous Rif ^r derivative of 2043	This study
L15	Natural isolate; Canada	R.W. Miller and G. Kristjannsen
GS1020	Spontaneous Rif ^r derivative of L15	This study
102F51	Natural isolate; Uruguay	J.C. Burton, Nitragin Co.
DMG177	Natural isolate; origin not known	E.A. Peterson
Ottawa HB5	Natural isolate; Canada	As above
54033	Natural isolate; Canada	As above
SU47	Natural isolate; Australia	E.R. Signer (Brockwell and Hely, 1966)
<u><i>R. leguminosarum</i></u>		
128C52	Natural isolate; Wisconsin, U.S.A.	L.M. Nelson, Prairie Regional Lab Canada, via G. Strur (Nelson and Child, 1981)
GS1400	Spontaneous Rif ^r derivative of 128C52	This study
175G16	Natural isolate; West Virginia, U.S.A.	L.M. Nelson (Nelson and Child, 1981)
GS1401	Spontaneous Rif ^r derivative	This study
128C53	Natural isolate; Wisconsin, U.S.A.	N.J. Brewin, John Innes Institute, U.K. (Brewin et al. 1980)
GS1402	Spontaneous Rif ^r derivative	This study
<u><i>R. trifolii</i></u>		
54027	Natural isolate; Canada	E.A. Petersen
GS1403	Spontaneous Rif ^r derivative	This study

^a Derivatives of these strains have been described in the text

^b This strain has two indigenous plasmids (approximate size: 350 Mdal, and 140 Mdal). A derivative of this strain, cured of the smaller plasmid has been isolated (Adachi et al., 1983). The latter strain, JJ1C10 is intensively studied in our laboratory.

Table 5

Plasmids

Plasmid	Relevant Characteristics			Source and Reference ^a
	Replicon	Transfer	Antibiotic-resistance markers	
pCU1	N	Tra ⁺ ; N-type	Sp ^r , Sm ^r , Cb ^r	Konarska-Kozłowska and Iyer (1981)
pCU29	N	As above	Sp ^r , Sm ^r , Cb ^r , Nm ^r (Tn5)	Thatte and Iyer (1983)
pCU101	p15A	Tra ⁺ , N-type	Cm ^r	As above
pCU51	N	Tra ⁻	Nm ^r	V. Thatte; unpublished construct
pGS6	p15A	As above	Cb ^r	This study
pGS15	N	As above	Cb ^r , Nm ^r (Tn5)	This study
pGS9	p15A	As above	Cm ^r , Nm ^r (Tn5)	This study
pGS18	p15A	As above	Cm ^r , Cb ^r (Tn1)	This study
pGS27	p15A	As above	Cb ^r , Cm ^r (Tn9)	This study
pGS20	p15A	As above	Cb ^r , Nm ^r	This study
pGS36	P	As above	Tc ^r	This study
pACYC177	p15A	Tra ⁻	Cb ^r , Nm ^r	S.N. Cohen; (Chang and Cohen, 1978)
pACYC184	p15A	As above	Cm ^r , Tc ^r	As above
pGS33	p15A	As above	Cm ^r	This study
pGS38	p15A	As above	Cm ^r , Nm ^r (Tn5)	This study
pGS39	p15A	Tra ⁻ , Mob ⁺ _p	Cm ^r , Nm ^r (Tn5-A1)	This study
pGS12	p15A; P	Tra ⁻	Cb ^r , Nm ^r , Tc ^r	This study
pJB4J1	P	Tra ⁺ ; P-type	Gm ^r , Nm ^r (Tn5)	J.E. Beringer (J.E. Beringer <i>et al.</i> 1978)
pRK248	P	Tra ⁻	Tc ^r	D.R. Helinski (Kahn <i>et al.</i> , 1979)
pRK2501	P	Tra ⁻	Tc ^r , Nm ^r	As above
RK2	P	Tra ⁺ (P-type)	Tc ^r , Nm ^r , Cb ^r	As above
pRK290	P	Tra ⁻ , Mob ⁺ _p	Tc ^r	Ditta <i>et al.</i> (1980)
pGS28	N	Tra ⁻ , Mob ⁺ _p	Cb ^r	This study
pCU32	N	Tra ⁻	Cb ^r	Konarska-Kozłowska and Iyer (1983)
pRK404	P	Tra ⁻ , Mob ⁺ _p	Tc ^r	G. Ditta via L. Barran
pGS48	P	Tra ⁻	Tc ^r , Nm ^r	This study
pGS49	P	Tra ⁻	Tc ^r	This study
pGS46	P	Tra ⁻ , Mob ⁺ _N	Nm ^r	This study
pGS47	P	Tra ⁻ , Mob ⁺ _N	Nm ^r	This study
pGS51	P	Tra ⁻	Tc ^r	This study
pGS63	p15A	Tra ⁺ (N-type), Mob ⁺ _p	Cm ^r , Nm ^r (Tn5-A1)	This study
pRK2013	ColE1	Tra ⁺ (P-type)	Nm ^r	Ditta <i>et al.</i> (1980)

^aPlasmids derived in this study have been described in the text.

When growth on a defined medium was warranted, M9 minimal medium (Miller, 1972) was used for all bacterial strains used in this study. Supplementing the medium with a mixture of biotin, pantothenate and thiamine at a final concentration of 0.1 ug/ml enhanced the growth of Rhizobium strains. The composition of M9 minimal medium is as follows. Na_2HPO_4 (0.6%), KH_2PO_4 (0.3%), NaCl (0.05%), NH_4Cl (0.1%); 10 ml of a 0.01M solution of CaCl_2 and 1 ml of a 1M solution of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ are added after autoclaving. When required, the media were solidified with agar (1.5%). The amino acids, when required, were added at a final concentration of 40 ug/ml. The final concentration of vitamin supplements in the case of mutants requiring them was 1 ug/ml.

The concentration of antibiotics used in solid medium was as follows (ug/ml): ampicillin, Ap, 40; carbenicillin, Cb, 40; chloramphenicol, Cm, 50; gentamycin Gm, 10; kanamycin, Km, 40; nalidixic acid, Nal, 50 (for E. coli), 100 (for Rhizobium). In liquid media; half of these antibiotic concentrations were used, unless otherwise stated. The antibiotics were from Bristol-Myers Canada Ltd. and Sigma Chemical Corp.

Bacterial matings

The following methods were used. Spot matings between 10^6 donors and recipients were done on appropriate selective plates. Alternatively, the donors and the

recipients (10^6 cells each) were mixed on TY or TYS agar; following incubation at 30 C (for all matings involving Rhizobium) or at 37 C (for E. coli) for 12-16 hrs, the mating mixtures were streaked on selective plates. Bacterial matings on membrane filters were done as follows. 10^8 - 10^9 cells of donors and recipients were mixed and collected on a sterile membrane filter (porosity, 0.45 μ m; Millipore Corp.). The filters were placed on TY or TYS agar and following an incubation at 30 C for 12-16 hrs, the cells were resuspended in 5 ml of PBS (Na_2HPO_4 , 0.7%; KH_2PO_4 , 0.3%; NaCl , 0.4%). Appropriate dilutions, made in PBS, were plated on selective plates.

Bacterial transformation

E. coli. Genetic transformation of E. coli was carried out by the method of Cohen et al. (1972) with a minor modification. A 20-ml culture from late log phase was spun for 2 min at 17,000 $\times g$ in a Sorval SS34 rotor (the rotor was pre-chilled at 4 C). The pellet was washed with 10 ml of pre-chilled 0.03M CaCl_2 and was resuspended in 10 ml of the CaCl_2 solution. The cell suspension was held in an ice-bath for 30 min. The cells were then spun down at 15,000 $\times g$ for 1 min in SS34 rotor. The pellet was gently resuspended in 1 ml of pre-chilled 0.03M CaCl_2 . To 0.2 ml of this cell suspension, 1-10 μ l (about 0.2 μ g) of plasmid DNA preparations were added, and the mixture was held in

an ice bath for 1 h. The cells were then subjected to a heat pulse at 37°C for 10 min. 5 ml of TYS broth was added to the cells and the tubes were chilled briefly in an ice-bath. After 1 min the tubes were transferred to a 37°C water-bath where it was aerated for 2-3 h to allow phenotypic expression. Appropriate dilutions were plated on selective plates.

R. meliloti. The procedure used for the genetic transformation of R. meliloti was developed as a part of this study and it has been described in detail in Chapter I (page 78). The yield of transformants was greater, if fresh preparations of purified plasmid DNA were used. Plasmid DNA isolated by rapid methods (e.g. Holmes and Quigley, 1981), but not purified, also yielded transformants. However, the yield which was variable depending on the size of the plasmid, was lower. For strain construction the latter preparations of DNA were adequate.

Bacteriophage procedures

Bacteriophages λ ::Tn1, λ ::Tn5 and PlCm were grown on E. coli HB101 or E. coli C600 cells as follows. 0.1 ml of different dilutions of a bacteriophage lysate (in TYS broth) was added to 1 ml of the host strain grown in TYS broth. CaCl_2 was added to this mixture at a final concentration of 5 mM. After the incubation of this mixture for 15 min at room temperature, 2.5 ml of molten

TYS soft agar (TYS broth + 0.8% agar + 5 mM CaCl_2) was added and the contents were overlayed on TYS bottom agar plates (same as TYS soft agar, but with 1.5% agar). The plates were incubated at 37 C for 12 h. The soft agar layer from the plates showing confluent plaques was pooled to which few drops of CHCl_3 were added. The contents were mixed thoroughly, and held at 4 C for 30 min. The agar and cell debris were spun down at 25,000 xg for 20 min. Few drops of CHCl_3 were added to the supernatant which usually contained 10^{10} - 10^{11} PFU/ml. Despite the use of CHCl_3 , occasionally the lysates were contaminated with viable bacteria. Therefore, prior to using a lysate, its sterility was ascertained.

Derivation of transposon-insertions in *Rhizobium* strains

The protocol which was developed as a part of this study has been described in detail in Chapter II (page 91). Following the discovery of Tn5-specified Sm-resistance in *Rhizobium* (Chapter III), Sm-resistance was used as the selective marker while isolating Tn5-insertion derivatives of *R. meliloti*.

Identification of auxotrophic mutants

Following the isolation of random insertion derivatives of *R. meliloti*, individual strains were tested for auxotrophy by replica-plating them on M9 minimal agar medium and TY agar. The nutritional deficiency of individual auxotrophs was identified by replica-plating

the mutants on a total of 11 M9 minimal agar plates containing various combinations of supplements as formulated by Holliday (1956). The phenotype was confirmed using particular nutritional supplements, deemed necessary from the Holliday test.

Isolation of prototrophic revertants

Prototrophic revertants of the auxotrophs of R. meliloti were isolated by plating $10^{10} \times 10^{11}$ cells on large Petri plates (150 X 25 mm) containing M9 minimal agar. Revertants, if present formed colonies after 1 week at 30 C.

Identification of UV-sensitive and MMS-sensitive mutants.

R. meliloti strains were grown in TY broth. The cell density was about 10^8 - 10^9 cells/ml. 0.5 ml of the cell suspension of different strains was transferred to individual compartments in a pre-sterilized dish that has 25 compartments. Using a suitable multiprong inoculating device (Rodriguez-Iglesias, 1981), approximately 0.02 ml of the cell suspensions were transferred to TY agar plates and to freshly prepared TY agar containing 0.02% (v/v) of methylmethane sulphonate (Kodak Chemical Co.). For testing the UV-sensitivity of the strains, one set of TY plates, seeded with bacteria was irradiated with short-wave UV (UVS-11; Ultra-Violet Products Inc.) at a

distance of 9 cm. An exposure of 15 seconds was considered to be adequate, since further exposure to the radiation was lethal to the "wild-type" strain, JJ1.

The UV-sensitivity of potential UV-sensitive mutants was confirmed as follows. 2×10^8 cells of each strain were washed and resuspended in 4 ml of PBS. The cell suspension was transferred to a compartment in a sectored Petri plate (Falcon). The shallow layer of cell suspension was irradiated as described above for different duration of exposure. The relative survival of the strains at different doses of UV radiation was compared to that of the "wild-type" strain.

The MMS-sensitivity of potential MMS-sensitive mutants and the UV-sensitivity mutants was re-tested by spotting 100 μ l of different dilutions of the strains on freshly prepared TY agar containing various concentrations of MMS. Since the strain JJ1 and its derivatives were non-mucoid, individual colonies could be visualized at appropriate dilutions. Up to 5 spots could be accommodated on a standard Petri plate (15 X 100mm).

Determination of minimal inhibitory concentrations of antibiotics

About 10^4 cells were added to 5 ml batches of TY or TYE broth containing various concentrations of the antibiotic. For Sm, a range of 2 to 150 μ g/ml was used with a difference of 5 or 10 μ g/ml between successive

concentrations. A concentration at which the strain did not grow, but below which it grew in a comparable manner to the antibiotic-free culture was taken as the minimal inhibitory concentration. This was determined after 2 days of incubation, unless otherwise stated.

Assay of Nod⁺ and Fix⁺ phenotypes of *R. meliloti* strains

Alfalfa (cultivar, Saranac) seedlings, germinated from surface sterilized seeds were used. The seeds were surface sterilized by immersing them in a 15% Javex solution, stirring the contents for 15-30 min followed by a wash with 90% ethyl alcohol. The seeds were germinated on Petri plates containing 25% TY medium solidified with 1% agar. Despite the surface-sterilization, some seedlings showed bacterial and/or fungal contamination. These were discarded. 3 day-old seedlings were then transferred to 25X 150 mm Petri plates or 25 X 150 mm tubes containing Thornton's Nitrogen-free agar medium (Vincent 1970; described below). 10^6 - 10^7 cells of *R. meliloti*, washed and resuspended in PBS were added to the surface of the medium. The inoculated seedlings were then grown at 21 °C under an illumination of 900 foot-candles (16 hr day regime). Nodule-like structures started to appear, in the case of Nod⁺ strains, after 7-10 days, and the effect of N-starvation (lack of N₂-fixation) was clear at the end of 3-4 weeks. Plants that were green and lush were considered to be Fix⁺. Small plants, with yellowish

leaves that were withering away at the end of 4 weeks of growth were considered to be Fix^- . The Fix^- ones were comparable in growth to plants that were not inoculated. Plants showing intermediate growth are referred to as $\text{Fix}^\pm$. Inoculated plants that did not nodulate were similar to the Fix^- ones in growth and appearance. These are referred to as Nod^- plants. The plant assays were done in quadruplicate unless otherwise stated. Since the bacterial component of these assays was the variable, the phenotypes with respect to nodulation, and N_2 -fixation were ascribed to the bacterial strains.

A modified version of Thornton's N-free medium was prepared as follows. A 1000 ml. stock (2X concentrated) containing $\text{Ca}_3(\text{PO}_4)_2$, 4.0g; K_2HPO_4 , 1.0g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4g; NaCl , 0.2g; FeSO_4 , 2.0g; and FeCl_3 , 0.02g was prepared by stirring the contents for 1-2 h. 1 ml of 10N NaOH was added and the solution was autoclaved. A solution of trace elements, (per 100 ml: boric acid, 0.01g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.005g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05g; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01g; Sequestrene (CIBA-Geigy), 0.1g; CrCl_4 , 0.01g; CuCl_2 , 0.02g and NiCl_2 , 0.02g) was prepared separately and autoclaved. 2 ml of this solution was added to 100 ml of the former, and the contents were mixed with equal volume of 3% agar (in water).

Determination of the plasmid-composition of
bacterial strains

The analytical procedure of Eckhardt (1978), modified as described below was used to determine the plasmid composition of R. meliloti strains. 10^7 - 10^8 cells, washed and resuspended in 50 μ l of lysozyme mixture {20% Ficoll, lysozyme (7500 u/ml), RNase (0.3 u/ml) and 0.01% bromophenol blue; in Tris-acetate buffer (40 mM Tris, 20 mM sodium acetate, 10 mM EDTA, pH 8.3)}. The cell suspension was chilled on ice for 15-30 min. Then it was transferred to individual slots in a vertical gel {0.9% Agarose (Sigma, TypeV) in Tris-Acetate buffer} whose dimensions were 130 x 140 x 3 mm. 50 μ l of lysis mixture (2% sodium lauryl sulphate in Tris-acetate buffer containing 10% Ficoll) was layered atop the cell suspension. Alternatively, 100 μ l of the lysis mixture was added to the gel slots, before adding the cell suspension. The cell suspension was then added dropwise which sank to the bottom of the slot. The slots were then filled up with an overlay mixture (same as lysis mixture, but with 5% Ficoll), and sealed with molten agarose that was used to cast the gel. The gels were first run at 2 Volts for 30 min at room temperature, and then at 50 Volts for 16-18 h at 4 C. Tris-borate was used as the electrophoresis buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). At the end of the run, the gels were stained with ethidium bromide (EtBr; 0.5 μ g/ml in water)

for 30 min to 1 h. The gels were then washed with water and photographed under illumination with short-wave UV. Different strains containing one of the following plasmids were used as standards: pTA2, 140 Megadaltons (Adachi et al., 1983); pJP4, 52 Mdal (Don and Pemberton, 1981); RK2, 36 Mdal (Meyer et al., 1977); pCU101, 16.5 Mdal (Thatte and Iyer, 1983); ~~p~~PK248, 6.4 Mdal (Kahn et al., 1979).

Isolation of plasmid DNA

Preparative isolation and purification of plasmid DNA was done by either of the two methods described below. For the isolation and purification of plasmid DNA from R. meliloti, the cleared-lysate procedure of Clewell and Helinski (1969) was used with the incorporation of a step in which the plasmid DNA was concentrated by the use of Polyethylene glycol (PEG) as devised by Humphreys et al. (1975). The procedure of Davis et al. (1980) with the modifications described by Selvaraj and Iyer (1983) was used for the isolation of plasmid DNA from E. coli.

Isolation of plasmid DNA from R. meliloti. This procedure was used with plasmids ranging in size from 6.4 to 36 Mdal. The indigenous plasmids (140-350 Mdal) of R. meliloti do not lend themselves to isolation by this procedure. A 1200 ml culture of R. meliloti strains was grown to mid-log phase in TY medium containing an antibiotic to which the plasmid of interest specified resistance. The cells were spun at 9,000 xg for 2 min in

a GSA rotor (Sorval). The pellet was resuspended in 25 ml of ST (50mM Tris-HCl, pH 8.2; 25% sucrose). The cell suspension was chilled in an ice bath. 5 ml of lysozyme (0.01g in 10mM Tris-HCl, EDTA, pH 8.3) was added to the cell suspension. After 5 min (in an ice bath) 10 ml of pre-chilled 0.25 M EDTA (pH 8.2) was added to the cell suspension. The contents were divided into two equal portions and were transferred to two pre-chilled SS34 (Sorval) tubes. The tubes with their contents were chilled on ice for 10 min and to each tube, 15 ml of a detergent mixture (1% Brij, 0.4% sodium deoxycholate, 62.5 mM EDTA in 50 mM Tris-HCl, pH 8.3) was added. Usually there was a visible increment in the viscosity of the suspension. If not, few drops of SDS (10% in water) were added and the contents were spun for 45 min at 30,000 xg at 4 C. The supernatants were pooled and transferred to a GSA tube. PEG was added, from a stock solution of 100% (w/v) to a final concentration of 10%; in addition, NaCl was added to a final concentration of 3%. The contents were gently mixed and held at 4 C for 6-16 h to allow the precipitation of DNA. The precipitate was then dissolved in 7.5 ml of TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.2). 8.2g of cesium chloride (CsCl) was added to the solution. After the CsCl was dissolved, 0.5 ml of EtBr (10 mg/ml, in TE) was added and the contents were mixed gently. The tubes (GSA) containing the solution were held in dark for 30 min to 1 h. The precipitated cell debris were removed by

centrifugation at 9,000 xg for 20 min or by filtration through siliconized glass wool. The solution was then spun at 40,000 RPM in Ti 50 rotor (Beckman) for 40 h. The run temperature was 15 °C. The lower band (visualized with long-wave UV) containing the plasmid DNA was re-run for 4 h in a 50% (w/w) CsCl solution at 300,000 xg in a VTi80 rotor (Beckman). The plasmid DNA was extracted thrice with CsCl-saturated isopropanol, and it was precipitated by the addition of chilled ethyl alcohol. The CsCl crystals that formed following the addition of alcohol was re-dissolved by dropwise addition of TE. The contents were then left at -20 °C for 12 h. The precipitated DNA was collected by centrifugation at 2000 xg for 20 min and was dissolved in 0.2 ml of TE. The contents were transferred to an autoclaved microfuge tube (1.5 ml capacity), and the DNA was re-precipitated at -20 °C with the addition of 1.5 volume of ethyl alcohol. After 12 h, the DNA was collected by centrifugation at 15,000 xg for 5 min, and was dissolved in 50-100 µl of TE. The DNA samples were stored at 4 °C.

Isolation of plasmid DNA from E. coli. 600 ml of an overnight culture was centrifuged at 4 °C at 9,000 xg in a Sorval GSA rotor and the pellet was resuspended in 4 ml of chilled STE (15% sucrose, 0.05 M Tris-HCl, pH 8.3, 0.05 M EDTA); 0.5 ml of freshly prepared lysozyme solution (Sigma, 10 mg/ml in STE) was added. The cell suspension

was held at room temperature for 5 min and then on ice for a further 5 min. 5 ml of Triton X-100 solution (0.1% in 0.05 M Tris-HCl, pH 8.3, 0.005M EDTA) was added and the contents were gently mixed once. If there was no increment in viscosity of the cell suspension, a few drops of SDS (10% in distilled water) was added and the lysate was spun at 25,000 xg for 1 h at 4 C. To the supernatant, cesium chloride was added to a final concentration of 45% (w/v); once it was dissolved, 1 ml of ethidium bromide (10 mg/ml in distilled water) was added. The contents were held at 4 C in the dark for about 30 min and the precipitated debris was removed as described above. The rest of the operation was the same as described above.

Rapid isolation of plasmid DNA

The method of Holmes and Quigley (1981) was used for isolating small plasmids (15 Mdal) from E. coli strains. The DNA preparations were suitable for restriction analysis, and for use in genetic transformation. A 5 ml-culture was spun at 2,000 RPM for 10 min. The cells were resuspended in 0.35 ml of STET buffer (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl, 50 mM EDTA, pH 8.0). 25 ul of a 10 mg/ml stock of freshly prepared lysozyme (in TE) was added. The contents, in a microfuge tube, were mixed once and were immediately transferred to a boiling water bath where tubes were held for 40 seconds. The lysate was then chilled in an ice-bath for 5 min and was spun at

15,000 xg for 15 min at 4°C. The pellet was removed with a tooth pick, and an equal volume of isopropanol was added to the supernatant. The precipitated DNA was washed with 70% ethyl alcohol and was dissolved in 50 ul of distilled water.

Plasmid DNA manipulations. The restriction enzymes were purchased from New England Biolabs or Boehringer-Mannheim, and were used as recommended in Davis et al. (1980).

Plasmid DNA were digested to completion with appropriate enzymes in suitable reaction buffers recommended by Davis et al. (1980). The duration of the reaction was 4-6 h at 37°C or 10-12 h at 30°C. When digestion with two or more enzymes that required different buffers was necessary, the DNA was precipitated with ethyl alcohol and redissolved in appropriate buffer. DNA ligations were performed with T4 DNA ligase purchased from New England Biolabs in a buffer recommended by the supplier. The reaction temperature was 17°C and the duration was 2-3 days. These techniques have been described in detail in Davis et al. (1980) and Maniatis et al. (1982).

Agarose gel electrophoresis was performed at 20 Volts for 12-15 h with 0.9% gels cast in Tris-acetate buffer. Either a vertical gel or a horizontal gel was used. The Tris-acetate buffer was used as the electrophoresis buffer. λ DNA cut with HindIII was used as the size standards (Murray and Murray, 1975).

Colony-hybridization: ^{32}P -labelled DNA of the plasmids pACYC104, pCU1, and ColE1::Tn5 were used as the probes in colony-hybridization as described by Grunstein and Hogness (1975). The in vitro labelling (nick-translation) of the plasmid DNA was performed according to the procedure of Rigby *et al.* (1977). The reaction mixture (25 μl) contained 2.5 μl of NT buffer (5000 mM Tris-HCl, pH7.5, 100 mM MgSO_4 , 10 mM DTT, and 500 $\mu\text{g/ml}$ of BSA), 2.5 μl of a solution of deoxyribonucleoside triphosphates (dCTP, TTP, and dGTP, 0.2 mM each), 1.0 μl of dATP (0.2 mM stock), 2.0 μl of the plasmid DNA (750 $\mu\text{g/ml}$), 1 μl of DNAase I (Sigma, pH7.5), and 2.0 μl of DNA polymerase I (Boehringer-Mannheim, 1 mg/ml stock), 1-5 μl of α - ^{32}P dATP (about 50 uCi; New England Nuclear Corp.), and double-distilled water to make up the volume to 25 μl . After 3 h of incubation at 18°C, the reaction was stopped by the addition of 10 μl of NT-stop buffer (50 mM EDTA, 5 mg/ml of sheared salmon sperm DNA and 0.5% SDS).

The ^{32}P -labelled DNA was separated from the radioactive dATP by passage through a column of Sephadex G-50 (medium). The size of the column was 80 X 10 mm. The Sephadex beads were swollen in TE buffer (10 mM Tris-HCl, pH7.5; 1 mM EDTA), and the column was equilibrated with TE buffer. The radioactive counts in 3-drop fractions of the effluent, collected by the addition of TE buffer were determined by "Cerenkov counting" in a liquid scintillation counter. The

fractions corresponding to the first peak were pooled and used as the probes in hybridization.

"Colony-blots" were prepared as follows. About 0.02 ml of mid-log phase cultures grown in sterile 25-well dishes (Rodriguez-Iglesias 1981) was spotted on Nitrocellulose sheets (Schleicher and Schuell, BA85) using a multiprong inoculator. This device was suitable for simultaneous transfer of 25 cultures. The seeded sheets were placed on ethanol-sterilized plastic trays containing TY agar, and were incubated for 6-12 h, until a film of growth was visible in the spotted zones. The filters were then treated with different solutions by placing the filters on a series of Whatman No. 1 chromatography sheets soaked with the following solutions.

(a) 25% sucrose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1.5 mg/ml lysozyme (10 min). (b) 0.2% Triton X-100, 0.5 N NaOH (10 min). (c) 0.5 N NaOH (10 min). (d) 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl (10 min). The filters were then baked at 80°C for 2-2.5 h in a vacuum oven, and were then stored between layers of Whatman No. 1 sheet at 4°C.

The hybridization reactions were essentially as described by Davis et al. (1980). The nitrocellulose sheets carrying colony-blots were pre-treated at 42°C with a pre-hybridization solution containing 5x SSPE (1x SSPE = 180 mM NaCl, 10 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 mM Na_2EDTA , pH 7.0) 50% formamide, 5X Denhardt's reagent (1X = 0.02% BSA, polyvinyl pyrrolidone and Ficoll), 1% glycine, and 100 ug/ml of

denatured salmon sperm DNA. The duration of incubation was 2-3 h. Following the removal of the Pre-hybridization solution, the hybridization solution containing 50% formamide, 1X Denhardt's reagent, 5X SSPE, 100 ug/ml of denatured salmon sperm DNA, 0.3% SDS, 10% sodium dextran sulphate and a sufficient amount of the probe (10^5 CPM) was added. The hybridization reaction was carried out at 42°C for 24-40 h. The nitrocellulose sheets were then washed thrice with 2X SSPE containing 0.2% SDS at 60°C. They were then covered with Saran Wrap and an autoradiogram was obtained by using a Kodak X-omat RP film, and a Dupont intensifying screen.

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CHAPTER I: GENETIC TRANSFORMATION OF *Rhizobium*
meliloti BY PLASMID DNA

Plasmid DNA-mediated genetic transformation is important in both recombinant DNA technology and in the study of plasmids. It is a widely used method by which both native plasmid DNA as well as manipulated DNA is introduced into bacterial cells (see Timmis et al., 1978 and also a collection of reviews on "gene cloning", edited by Hofschneider and Goebel, 1982). The observations that plasmids play a major role in symbiotic nitrogen-fixation by R. meliloti further emphasized the potential value of developing a procedure to transform R. meliloti by plasmid DNA. The earlier literature on transformation of R. meliloti by total DNA has been reviewed by Balassa (1963) and Gabor-Hotchkiss (1972), and a summary of it has been presented in the Literature Review.

Plasmid DNA-mediated transformation in other species of Rhizobium has been reported (O'Gara and Dunican, 1973; Dunican and Tierney, 1973; Bullerjahn and Benzinger, 1982; Berry and Atherly, 1982). However, the method to transform R. meliloti with plasmid DNA, developed as a part of this project is the first one to be described for this species (Selvaraj and Iyer, 1981).

Results and Discussion

Choice of plasmids

Plasmids of the P1 incompatibility group (abbreviated IncP) have a wide host-range (Datta and Hedges, 1972; Olsen and Shipley, 1973, Beringer, 1974).

Kondorosi et al. 1977a; Meade and Signer, 1977). From one of these plasmids, RK2, smaller cloning vehicles have been derived (Kahn et al. 1979). These plasmids, pRK248 and pRK2501, are non-conjugative and specify resistance to tetracycline (Tc), and to Tc and kanamycin (Km) respectively. Plasmid RK2, a self-transmissible plasmid, was transferable from E. coli donors into a number of R. meliloti strains including strain JJ1 (see Table I-2) at a frequency of 10^{-2} (per donor cell). These results indicated that the host-range of RK2 include those strains listed in Table I-2, and that the Tc^r and Km^r markers associated with RK2 are expressed in these strains. Therefore RK2 and its derivatives were chosen as candidates for introduction into R. meliloti via plasmid DNA-mediated transformation.

Attempts to prepare bacterial cells capable of DNA-uptake

Cohen et al. (1972) described the first efficient method for introducing plasmid DNA into E. coli cells. This method involved pre-treating cells with 0.03M $CaCl_2$ that induced "competence", as physiological state for DNA uptake, in the cells. This method has been later modified to transform other Gram-negative bacteria (Lederberg and Cohen, 1973; Chakrabarty et al., 1975; Cannon et al., 1977). In our initial attempts to transform R. meliloti strain JJ1, chosen on the basis of its sensitivity to Tc and Km, we used the DNA of plasmid RK2, and the procedure of Cohen et al. (1972). Several attempts did not yield

any transformants ($< 5 \times 10^{-1}$ per μg of DNA). The use of different concentrations of CaCl_2 , ranging from 0.01 M to 0.3 M also failed to yield transformants. Therefore we resorted to another procedure that was originally used for transfecting *E. coli* (Dityatkin *et al.*, 1972) and for transforming *A. tumefaciens* with plasmid DNA (Holsters *et al.* 1978). According to this method, bacteriophage DNA or plasmid DNA is added to frozen cells and upon thawing, the cells take up the DNA to yield transfectants or transformants.

Transformation of *R. meliloti* strains by the 'freeze-thaw' method

The following is an adaptation of the method of Dityatkin (1972) and Holsters *et al.* (1978) that proved to be successful in transforming *R. meliloti*. 100 ml of a late-exponential phase culture ($A_{550}=0.7$) of *R. meliloti* grown in TY medium (see "Experimental Procedures", page 57) was harvested by centrifugation at 14,000 $\times g$ for 20 min at 4°C followed by washing with 1 vol. of 0.01 M Tris-HCl pH 7.5. The cells were resuspended in 1 ml of TY medium. To 0.02 ml of this cell suspension, a desired amount of plasmid DNA in a volume of 5-10 μl was added. The mixture was rapidly frozen in a dry ice-ethanol bath and held frozen for 5 min. The mixture was then transferred to 30°C to permit DNA uptake. 10 ml of TY medium was added at 60 min after the transfer and the cells were further

incubated at 30 C to allow phenotypic expression of the plasmid-associated resistance markers. Viable counts were determined on TY agar and the transformants were selected on TY agar containing an appropriate antibiotic. The result of a representative experiment involving various concentrations of plasmid DNA is shown in Fig I-1.

The Tc^r transformants of JJ1 that were obtained were purified and confirmed to be JJ1-derivatives on the basis of their resistance to rifampicin (rif) and their ability to nodulate alfalfa. With Tc present in the medium, the plasmid was maintained stably. Plasmid DNA reisolated from the transformants using the cleared lysate method (see experimental procedures) was 9.6 Kilo base pairs (kb) in size, and had single restriction sites for EcoRI, SalI and BglII as expected [see Meyer (1979) for a physical map of pRK248]. The indigenous large plasmids of strain JJ1 do not lend themselves to isolation by the cleared-lysate method (Adachi and Iyer, 1980; Adachi et al. 1983), and therefore did not interfere with the procedure used here for re-isolating pRK248 from the transformants.

A distinct feature of the plasmid DNA transformation in R. meliloti was the absence of saturation for DNA concentrations up to 7 ug (Fig. I-1). In contrast, A. tumefaciens is transformed with the same efficiency, irrespective of whether 0.03 ug or 3 ug

Fig.I-1. Transformation of *R. meliloti* JJ1 by PRK248 DNA. To 5×10^9 cells resuspended in 0.2 ml of TY medium, the indicated amounts of plasmid DNA in a volume of 10 μ l were added, and the mixture was frozen in a dry ice- ethanol bath for 5 mins. The tubes were then incubated at 30°C for 1 hr. followed by the addition of 10 ml of TY medium and a further incubation of the suspension at 30°C for 1 hr. The cells were concentrated by centrifugation at 5000 Xg for 20 min. and the concentrated samples and their appropriate dilutions were plated on TY agar plates containing tetracycline (15 μ g/ml)

of plasmid DNA is added, indicating that in this organism a saturation point is reached at or below 0.3 ug of DNA in the transformation mixture (Holsters et al. 1978).

If R. meliloti cells were frozen without the plasmid DNA and the DNA added to the frozen cells at different times during the thawing period, there was an initial decline in the frequency of transformants (Fig. I-2), presumably because of an uneven distribution of the DNA and the inaccessibility of this DNA to all of the cells in the population. However, upon thawing, the cells were transformable, but lost this ability after further incubation (Fig. I-2). At the end of 30 min. at 30 C, the cells had completely lost the ability to yield transformants. It is possible that the uptake of plasmid DNA by the cells is facilitated during the transient period when cells are recovering from the exposure to freezing temperatures. The freeze-thaw step did not have any appreciable effect on cell viability, which was >90%.

The earlier literature on genetic transformation of R. meliloti by total DNA suggested that a physiological state of competence may exist in these organisms and that this may be dependent on the age of the culture. For the procedure described above, this was not the case. Cells harvested at different stages of growth in the exponential or stationary phase (A_{550} , 0.01 to 2.2) were equally susceptible to the transformation procedure. If the cells were not frozen after the addition of plasmid DNA,

Fig. I-2. Effect of addition of pRK248 DNA to *R. meliloti* JJ1 at different times (t) during the freeze-thaw step. 1 μ g of pRK248 DNA was added to 6.0×10^9 cells at times shown on the abscissa. t = 0, DNA was added to 0.2 ml of cell suspension prior to freezing the cell suspension.; t = 5-35, the cell suspension was frozen and DNA was added soon after the removal of the cells from the dry ice-ethanol bath (t = 5), or at the indicated times (t = 10-35). The cell suspension had completely thawed at t = 10. Other details of the procedure are described in the text. Transformants were selected on TY agar containing 15 μ g/ml of tetracycline.

transformants could not be obtained. Therefore, unlike R. trifolii (O'Gara and Dunican, 1973; Dunican and Tierney, 1973), R. meliloti cells when grown under these conditions do not exhibit "competence" for plasmid DNA uptake.

pRK248 DNA is 9.6 kb in size (Kahn et al., 1979). DNA of related but larger plasmids (pRK2501, 11.1 kb; RK2, 56.4 kb) could also be successfully used in the transformation experiments (Table I-1). This implies that if a vector such as pRK248 is used in molecular cloning experiments, inserts as large as 46 kb can be successfully introduced into R. meliloti cells by transformation. The ability of frozen cells to take up plasmid DNA during the thawing period (see Fig. I-2) suggested that batches of frozen cells kept in storage could be used in transformation experiments. R. meliloti cells frozen without plasmid DNA in dry ice-ethanol bath were stored at -20 C as frozen batches of 0.2 ml vol., and these cells did not show any loss in transformability over a period of 10 days. This feature of the method has been adapted for transforming a large number of batches of A. tumefaciens cells with plasmid DNA (Iyer et al. 1982).

Transformation of other R. meliloti strains of diverse origin.

In addition to strain JJ1, the transformation procedure could be used successfully on six other natural isolates of R. meliloti (Table II-2). The efficiency of

Table I-1
Transformation of *E. coli* strain J31 with plasmids of different sizes^a

Plasmid	Size (Kb)	Relevant markers	Number of transformants per viable cell ^b		
			Expt. I	Expt. II ^c	Expt. III
pRK248	9.6	Tc ^r	6.6×10^{-9}	8.3×10^{-9}	2.0×10^{-8}
pRK2501	11.1	Tc ^r Km ^r	1.0×10^{-8}	2.0×10^{-8}	3.6×10^{-8}
RK2	56	Tc ^r Km ^r Cd ^r	3.0×10^{-9}	5.0×10^{-9}	2.0×10^{-9}

^aSee the text for a detailed description of the method

^bIn the three different experiments, for pRK248, the DNA concentrations used were 0.3, 0.5, and 1.5 μ g respectively.

^cThe concentrations of two other plasmid DNAs used were greater in proportion to their size. The occurrence of spontaneous Tc^r colonies was $<10^{-10}$.

Table I-2

Transformation of different isolates of *R. meliloti* with
pRK248 DNA^a

Strain	Origin	Transformation frequency ^b	
		<u>per ug of DNA</u>	<u>per viable cell</u>
102F51	Uruguay	3.7×10^3	1.0×10^{-6}
DMG177	Unknown	5.0×10^2	1.5×10^{-7}
54033	Canada	4.0×10^2	3.3×10^{-7}
SU47	Australia	3.0×10^2	1.3×10^{-7}
Rhodesia304	Zimbabwe	2.6×10^2	7.0×10^{-8}
OttawaMB5	Canada	7.0×10^1	2.0×10^{-8}

^a To about 10^9 cells in 0.2 ml of TY medium, 1.0 μ g of pRK248 DNA was added and the transformants were selected on TY agar containing 15 μ g/ml of tetracycline. See the text for the methods.

^b The occurrence of spontaneous tetracycline-resistant colonies was $< 10^{-10}$.

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transformation was variable; strain 102F51 yielded the highest number of transformants. A few other R. meliloti isolates are also transformable at a frequency comparable to the one described for JJ1 (R.J. Watson, personal communication).

Kiss and Kalman (1982) have subsequently demonstrated transformation of R. meliloti strain Rm41 with an IncW-group plasmid. They employed a procedure described by Mandel and Higa (1970) for transfecting E. coli, and obtained 10-50 transformants per ug of DNA. Recently Bullerjahn and Benzinger (1982) have reported their independent observation that using a "freeze-thaw" procedure a strain of R. leguminosarum (strain 300) can be transformed by plasmid DNA. The yield of transformants is low and is comparable to that obtained with R. leguminosarum strain 128C53 in our laboratory (Selvaraj and Iyer, unpublished results). Thus it seems that the "freeze-thaw" method is potentially applicable to other bacterial species, since it has been successfully used to transfect E. coli and to transform A. tumefaciens, R. meliloti and R. leguminosarum.

Potential uses of the procedure

The method described in this study can be used to introduce non-conjugative plasmids into R. meliloti. It can also be used in molecular cloning, provided the host strain is of a type (see Table I-2) that yields a large

number of transformants. This method has also been used to selectively clone replication regions of indigenous plasmids of R. meliloti (R.J. Watson, personal communication).

CHAPTER II: SUICIDE VEHICLES FOR INSERTION

MUTAGENESIS IN Rhizobium meliloti

Transposons are discrete genetic elements that ensure their own maintenance by inserting into other autonomously maintained genetic elements of bacteria (Kleckner, 1981; Starlinger, 1980). Such insertions lead to mutations. An added and very useful feature of transposons is that they often encode phenotypes such as antibiotic-resistance by which their presence may be recognized. Furthermore, they can and have also been used for genetic manipulations such as deletions, inversions and fusions. These applications have been reviewed (Kleckner et al. 1977). Although the use of transposons was pioneered in E. coli and closely related bacteria, they can be used in other bacteria as well. We describe a procedure for doing this in R. meliloti.

Since transposons themselves are not autonomous replicons, it is reasonable to expect that if they are delivered efficiently into a cell by means of a plasmid vehicle that will not itself be maintained (a suicide vehicle), this would allow the selection of the rare transposon-insertion derivatives using the antibiotic-resistance marker contained within the transposon. In practice, the efficiency of delivery of the vehicle into cells of interest, the efficiency with which it is lost from the cells and an assurance that the transposon-insertion is the only genetic event that has occurred in a large majority of the selected colonies, are all important considerations. For members of the

Rhizobiaceae family, the suicide vehicles that have been used so far are cointegrates of a conjugative, P-incompatibility group plasmid and bacteriophage Mu, the Mu genome conferring the suicidal property to the plasmid by mechanisms that are not well understood (Beringer et al., 1978; Casades et al. 1980; Van Vliet et al. 1978). Our experience and the results of other groups studying R. meliloti have shown that such Mu-containing vehicles induce secondary genetic changes, presumably incited by Mu itself (Meade et al. 1982). These observations prompted the construction of a set of suicide plasmid vehicles of a different type that proved useful for R. meliloti, and is potentially useful for other Gram-negative bacteria. These plasmids contain one of the transposons Tn1, Tn5 or Tn9 and are composed of a pl5A-type of replicon and an N-type of bacterial mating system. The construction, use and efficiency of these vehicles is described in this chapter. A report of a part of this study is in the press for publication. (G. Selvaraj and V. N. Iyer, J. Bacteriol. Dec. 1983).

Results and Discussion

Behaviour of bacteriophage Mu-based suicide vehicles in *R. meliloti* strain JJ1.

Plasmids pJB4JI and RP4::Mu are the prototype conjugative suicide vehicles that have been developed for insertion mutagenesis of members of the Rhizobiaceae family (van Vliet et al. 1978; Beřinger et al. 1978). Although these Mu-carrying vehicles have been used in the past, several difficulties have been encountered. In our attempts to generate Tn5-insertion mutants of *R. meliloti* strain JJ1, the recipient strain was mated with *E. coli* 1830 that carries pJB4JI. Neomycin-resistant (Nm^r , a phenotype specified by Tn5) colonies were obtained at a frequency of about 10^{-5} . About 50% of these had gentamycin-resistance, a marker associated with the plasmid. One auxotroph (Trp^-) was found among the remainder (1/1000), but when this auxotroph reverted to prototrophy, it remained Nm -resistant, suggesting that the mutation was not a result of a simple Tn5-insertion event. There is also evidence to suggest that the transient presence of Mu DNA in *R. meliloti* can cause phenotypic alterations, the basis of which is not understood (Patel et al., 1982). Recently Meade et al. (1982) have reported that all or part of Mu DNA can transpose from pJB4JI onto the genome of *R. meliloti*. These observations suggested that it would be useful to develop a suicide vehicle that

did not contain Mu DNA. We sought to identify or construct a plasmid which had a wide host range of efficient conjugal transmission, but with a capacity of replication and maintenance limited to E. coli and closely related bacteria. Such a plasmid carrying a transposon would be suicidal upon entry into R. meliloti. Selection for a transposon-associated marker would then yield derivatives carrying transposon-insertions.

Transmission of IncN-group plasmids into R. meliloti

Of the different bacterial conjugation systems that have been studied in E. coli, N, P and W systems are of wide host-range (Datta and Hedges, 1972; Beringer, 1974; Jacob et al., 1977; Tardif and Grant, 1980). Leemans et al. (1981) have reported that N plasmids could be used as suicide vehicles for insertion mutagenesis in Agrobacterium tumefaciens, a member of Rhizobiaceae that is closely related to R. meliloti. pCU1 is a prototype N group plasmid whose structural and functional organization is known (Konarska-Kozłowska et al. 1981). pCU29 is a derivative of this plasmid carrying Tn5 at a non-essential site (Thatte and Iyer, 1983). The results shown in Fig. II-1 suggest that this plasmid could be transmitted to R. meliloti but that putative Tn5-carrying derivatives of R. meliloti also inherit some of the other antibiotic-resistance markers of the pCU1. Upon further growth under non-selective conditions, there was a

Legend to Fig. II-1

- A. Derivation of an N-group plasmid pCU29 carrying transposon Tn5 (Thatte and Iyer, 1983). Cb, carbenicillin; Sm, streptomycin; Rep, replication and maintenance; Nm, neomycin; Tra, conjugal transfer.
- B. Transfer of pCU29-associated antibiotic-resistance markers to R. meliloti. The matings were carried out on membrane filters. The transconjugants of R. meliloti from TY agar containing rifampicin (Rif) and a selective antibiotic (Nm, Sm or Sp) were screened for unselected markers by replica plating on TY agar containing an appropriate antibiotic. Patches of growth from TY + Nm plates were further streaked on TY agar (non-selective conditions) and one colony from each streak was examined for inheritance of the different plasmid markers. The asterisk indicates that transconjugants selected on TY+Rif+Sm or TY+Rif+Sp also underwent a segregation. It is likely that in the majority of the Nm^r Sm^r transconjugants, streptomycin-resistance is due to the presence of str, a gene of transposon Tn5, conferring resistance to Sm in Rhizobium (See Chapter III)

B. *E. coli* WA 803 / pCU 29 X *R. meliloti* JJ1

Nm ^r (10^{-4})	Sm ^r (10^{-4})	Sp ^r (10^{-5})
[SCREENING FOR UNSELECTED MARKERS]		

15% Cb^r Sp^r Sm^r
 4% Cb^s Sp^r Sm^r
 81% Cb^s Sp^s Sm^r

[GROWTH UNDER NON-SELECTIVE
 CONDITIONS AND SCREENING
 FOR MARKERS]

6% Cb^s Sp^r Sm^r Nm^r
 4% Cb^s Sp^s Sm^s Nm^s
 1% Cb^s Sp^s Sm^r Nm^s
 89% Cb^s Sp^s Sm^r Nm^r

continual segregation of these markers. Thus it seemed that N plasmids might not be completely unstable in R. meliloti and that some stability might be achieved by continuous selection for their markers. Recently it has been shown that pCU1 DNA has inverted repeats, structures that are often associated with transposable elements (Konarska-Kozłowska et al. 1983): The regions of pCU1 (and its derivative pCU29) encoding resistance to spectinomycin (Sp) and streptomycin (Sm) are on such structures. Perhaps the continual instability of pCU29-associated markers reflect this feature of the N plasmid.

To determine whether a simpler N-plasmid would be completely unstable in R. meliloti, pCU51, a derivative of pCU29 was introduced into strain JJ1 by genetic transformation (see Chapter I). This plasmid is a deletion derivative of pCU29 generated by BamHI cleavage and ligation of the fragment that carries the maintenance region and the Nm^r -marker of pCU29 (V. Thätte, unpublished results; see Fig. II-1 for a map of pCU29). Nm^r transformants of strain JJ1 were obtained at a frequency of 400/ μ g of DNA, a frequency that is comparable to that observed with IncPl-group plasmids Chapter I; see Fig. II-2). However, pCU51 was lost in all of the 100 transformants when they were grown on TY agar lacking Nm . In contrast, the plasmid was maintained if the transformants were grown in media containing Nm . These

Plasmid	Transformants ^a		Co-inheritance of unselected marker ^b	
	Nm ^r	Tc ^r	Nm ^r	Tc ^r
	$<5 \times 10^{-2}$	-	-	-
	$<2 \times 10^{-1}$	350	1%	-
	400	350	100%	100%

Legend to Fig. II-2

Transformation of *R. meliloti* strain JJ1 with plasmid DNA. pACYC177 is a derivative of *E. coli* plasmid p15A (Chang and Cohen, 1978) and pRK248 is a wide host-range plasmid derived from the IncP1-group plasmid RK2 (Kahn et al., 1979). The recombinant plasmid pGS12 was constructed by cleaving the parental plasmids with BglII and BamHI, and ligating the protruding complementary cohesive ends. The composite plasmid was maintained stably by *E. coli* and *R. meliloti* on media containing any one of the three antibiotics (Tc, Nm or Cb).

^a Number of transformants per ug of plasmid DNA

^b The transformants were replica-plated on TY agar containing an appropriate antibiotic.

results indicate that N replicons are not suitable vehicles for R. meliloti unlike as in the case of A.

tumefaciens for which a N group plasmid, RN3, has been reported to be suicidal (Leemański et al., 1981).

Instability of pl5A-replicons in R. meliloti

Plasmid pl5A is a cryptic, non-conjugative plasmid of E. coli from which cloning vectors have been derived that are extensively used in this host (Chang and Cohen, 1978). These plasmids have not been used in hosts other than E. coli and closely related bacteria, presumably due to their limited host-range. We wished to take advantage of their narrow-range in constructing a suicide plasmid for the delivery of transposons into R. meliloti. Initially, the instability of plasmid pACYC177, a derivative of pl5A, in this host was assessed. pACYC177 was introduced into R. meliloti JJ1 by transformation. As shown in Fig. II-2 transformants of R. meliloti JJ1 could not be obtained with this plasmid, in contrast to results with pRK248. When pACYC177 was fused to pRK248 by ligation of the linear molecules generated by BamHI-cleavage of pACYC177 and BglII-cleavage of pRK248, a composite plasmid specifying the resistance phenotypes of both plasmids was obtained. This plasmid, pGS12, could be transformed into R. meliloti JJ1 and the transformants maintained the plasmid stably (Fig. II-2). Restriction analysis of the plasmid re-isolated from the transformants

showed single sites for EcoRI, SalI and HindIII and the size of the plasmid was unaltered (13.3 kb). Thus these results confirmed the physical stability of the composite plasmid. These results also suggested that pl5A-derivatives might be unstable in R. meliloti, unless fused to a stable replicon. Our unsuccessful attempts to transform R. meliloti with another pl5A-derivative, pACYC184, and the results of Ruvkun et al. (1982) who could transfer a fusion derivative of pACYC184 and an IncPl-group plasmid into R. meliloti provide additional evidence for the instability of pl5A-derivatives in R. meliloti. Therefore pACYC184 and pACYC177 were chosen for the construction of suicide vehicles.

Construction of a pl5A-group plasmid with N-transfer system and transposon Tn5

Although plasmids ranging in size from 9.6 to 56 kb could be introduced into R. meliloti by transformation (Chapter I), this procedure would be relatively less efficient than bacterial mating to derive strains carrying transposon-insertion from a suicide vehicle.

Transformation of R. meliloti strain JJ1 with pGS38, a derivative of pACYC184 carrying Tn5, yielded only 3 Nm^r transformants per 15 μg of DNA (10^{-10} per viable cell). Therefore we developed a conjugative suicide vehicle. Recently Thatte and Iyer (1983) have cloned a region of the N-group plasmid pCU1, specifying conjugal transfer

(tra genes) into pACYC184. This region of pCU1 does not include any transposon-like structures (Konarska-Kozłowska et al., 1983). It was reasoned that this recombinant plasmid pCU101 would be conjugative in interspecific matings between E. coli and R. meliloti, but unstable in the latter host. E. coli strain HB101/pCU101 was infected with λ ci857 b221 Pam Oam rex::Tn5 (Shanabruch and Walker, 1980) and the resultant Nm-resistant colonies were mated en masse with E. coli strain PB3. The Nm-resistant transconjugants of PB3 were further tested for conjugal transmission of Nm^r and chloramphenicol-resistance (Cm^r; pCU101-associated marker). Plasmid DNA from one such derivative that transferred both Nm^r and Cm^r at a Frequency of 1 (per donor) was analysed by restriction analysis for the presence and location of the transposon. This plasmid was designated as pGS9 (see Fig. II-3). Its characteristics with respect to conjugal transfer and instability are shown in Table II-1. The results show that the yield of Nm^r transconjugants R. meliloti (potential Tn5-insertion derivatives) is much higher compared to Tn-mutagenesis with the non-conjugative vehicle, pGS38. Thus in pGS9 we seem to have combined the advantageous features of pACYC184 and pCU1, namely replicon instability and transfer proficiency.

Legend to Fig. II-3

Construction of a conjugative plasmid (pGS9) composed of p15A-replicon, N-transfer system and transposon Tn5. The sizes of pGS9 and its parental plasmids as determined by restriction analysis are: pACYC184, 4.0 kb; pCU29, 44.2 kb; pCU101, 24.5 kb, and pGS9, 30.5 kb. Ap, ampicillin (■ Cb, carbenicillin); Cm, chloramphenicol; Ori, origin of replication; Tc, tetracycline; other symbols have been described in Fig. II-1.

Table II-1

Conjugal transfer of pGS9^a

Recipient strain	Transfer frequency		Co-inheritance of unselected marker ^b	
	<u>Nm</u>	<u>Cm</u>	<u>Nm</u>	<u>Cm</u>
<u>R. meliloti</u>				
JJ1	4×10^{-3}	$<1 \times 10^{-7}$	-	3%
GS1002	4×10^{-4}	$<1 \times 10^{-7}$	-	3%
GS1003	1×10^{-4}	$<1 \times 10^{-7}$	-	2%
<u>E. coli</u>				
GS500	1	1	100%	100%

^a Matings between 10^8 cells of E. coli WA803/pGS9 and the recipients were done on membrane filters placed on TY or TYS agar at 30°C. The duration of matings was 2 h for GS500, 4 h for JJ1 and 12-16 h for GS1002 and GS1003. Transconjugants were sought on TY or TYS agar containing Rif and appropriate antibiotics.

^b 100-800 transconjugants were screened on TY agar containing the antibiotics.

Protocol for insertion mutagenesis of Rhizobium with pGS9

The protocol recommended for the isolation of Tn-insertion mutants of R. meliloti is as follows.

E. coli strain WA803/pGS9 is mated with the recipient at an equal cell density of donors and recipients (10^8 - 10^9 cells) on sterile membrane filters placed on TY agar. The optimal duration of mating varies with the recipient. For R. meliloti strain JJ1, this was 4 hrs, and for other strains it varied from 12-24 hrs. Following mating, dilutions of cell suspensions in TY broth are plated on TY + Rif + Nm. The Nm-resistant colonies that arise after 4-5 days of incubation at 30 C are purified on plates of the same composition and screened for Cm-resistance or sensitivity and auxotrophy.

Analysis of putative Tn5-insertions

20 Nm^r colonies chosen at random were analysed for plasmids and for conjugal transfer of Nm-resistance. None of these displayed any plasmids other than their indigenous plasmids (500 kb and 200 kb) in Eckhardt-type gels (Eckhardt, 1978; see Behki et al., 1983 for a plasmid profile of strain JJ1). Transfer of Nm-resistance to a tetracycline derivative of E. coli WA803 could not be detected in spot matings. These results suggest that the suicide vehicle did not establish itself as an independent plasmid in these R. meliloti transconjugants. However, of the total of 800 independent Nm^r colonies, 3% were

chloramphenicol-resistant indicating the presence of pGS9 DNA sequences in addition to Tn5 in this class of transconjugants. Five randomly chosen Nm^r Cm^r colonies also displayed the characteristics described above. It is likely that this rare class of transconjugants could have arisen by Tn5-promoted insertion of the entire plasmid (replicon fusion) into an indigenous genetic element of R. meliloti.

The remainder of the transconjugants (Nm^r) were screened for auxotrophic mutations. About 3% of these colonies exhibited auxotrophy with different-nutritional requirements (see chapter IV), suggesting that mutagenesis with pGS9 yields different mutations in R. meliloti. Spontaneous prototrophic revertants of these auxotrophs were sought. These should give rise to Nm^s colonies, if Tn5 was inserted at the mutated site. The frequency of reversion was very low (10^{-10}). When revertants were isolated, they were of two types: those that were now Nm^s (5 out of 6 auxotrophs), and those that continued to be Nm^r (1/6). The appearance of Nm^s revertants in a majority of the mutants that yielded revertants gave assurance that these mutations were indeed caused by Tn5. Since this analysis depended on the precise excision of Tn5, which is a rare event, it could not be extended to all of the Tn5-insertion mutants. In one of the mutants (Adenosine⁻) that gave rise to Ade⁺ Nm^r revertants, the reversion event could have occurred due to activation of an alternative

metabolic pathway. Alternatively, the ade mutation could have been caused by an indigenous insertion element similar to ISRml (Ruvun et al. 1980; 1982).

Further assurance that no other part of the suicide vehicle was inserting independent of Tn5 was obtained by colony-hybridizing the Nm^r Cm^s transconjugants (including all auxotrophs) with ^{32}P -labelled probes of the parental plasmids of pGS9, pCU1 and pACYC184 (see legend to FigII. 3) that are devoid of Tn5. Under the experimental conditions employed, we estimate that we would have detected a sequence homology of ≥ 1 kb. Of the 233 colonies thus screened only 2 exhibited DNA sequence homology to the probes. Thus it appears that a very large fraction of the mutants isolated following mutagenesis with pGS9 arise by bona fide Tn5-insertions. In summary, 97% of the Nm^r transconjugants of R. meliloti derived from a mating with E. coli carrying pGS9 were Cm^s , of which about 3% were auxotrophic mutants. Among these >98% did not exhibit a homology to the parental plasmids of the suicide vehicle.

Recently Simon et al. (1982) have described the construction of a suicide vehicle suitable for Tn5-mutagenesis of R. meliloti. This plasmid, a derivative of pACYC184, is mobilizable into R. meliloti from an E. coli strain that carries RP4::Mu in its chromosome.

Suicide plasmids carrying Tn1 and Tn9.

It has been reported that Tn5 exhibits some regional insertion-specificity in E. coli (Shaw and Berg, 1979) and in R. meliloti (Meade et al. 1982). The proportion of different types of auxotrophic mutants of R. meliloti JJ1 and its derivatives following mutagenesis with pGS9 (for example, Ade⁻, 20%; Ilv⁻, 4%; His⁻, 10%; Thr⁻, <1%; Ala⁻, <1%) also support these observations (discussed in detail in Chapter IV). It is therefore clear that the use of Tn5 alone would not saturate a given large segment of the genome of R. meliloti. It should be possible to overcome this limitation by insertion mutagenesis with other transposons such as Tn1 and Tn9. Modelled after pGS9, we have constructed pGS18 (Tn1) and pGS27 (Tn9) as shown in Fig. III-4. Transposons Tn1 and Tn9 are well-characterized transposons that specify resistance to carbenicillin (Cb) and chloramphenicol (Cm) respectively (Heffron et al. 1977; Alton and Vapnek, 1979). These two suicide vectors were also found to yield Tn1 and Tn9 insertion mutants in R. meliloti strain JJ1. The vectors carrying these transposons were also found to yield Tn1 and Tn9 insertion mutants in R. meliloti strain JJ1.

It is perhaps worth noting that Tn10, another well-studied transposon that specifies resistance to tetracycline (Tc^r), did not transpose in R. meliloti. A suicide vehicle similar to pGS9 but carrying this transposon (pGS16) was transmissible among E. coli strains

Legend to Fig. II-4

Construction of self-transmissible suicide vehicles, carrying Tn1 (pGS18), and Tn9 (pGS27). These plasmids are composed of p15A-replicon and N-transfer system. The addition of Tn9 to plasmid pCU101 was done in a manner analogous to the construction of pGS9 as described in the text. pGS27 was constructed by mating P1Cm lysogens of *E. coli* HB101/pGS6 with *E. coli* CR34 followed by selection for chloramphenicol-resistance (Tn9-associated marker). *E. coli* CR34 did not adsorb bacteriophage P1Cm (data not shown) and, therefore the Cm-resistant transconjugants arose due to the transfer of pGS6 carrying Tn9. Plasmid DNA from one such transconjugant was characterized further and designated as pGS27. The sizes of pGS18; pGS27 and their parental plasmids as determined by restriction analysis are: pGS18, 29.5 kb; pGS27, 27.0 kb; pACYC177, 3.7 kb; pGS6, 24.2 kb and pCU101, 24.5 kb. The symbols have been described in Fig. II-3

at a frequency of about 10^{-2} . Assuming that the transfer frequency of the plasmid is the same in E. coli X R. meliloti JJ1 crosses, Tc^r insertions of R. meliloti, if they occurred, should have been detected. We did not observe them ($< 2 \times 10^{-9}$).

Other uses of suicide vehicles described in this study

Tn5 has unique sites for SalI and BamHI (Jorgensen et al. 1979). Cloning of DNA into these sites does not abolish the transposition proficiency of the resultant derivatives of Tn5 or its Nm^r -marker (Selvaraj and Iyer, unpublished results; see Chapter III). By cloning exogenous DNA into these sites it should be possible to transpose these sequences to different targets in R. meliloti (and other bacteria). We have constructed a derivative of Tn5 that carries the "mob" (oriT) region of the wide host-range plasmid, RK2 (Fig III-5). This transposon, Tn5-A1 was transposable in R. meliloti. Suicide vehicles carrying such modified transposons should facilitate further genetic manipulations of R. meliloti and related organisms. The suicide vehicles described in this study are sufficiently small (about 30 kb) to permit their in vitro manipulations.

Use of the suicide vehicles in other bacteria

We have found the plasmid pGS9 to be effective for all of the three strains of R. leguminosarum (128C53, 128C52 and 175G16). It is also effective in inducing

mutants of fast-, and slow-growing strains of R. japonicum (D.P.S. Verma, communication of unpublished results). The plasmid pGS18 has been used in inducing random mutants of Azotobacter chroococcum (R.L. Robson, personal communication). Thus the suicide vehicles constructed in this study are potentially useful for other bacteria as well.

Legend to Fig. III-5

Construction of a derivative of transposon Tn5 carrying the "mob" (oriT) region of P group plasmid RK2.

The linear molecules of pACYC184 generated by BamHI cleavage were treated with S1 nuclease (Boehringer Mannheim) to generate blunt-ended molecules. Using this DNA, Cm-resistant transformants of *E. coli* strain HB101 were obtained. 2% of the transformants were sensitive to Tc. Plasmid (pGS33) from one such clone was found to have suffered a deletion encompassing a region that includes the BamHI and Sall sites of pACYC184. A Tn5-carrying derivative of pGS33 was obtained (plasmid pGS38). The fragments of pGS28, generated by partial digestion by Sau3A, were cloned into the unique BamHI site of pGS38. A recombinant plasmid (pGS39) carrying the "mob" region of pGS28 was identified based on its mobilizability by a P group plasmid in *E. coli* X *E. coli* matings. pGS39 was also mobilizable from *E. coli* to *R. meliloti*. In the latter host, it induced insertions of Tn5-A1. A self-transmissible plasmid, similar to pGS9 (Fig. III-3), but carrying Tn5-A1 was constructed by transposing Tn5A1 from pGS39 onto pCU101 (see Fig. III-3 for description of pCU101). This latter plasmid, pGS63 was comparable to pGS9 in its ability to induce transposon insertion mutants in *R. meliloti* strains.

PRK290, the primary source of "mob" in this study, is a derivative of the P-group plasmid RK2 (Ditta *et al.*, 1980). P group plasmid pGS28 carrying the "mob" region of PRK290 was constructed as shown in the Fig. The size of the insert DNA ("mob") in pGS28 and Tn5A1 is 1.8 kb and 1.6 kb respectively.

CHAPTER III: TRANSPOSON Tn5 SPECIFIES STREPTOMYCIN
RESISTANCE IN *Rhizobium meliloti*

Transposon Tn5 is a well-studied representative of a class of movable genetic elements (Berg et al., 1975; Jorgensen et al., 1979). In E. coli, a host in which Tn5 has been intensively studied, its neo gene encodes resistance to neomycin, kanamycin and related aminoglycoside antibiotics (Davies et al., 1977). Its selectable resistance phenotype, its frequency of transposition and some degree of randomness in insertion specificity have made Tn5 the most widely used transposon in genetic manipulations in different bacteria (Berg, 1977; Mass et al., 1981; Kuner et al., 1981; Shaw and Berg, 1979; Beringer et al., 1978; Meade et al., 1982).

We have been interested in Tn5 because of its versatility as a genetic tool. A suicide delivery vehicle carrying it has been developed; using this vehicle, insertion mutants of R. meliloti could be isolated (Chapter II). While using this plasmid to induce Tn5-insertions in a streptomycin-sensitive (Sm^S) strain of R. meliloti, we noticed that all kanamycin-resistant (Km^R) colonies were also resistant to streptomycin (Sm). This initially raised a suspicion that Tn5 was inserting into a specific locus causing the Sm^R -phenotype. Further experiments have not supported this suspicion. Instead, they have led to the conclusion that Tn5 itself confers Sm -resistance to R. meliloti, but not to E. coli. We have identified and localized a gene that encodes this phenotype and have shown that this gene and neo are part

of a single transcriptional unit. We have also shown that this phenotype is useful as a genetic marker.

Tn5 is 5700 bp (base pairs) long with a central unique region of about 2700 bp flanked by two nearly identical inverted repeats designated as IS50L and IS50R (Auerswald et al., 1980; Berg et al., 1980; Jorgensen et al., 1979). The inverted repeats and a part of the central region of Tn5 have been sequenced (Auerswald et al., 1980; Rothstein and Reznikoff, 1981; Beck et al., 1982). RNA polymerase binding assays, analysis of transcripts of cloned pieces of DNA and genetic analysis of deletion, insertion and inversion mutants of Tn5 reveal that it has at least three promoters, one at end of each of the two repeats and one at the inside end of IS50L (see Fig. III-1; Rothstein et al., 1980; Johnson and Reznikoff, 1981). The two promoters that are distal to the central region are part of genes concerned with the transposition process itself and third promoter is utilized for transcription of the neo gene that encodes neomycin (kanamycin) phosphotransferase Type II, NPT II (Rothstein et al., 1980). Consistent with this data, a total of five Tn5-specified polypeptides are seen in E. coli. One of these is NPT II and the rest have been designated as Protein 1, 2, 3 and 4 (Rothstein et al., 1980; see Fig. III-1). The results presented in this Chapter suggest that in R. meliloti an additional gene product confers Sm-resistance.

Results

R. meliloti acquires streptomycin-resistance following the insertion of Tn5

Several independent Tn5-insertions derivatives of R. meliloti strain JJ1 were isolated with the aid of a Tn5-carrying plasmid suicide vehicle, pGS9. The construction of this plasmid and related ones have been described in detail (Chapter II). The plasmid pGS9 is a Tn5-carrying derivative of pCU101; the latter being a construct of the N-type conjugal transfer system of pCU1 and the replicon of the p15A-derivative, pACYC184 (Thatte and Iyer, 1983). Neither pCU101 nor the plasmid-free E. coli strain hosting the plasmid (strain WA803) carried a Sm-resistance marker. WA803/pGS9 is also Sm-sensitive. Matings between WA803/pGS9 and R. meliloti JJ1 followed by selection on Tryptone-Yeast extract (TY) agar containing Rif (rifampicin) and Km yielded transconjugants, over 95% of which are mutants caused by the insertion of Tn5. Replica-plating of 650 such independent Tn5-insertion derivatives on TY + Sm (50 ug/ml) showed that all of them were resistant to Sm whereas the parental R. meliloti was sensitive. These insertion mutants included a range of different auxotrophs (Chapter II; e.g. met::Tn5, gly::Tn5) indicating that the acquisition of Sm-resistance by R. meliloti could be associated with Tn5-insertions into several different sites in the R. meliloti genome. When

prototrophic revertants of some of the auxotrophs (met::Tn5; gly::Tn5; ilv::Tn5) were isolated (5 of each), all of them had lost both Km-resistance and Sm-resistance.

Acquisition of Tn5-associated streptomycin-resistance does not require an insertion event

The plasmid pHM4021 is an IncP-group plasmid into which Tn5 has been inserted (Meade et al., 1982). IncP-group plasmids can be transferred efficiently by conjugation into R. meliloti strains and they are stably maintained in this host (Meade and Signer, 1977; Ditta et al., 1980). When pHM4021 from E. coli W3115/pHM4021 was introduced into R. meliloti strain JJ1 and the transconjugants selected for Km-resistance, all such colonies (100) were also found to be Sm-resistant. Thus the ability of R. meliloti to express the Tn5-associated Sm-resistance does not require a transposition event to have occurred in R. meliloti and was independent of the location of Tn5 with respect to a plasmid or a chromosome.

Expression of Tn5-associated streptomycin-resistance in diverse fast-growing Rhizobium strains

Plasmid pHM4021 was introduced via conjugation into Rif^r-derivatives of a number of fast-growing natural isolates of R. meliloti and the R. leguminosarum sub-group that includes R. trifolii (see Table III-F). These Rhizobium strains were initially chosen on the basis of their sensitivity to Km and Sm. Table III-1 shows the

Sm-resistance of their pHM4021-carrying derivatives (selected as Km-resistant transconjugants) in comparison to their respective parental strains. With one exception, strain GS1020 (and its parent wild-type strain L15), all other Rhizobium strains that had acquired pHM4021 had also acquired Sm-resistance (Table III-1). pGM102, the parental plasmid of pHM4021, which is devoid of Tn5 did not confer Sm-resistance. There was host-specific variation in the magnitude of resistance to Sm in different pHM4021-carrying strains. In the case of L15 (and its Rif^r derivative, GS1020) the absence of Tn5-specified Sm-resistance was confirmed by replica-plating 100 separate pHM4021-carrying transconjugants on TY + Sm. Similar results were obtained with E. coli strains carrying Tn5 in which Sm-resistance was not expressed (data shown for only one strain) Table III-1.

The neo gene of Tn5 is insufficient for the expression of streptomycin-resistance in R. meliloti

Tn5 encodes an aminoglycoside phosphotransferase classified as neomycin phosphotransferase II (NPT II) which in addition to phosphorylating neomycin/kanamycin, is known to inactivate paramomycin, paramomine, neamine, ribostamine and butirosin (Umezawa, 1974). To determine whether the genetic region on Tn5 coding for this enzyme is responsible for the Sm-resistant phenotype (in

Table III-1

Expression of streptomycin-resistance in different Rhizobium strains carrying Tn5^a

Strain	Presence of Tn5 and its location	Minimal inhibitory concentration (MIC) of streptomycin (ug/ml)
<u>R. meliloti</u>		
JJ1	-	2
JJ1/pGM102	-	2
JJ1/pHM4021	+, plasmid	125
WS1002	+, chromosome	100
WS1101	-	2
GS1002	-	5
GS1002/pHM4021	+, plasmid	125
GS1003	-	10
GS1003/pHM4021	+, plasmid	125
GS1020	-	5
GS1020/pHM4021	+, plasmid	5
<u>R. leguminosarum</u>		
GS1400	-	5
GS1400/pHM4021	+, plasmid	50
GS1401	-	5
GS1401/pHM4021	+, plasmid	50
GS1402	-	2
GS1402/pHM4021	+, plasmid	40
<u>R. trifolii</u>		
GS1403	-	5
GS1403/pHM4021	+, plasmid	100
<u>E. coli</u>		
GS501	-	2
GS501/pHM4021	+, plasmid	2

^a The MIC was determined in TY broth after 2 days of incubation. The plasmid-carrying strains were constructed by mating of appropriate strains with E. coli W3115/pGM1102 or W3115/pHM4021. pHM1021 is a Tn5-carrying derivative of pGM102. WS1002 is a put::Tn5 derivative of R. meliloti strain JJ1, and WS1101 is a prototrophic revertant that is Km-sensitive. The pGM102-carrying derivatives were as sensitive to streptomycin as the parental strains. In all cases, the plasmid-carrying derivatives were found to maintain the plasmid stably.

Rhizobium), a plasmid construct carrying only the left part of Tn5 (see Fig. III-1) was used. This part, referred to as "BamHI-1", contains the entire sequence of the neo gene. A convenient source of this segment of Tn5 was pCU51, an IncN group plasmid generated by in vitro deletion of a larger plasmid carrying Tn5 (V. Thatte, unpublished construct; see Fig. III-2). N replicons like pCU51 are stable in R. meliloti, provided there is a continuous selection for their maintenance (Chapter II). Strain JJ1 of R. meliloti was transformed by pCU51 DNA and transformants were selected on TY + Km. Three hundred Km^r transformants were then screened for Sm-resistance and a randomly chosen one was used to determine the minimal inhibitory concentration (MIC) of Sm. The results (Table III-2) show that this region of Tn5 specifying Km-resistance to R. meliloti (and to E. coli) is unable to confer Sm-resistance.

The junction between "BamHI-L" and "BamHI-R"
is needed for the conferral of streptomycin-resistance

The procedure by which the BamHI-R region of Tn5 (see Fig. III-1) was introduced into R. meliloti is outlined in Fig. III-2. The largest BamHI fragment of pGS15 that contained "BamHI-R" was first cloned into the unique BamHI site of the non-conjugative plasmid pACYC177 (Chang and Cohen, 1978). The recombinant plasmid pGS20 (identified on the basis of its conjugal

Legend to Fig. III-1

Genetic and physical organization of Tn5. The length of Tn5 is taken as 5700 bp (Beck et al., 1982). This figure was drawn on the basis of information from the following papers: Beck et al. (1982); Berg 1980); Johnson et al. (1982) Jorgensen et al (1979); Rothstein et al. (1980) Rothstein and Reznikoff (1981). "BamHI-L" and BamHI-R" refer to the two regions of Tn5 separable by cleavage at the unique BamHI site, and "SalI-R" refers to the rightward SalI-segment. 'P' indicates a promoter and the solid wavy lines denote transcripts. The polypeptides known to be synthesized in *E. coli* are shown as solid coils. The jagged lines indicate translational reading frames. The dotted lines denote uncertainty about the termination site. NPTII is neomycin phosphotransferase Type II and No. 1 through No. 4 are proteins concerned with transposition. No. 3 and No. 4 are the truncated versions of No. 1 and No. 2 respectively. The nucleotide sequence of IS50L, IS50R and a part of the central region (up to the SalI site from the left boundary) is known (Auerswald et al., 1980; Rothstein and Reznikoff, 1981; Beck et al. 1982).

Legend to Fig. III-2

Plasmids carrying the entire Tn5 DNA or regions of it, designated as "BamHI-L" and "BamHI-R". These regions have been defined in Fig III-1. The wavy line in pGS36 denotes a physical rearrangement in the corresponding region of the plasmid. This reorganization of the plasmid does not abolish self-transmissibility, but reduces its efficiency. Plasmids pCU29 has been described previously (Thatte and Iyer, 1983;). pCU51 is an unpublished construct from the collection of Thatte and Iyer. pRK248 and pACYC177 are cloning vehicles derived from an IncPl-group plasmid and *E. coli* p15A plasmid respectively (Kahn et al., 1979; Chang and Cohen, 1978). B, BamHI; Bg, BglII; E, EcoRI; Sa, SalI; Cb, carbenicillin-resistance, Km, Kanamycin-resistance; Rep, replication region; Sm, streptomycin-resistance, Sp, spectinomycin-resistance; Tc, tetracycline-resistance; Tra, conjugal transfer.

self-transmissibility) was in turn used as the source of the BamHI-R fragment of Tn5. When pGS15 was directly used as the source, recombinant plasmids of desired phenotype (described below) could not be obtained. The larger BamHI fragment of pGS20 that contained the BamHI-R region was cloned into the unique BglII site of the non-conjugative wide host-range vector, pRK248 (Kahn et al., 1979). This was possible because of the complementarity of the protruding single-stranded DNA sequences (Roberts, 1983). Since the cloned fragment would contain all of the conjugal transfer region of pGS20, in addition to "BamHI-R", recombinant plasmids could be identified on the basis of the conjugal transfer of the pRK248-associated marker, tetracycline-resistance (Tc^r). Of three such plasmids obtained, all had undergone physical rearrangement at one of the boundaries of the insert DNA, as indicated in Fig. III-3 for one of the plasmids, pGS36. These plasmids also suffered a 200-fold reduction in their transfer frequency, relative to the transfer frequency of pGS20 in E. coli. However, they were transferable to R. meliloti, indicating that the host-range of the transfer system was not altered. Further experiments are needed to understand the basis of these observations. Since the BamHI-R region was unaltered in these plasmids, plasmid pGS36 was retained for introducing "BamHI-R" into R. meliloti. Plasmid, pGS36 was transferred to R. meliloti strain JJ1 by conjugation followed by selection for Tc^r.

Of ten independent transconjugants, none was resistant to Sm. The MIC of Sm for one of the derivatives is shown in Table III-2. Thus neither "BamHI-L" nor "BamHI-R" of Tn5 when cloned separately and introduced into R. meliloti could confer Sm-resistance. By introducing into R. meliloti these two segments of Tn5 as part of two separate replicons compatible with one another (pCU51 and pGS36), it was also observed that these two segments that span the entire Tn5 DNA (in trans) were unable to complement one another to restore the Sm-resistant phenotype (Table III-2). However, plasmid pGS15 that carried Tn5 DNA intact (i.e. "BamHI-L" and "BamHI-R" in cis) conferred Sm-resistance.

The inference that the junction of "BamHI-L" and "BamHI-R" is essential for the Sm-resistant phenotype was confirmed by showing that a mutant of Tn5 that carries an insert at the BamHI site did not confer Sm-resistance. Tn5-A1 is a derivative of Tn5 that contains the oriT sequence of the IncPl group plasmid RK2 (Guiney and Helinski, 1979) as an insert cloned at the unique BamHI site (see chapter II). When a derivative of pACYC184, a narrow host-range plasmid vector (Chang and Cohen, 1978), carrying Tn5-A1 (plasmid pGS39) was introduced into R. meliloti JJ1 by genetic transformation by a method described in Chapter II or by mobilization with pRK2013 (Ditta et al., 1980), insertion derivatives of R. meliloti JJ1 were obtained that were resistant to Km, but not to

Table III-2

Streptomycin-resistance levels in *R. meliloti* JJ1 carryingBamHI-segments of Tn5 DNA

Plasmid in the strain ^b	Region of Tn5 present on the plasmid ^a	Presence of <u>neo</u> (Km ^r) gene	Minimal inhibitory concentration ^c (μg/ml)	
			<u>streptomycin</u>	<u>kanamycin</u>
pCU51	"BamHI-L" region	+	2	> 200
PGS36	"BamHI-R" region	-	2	5
pCU51; pGS36	"BamHI-L" and "BamHI-R" in <u>trans</u>	+	2	> 200
pGS15	Complete Tn5	+	125	> 200

^a The "BamHI-L" and "BamHI-R" segments of Tn5 have been defined in Fig. III-1

^b The presence of individual plasmids in the strains was confirmed by the appropriate resistance phenotypes conferred by them. Their presence as separate entities was ascertained by the independent loss of phenotypes specified by each of them. The construction of these plasmids is outlined in Fig. III-2.

^c The MIC was determined after incubation for 2 days as described in Experimental Procedures. The MIC of Sm and Km for strain JJ1 were 2 μg/ml and 5 μg/ml, respectively. Incubation of the cultures for a further duration of 2 days did not show any difference in the MIC of Sm.

Sm. We therefore surmised that the putative gene conferring Sm-resistance (str) is located in the vicinity of the BamHI site of Tn5.

str is transcribed from the promoter of the neo gene

To identify the location of the promoter of the str gene, the central region of Tn5 containing the structural gene for NPTII, but not any promoters, was cloned downstream to a lac promoter in pRK404. pRK404 is a derivative of the IncPl group plasmid RK2 (G. Ditta, unpublished construct; see Fig. III-3). This plasmid carries a lacZ gene and insertions into this gene are identified on the basis of inability of the plasmid to complement an appropriate lac deletion mutant of E. coli (G. Ditta, unpublished results). The BglII fragment of Tn5 was cloned into the BamHI site of pRK404 as outlined in Fig. III-3. In one orientation (plasmid pGS48) in which the neo gene was proximal to the lac promoter, Km-resistance and Sm-resistance were expressed. In contrast, plasmid pGS49 that contained the insert DNA in the opposite orientation, did not express either of the resistance phenotypes in R. meliloti JIJ1 (Table III-3). Therefore it was reasoned that the central region of Tn5 does not have any "Rhizobium-specific" promoter that is utilized for the expression of str. Furthermore these results also show that the neo and str are expressed as a single transcriptional unit and that the latter is located

Legend to Fig. III-3

Expression of Km-resistance and Sm-resistance specified by Tn5 from the *lac* promoter of the IncP1-group vector, pRK404 (Ditta, unpublished construct). Plasmid pRK404 has the *lac* promoter and a gene encoding the *lacZ* peptide. Insertion of DNA into the *Bam*HI site (and other sites of the polylinker DNA sequence downstream to the *lac* promoter) inactivates the *lacZ* peptide and as a result Tc-resistant transformants of *E. coli* strain JM83 carrying such plasmids appear as white colonies on TYS plates containing 5-bromo-4-chloro-3-indolyl beta-D-galactoside. Colonies carrying intact plasmid DNA turn blue on these plates (Ditta, unpublished results, and this study). The direction of transcription is indicated by a solid arrow, and the alignment of the *neo* structural gene is indicated by a dotted arrow. "P" denotes a promoter. Other symbols have been described in Fig. III-2. The level of Km-resistance and Sm-resistance specified by the plasmids pGS48 and pGS49 in *R. meliloti* is shown in Table III-3

Table III-3

Expression of Sm-resistance in *R. meliloti* JJ1 carrying the central region of Tn5 fused to a lac promoter in plasmid pRK404

Plasmid in the strain ^a	Orientation of the Tn5 DNA relative to the <u>lac</u> promoter ^b	Minimal inhibitory concentration (ug/ml)	
		<u>streptomycin</u>	<u>kanamycin</u>
pGS 48	orientation I; <u>neo</u> coding region downstream to the promoter	> 100	> 150
pGS 49	orientation II; <u>neo</u> coding region inverted and distal to the promoter	2	5
pRK404	nil	2	5

^a The plasmids, pRK404, pGS48 and pGS49 are described in Fig.III-3 plasmids were stably maintained in this host.

^b See Fig.III-3 for a diagrammatic representation of the orientation of the Tn5 segment.

^c The MIC was determined after incubation for 2 days as described in Methods.

within the BglIII fragment of Tn5. Taken together with the data presented in the preceding section on Tn5-A1, it can be inferred that the neo promoter is used in an intact Tn5 for such expression.

Since it was evident that the N-terminal part of the str gene product would be upstream to the BamHI site on Tn5, we attempted to clone the "SalI-R" segment of Tn5 (Fig. III-1) that includes "BamHI-R" and about 380 bp upstream to the BamHI site.. This segment was cloned into the Sal site of pRK2501, an IncPl-group plasmid vector (Kahn et al., 1979). Since this site was within the tet gene of the plasmid, it was surmised that the inserted region of Tn5 would be 'read' from the tet promoter. If this occurred in an appropriate orientation, a fusion protein comprised of a part of tet protein and all or a part of str protein would be made (it was assumed that the str message is translated and therefore the str gene product is a protein). The method by which the "SalI-R" segment of Tn5 was cloned into pRK2501 is shown in Fig. III-4. The level of Sm-resistance in R. meliloti JJ1 carrying pRK2501 with the insert DNA in alternative orientations is shown in Table III-4. It is evident that the Sm-resistant phenotype is expressed, but at a lower level and only in one orientation (plasmid pGS46). The lower activity of the str gene product could be due to fusion to a part of the tet protein. (When the SalI-fragment of plasmid pGS48 (Fig. III-3) containing the

Legend to Fig. III-4

Fusion of the "SalI-R" region of Tn5 (see Fig III-1) to a part of the *tet* gene in pRK2501. The direction of transcription of the *tet* gene is indicated by an arrow. pRK2501 is a non-mobilizable plasmid vector derived from an IncP-group plasmid, RK2, (Kahn et al. 1979). The recombinant plasmids, pGS46 and pGS47 were mobilizable by a conjugative, Inc N- group plasmid, suggesting that the *mob* sequence of pGS15 is in the vicinity of "SalI-R". Ps, *Pst*I; S, *Sma*I. Other symbols have been described in Fig. III-2

Table III-4

Expression of Sm-resistance in *R. meliloti* JJ1 carrying the "SalI-R" region of Tn5 inserted in the middle of the tet (Tc^r) gene of pRK2501

Plasmid in the strain ^a	Orientation of the "SalI-R" segment	Minimal inhibitory concentration of streptomycin (μ g/ml) as determined after incubation for indicated duration ^b	
		2 days	4 days
pGS46	Orientation I; the unique region of Tn5 proximal to the <u>tet</u> promoter	10	50
pGS47	Orientation II (opposite to Orientation I)	2	2
pRK2501	nil	2	2

^a These plasmids are described in Fig. III-4 where the orientation of the "SalI-R" segment is shown. These plasmids were maintained stably by *R. meliloti* JJ1.

^b The MIC was determined as described in Methods.

neo gene was deleted in vitro by SallI-cleavage, a derivative of pGS48 in which "SallI-R" segment of Tn5 was fused to the lac promoter was obtained. The MIC of Sm for R. meliloti strain JJ1 carrying this plasmid pGS51, was >100 ug/ml. It is therefore concluded that the str gene product can function independent of the NPT II.

Tn5-associated streptomycin-resistance as a genetic marker in experiments with *R. meliloti*

The magnitude of Sm-resistance in Tn5-carrying derivatives of R. meliloti (Table III-1) suggested that this feature of Tn5 could be used as a genetic marker in experiments in R. meliloti. In matings of R. meliloti JJ1 with E. coli W3115/pHM4021, individual selections for any one of the markers of the plasmid (Tc^r , Km^r or Sm^r) yielded approximately the same number of transconjugants (10^{-4} per donor) which were cross-resistant to the other two antibiotics. In another series of experiments, Sm-sensitive natural isolates of R. meliloti IZ450 and Rhodesia 304 were mated with E. coli WA803/pGS9. Tn5-insertion derivatives of the R. meliloti strains could be isolated efficiently on TY + Sm. Since both the donor strain and the recipient strains were Sm-sensitive the use of a separate counter-selective antibiotic (such as Rif) was not necessary.

Discussion

We have shown that Tn5 encodes Sm-resistance which is expressed in different strains and species of Rhizobium. The streptomycin-resistance marker of Tn5 is not expressed in E. coli, K. pneumoniae (Gill, unpublished results) and, presumably, in other closely related Gram-negative bacteria. Recently Mazodier et al. (1982) have shown that a strain of Methylobacterium organophilum carrying Tn5 is also resistant to Sm and that Sm was phosphorylated to a significant level in such a strain. However, these authors also showed that an IncPl group plasmid that specifies neomycin phosphotransferase does not confer Sm-resistance in M. organophilum. Since the neomycin phosphotransferase specified by this plasmid is different (classified as Type I), these authors suggested as one of the possibilities, that the expression of Sm-resistance in M. organophilum could be related to the different (Type II) neomycin phosphotransferase specified by Tn5. As this Type II enzyme is known to inactivate a number of different aminoglycoside antibiotics (Umezawa, 1974), it seemed possible that some host-specific modifications of the Type II enzyme results in the extension of its activity to include streptomycin as a substrate. However, in this study we have shown that the presence of NPT II in R. meliloti cells was insufficient to confer Sm-resistance.

Beck et al. (1982) have sequenced approximately

one-half of the central region of Tn5 and have confirmed the location of the neo promoter at the inside end of IS50L. They have also identified the location of translational start and stop signals of the neo message transcribed from this region. The length of the intervening sequence between these signals is in agreement with the molecular weight of the NPT II protein (Matsushashi et al., 1976; Rothstein et al., 1980). Plasmid pCU51 carries this entire sequence and about 700 bp in addition, downstream to the C-terminus of NPT II enzyme (Fig. III-2 and see Fig. III-1 for Tn5 map). Since in R. meliloti, this plasmid specified Km-resistance but not Sm-resistance, we are able to conclude that the neo gene itself is insufficient for the expression of Sm-resistant phenotype.

Our observation that insertion of exogenous DNA at the unique BamHI site of Tn5 (downstream to neo) abolished the expression of Sm-resistance, but not that of Km-resistance raised a possibility that the genetic region on Tn5 specifying Sm-resistance (Sm^r), is downstream to neo in a single transcriptional unit. This possibility seemed more likely, since promoters had not been identified in the central region of Tn5 (Rothstein et al., 1980; Johnson and Reznikoff, 1981) and Berg (1980) had provided genetic evidence for the extension of transcription initiated at the neo promoter well beyond the central region. This supposition has been confirmed

by cloning the BglIII-generated segment of Tn5 in two orientations downstream to a lac promoter (Table III-3).

Recent data on the DNA sequence of a part of the central region encompassing the neo gene (Beck *et al.*, 1982) has revealed the following features. There are four reading frames in the message transcribed from the neo promoter, and of these the one that is immediately downstream to the promoter is the neo message (see Fig. III-1); two terminate within the "BamHI-L" region of Tn5. These cannot be responsible for Sm-resistance, since pCU51 that carries this region did not specify Sm-resistance. There is another translational start signal at position 2366 which is 318 bases upstream to the unique SallI site of Tn5, and there is no termination signal in this reading frame that has been sequenced up to this site. Our observation that the "SallI-R" segment of Tn5 (see Fig. III-1) cloned within the tet gene of pRK2501 (Kahn *et al.*, 1979) conferred a low but significant level of Sm-resistance (Table III-4) suggests that the functional domain of the str gene is present in the "SallI-R" segment of Tn5. This was confirmed by fusing the "SallI-R" segment downstream to a lac promoter. Therefore it is likely that the open reading frame starting at 2366 is the putative str message. If the translational reading frame starting at position 2366 is the str message, replacement of about 100 amino acids of the str gene product with the N-terminal sequence of the tet protein (in plasmid pGS46)

does not appear to abolish the activity of the str gene product but only to reduce it. Since the plasmid pGS51 carrying "Sall-R" downstream to a lac promoter conferred normal level of Sm-resistance, it is also likely that the ATG codon at position 2672 is the translational start signal for the str message.

The absence of expression of the str gene in E. coli is in agreement with the observation that in the E. coli maxicell system, only five polypeptides are detected which are accounted for by the transposition-proteins encoded by the inverted repeats, and the NPT II enzyme (Rothstein et al., 1980). Since in E. coli the mRNA synthesized from the neo promoter traverses across the str gene in the central region of Tn5 (Berg, 1980), the lack of str-expression in this host must be due to a post-transcriptional control. One of the isolates of R. meliloti (strain L15) that did not express Sm-resistant phenotype might be similar to E. coli in this respect. This variant isolate of R. meliloti indicates that it will be possible to study the contribution of host factors in the determination of the Sm-resistance. The observation that Tn5 is genetically more complex and that the host cell modulates its phenotype introduces an additional dimension to the understanding of the evolutionary role of transposable elements.

Tn5-specified Sm-resistance is useful as a selectable marker for Tn5 mutagenesis of Rhizobium strains

that are naturally resistant to kanamycin/neomycin and for which selection for resistance to these antibiotics is not feasible. Even for Rhizobium strains that are Km-sensitive, the use of Sm-resistance as the selective marker has a practical advantage because Sm-sensitive E. coli donors of Tn5 are more effectively eliminated by Sm than they are by other counter-selective antibiotics such as rifampicin. We have effectively used the Sm-resistance marker of Tn5 in transposon mutagenesis of R. meliloti (Chapter IV). The BamHI site of str in Tn5 can also be used for insertional inactivation in molecular cloning while using plasmids carrying all or the appropriate part of Tn5. { Recently, and after the completion of the preparation of this Chapter, Putnoky et al. (1983) have published their similar observations; these authors have also arrived at similar conclusions reported in this Chapter. }

CHAPTER IV: INSERTION MUTANTS OF *Rhizobium meliloti*

As evident from the literature review, random symbiotic mutants of R. meliloti are poorly represented in the collections of mutants of different R. phaseoli strains (Table 3, pp43-45). Some of these mutations were spontaneous and others were derived after mutagenesis with chemicals such as NTG or EMS. Genetic analysis of these mutants is relatively difficult, since the mutated phenotypes might not provide a handle in further experiments. There is also some uncertainty regarding the presence of other unidentified secondary mutations in these mutants. In contrast, there are distinct advantages in using transposons as mutagens as reviewed by Kleckner et al. (1977), and by Berg and Berg (1983), and these are summarized below.

In general, transposons induce single insertion mutations that are conveniently selected and identified by virtue of a transposon-associated antibiotic-resistance phenotype. The well-studied transposons, Tn5, Tn9 and ~~Tn10~~ can insert into man9 sites, despite some hotspots for insertions (see Berg and Berg, 1980). The drug-resistant phenotype associated with a mutation can serve as a marker in all genetic studies. The complete DNA sequence of some transposons is known and for many others partial sequence analysis and a detailed restriction map is available. Therefore they are also useful in the physical analyses of mutations. Insertion of transposons can cause polar mutations which is a very useful feature in identifying

units of genetic expression. Transposons also provide a region of portable homology in further manipulations such as chromosome or plasmid mobilization. In addition, transposons can be put to a variety of other uses such as generation of deletions, gene duplications and gene fusions. The versatility of transposons as genetic tools is further augmented by in vitro methods of manipulating DNA.

Meade et al. (1982) and Forrai et al. (1983) isolated random transposon-insertion mutants of R. meliloti strains Rm1021 and Rm41 respectively. In these studies, the plasmid pJB4JI, a suicide vehicle containing both the insertion elements Tn5 and Mu were used. Consequently, the majority of the mutants of interest had DNA sequences of Mu as well as that of Tn5. This complicated further genetic analysis. We chose to assemble a random collection of insertion mutants of R. meliloti strains, using the suicide vehicles described in Chapter II. The advantages of using these vehicles have been discussed in Chapter II. The R. meliloti strain JJ1 and its derivative were chosen for the reasons outlined below.

R. meliloti strain JJ1 is a spontaneous Rif-resistant derivative of the natural isolate IZ450. In a screening of many R. meliloti strains that included IZ450, the latter was found to be sensitive to a number of antibiotics including carbenicillin (Cb), chloramphenicol

(Cm), gentamycin (Gm), kanamycin (Km), nalidixic acid (Nal), streptomycin (Sm), and spectinomycin (Sp) (J. Johnson, unpublished results; Selvaraj and Iyer, unpublished results). This is a useful feature in many genetic experiments. In addition, this strain is transformable (Chapter I) and is amenable to mutagenesis with the suicide plasmids described in Chapter II. Another useful feature is the presence in them of only two indigenous plasmids (approximately 350 Mdal and 140 Mdal in size), unlike some strains of R. meliloti that harbour as many as six plasmids (see Behki et al., 1983; Puhler et al., 1982). Initial experiments on mutagenesis were performed with strain JJ1 until a derivative of it lacking the smaller plasmid was available (Adachi et al., 1983). This latter strain, JJ1C10 is as proficient at symbiotic nitrogen-fixation as its parent (Adachi et al., 1983). This strain is intensively studied in our laboratory and the majority of insertion mutants have been obtained with it.

The description of the mutants that constitutes this Chapter is organized as follows:

(a) Isolation of Tn5 and Tn9 insertion mutants of JJ1C10. In this section the frequency of auxotrophic and symbiotic mutants, and their characteristics are described.

(b) spontaneous auxotrophic and symbiotic mutants of strain JJ1C10; their occurrence and significance.

(c) Tn5 and Tn9 insertion mutants of strain JJ1; differences in the mutability of strains JJ1 and JJ1C10; spontaneous auxotrophic mutants of JJ1 and the comparison and significance of the differences in the pattern of auxotrophic mutants of the two strains.

Results

Tn5 and Tn9 insertion mutants of strain JJ1C10

A total of 9500 Tn5-insertion derivatives were isolated, employing the protocol described in Chapter II. Following the observation that Tn5 specified Sm-resistance in Rhizobium (Chapter III), a selection for Sm-resistance (instead of Nm-resistance) was employed, since it was also effective in counterselecting the donors. Tn9 insertion derivatives (a total of 560) were obtained using the conjugative suicide vehicle pGS27 (see Chapter II). From each of the matings of JJ1C10 with an E. coli donor carrying an appropriate suicide vehicle, only 20-30 transconjugants were retained for further studies, to reduce the inclusion of sibling colonies. The random mutants thus obtained were initially screened for the co-inheritance of other markers associated with the suicide vehicles, Cm-resistance in the case of pGS9 (Tn5)-derived mutants, and Cb-resistance for pGS27 (Tn9)-derived mutants [see Fig II-3, 4 (page 100) for a map of pGS9 and pGS27] 99% of the mutants did not show

co-inheritance of these phenotypes. These were screened for auxotrophy, and only the prototrophic clones were screened on alfalfa seedlings.

Auxotrophs of strain JJ1C10 induced by Tn5 and Tn9

The auxotrophs were sought for the following reasons. The distribution of different phenotypic classes would indicate whether or not the collection of mutants represent a random insertion of the transposon. In addition, the auxotrophs thus generated might be useful in studying the possible effect of alterations in vegetative metabolism on symbiotic functions. The reviews by Denarie et al. (1976), Schwinghamer (1977) and Kuykendall (1980) indicate that there are a number of auxotrophic mutants, (derived by conventional mutagenesis procedures) showing defective symbiosis. The isolation of new auxotrophic mutants that have not so far been reported (e.g. Asparagine⁻, Thr⁻, Bio⁻) will also enable the filling of gaps in the existing genome maps of R. meliloti (Meade and Signer, 1977; Kondorosi et al., 1977; Casadesus and Olivares, 1979a).

A total of 223 Tn5-induced auxotrophs were identified and the nutritional requirements of 101 of these were determined. These are listed in Table IV-1. Despite the large number of these mutants and the different phenotypic classes that were found, some mutants types were not encountered (for example, Lys⁻).

Auxotrophic mutants of strain JJ1C10 induced by Tn6

Strain	Phenotype ^a	Total number of mutants with stated phenotype ^b
WS 63, 64, 69, 70, 80-91	Ade ⁻	8
WS 25-29	Aro ⁻	5
WS 42, 98	Ala ⁻	2
WS 61, 62, 66, 74, 87	Asn ⁻	5
WS 96, 97	Biotin ⁻	2
WS 43, 44, 77-79, 81, 85, 86, 99, 101	Gln ⁻	10
WS 80, 82, 83, 93	Gln ⁻ /Asn ^{-c}	4
WS 58-60	Gly ⁻	3
WS162	Ser ⁻ /Gly ⁻	1
WS 45-54	His ⁻	10
WS 55-57	Ilv ⁻	3
WS 41	Leu ⁻	1
WS 13-23, 68, 71, 92, 95	Met ⁻	16
WS 24	Phe ⁻	1
WS40	PyrA ⁻	1
WS 75, 76	Ser ⁻	2
WS 1-12, WS 67	Sulphur ^{-d}	13
WS 72, 73	Thi ⁻	2
WS 30-33	Trp ⁻	4
WS 34-39, 65, 84	Ura ⁻	8

^a The abbreviations are as explained in the List of abbreviations (p.xi). Mutants requiring any of the following supplements were not found: Arg, Asp, Cys, Glt, Gua, Ile, Lys, Nic, Pan, Pro, Thr, Tyr, Thy

^b These are all independent mutants (total: 101 mutants); in addition, there were 122 auxotrophs with unknown nutritional deficiency

^c Indicates growth on media containing either of the supplements

^d Growth facilitated by addition of cysteine, methionine or sodium thiosulphate

Furthermore, the relative proportion of the mutants that were found is uneven. Such a distribution suggests that Tn5 does not insert at random into the genome of R. meliloti. Similar observations were reported by Meade et al. (1982) who isolated 20 auxotrophs of strain Rm1021, using pJB4JI as the suicide vehicle. In their collection mutants affected in sulphur metabolism (Sulphur⁻) had the largest representation (30%). Forrai et al. (1983) also isolated pJB4JI-induced mutants of strain Rm41. Among 20 auxotrophs, 30% were Sulphur⁻, and the rest showed different requirements. The proportion of sulphur mutants of strain JJ1C10 is 13% (Table IV-1)

The collection of Forrai et al. (1983) also included 2 Lys⁻ mutants which are not present in our collection or in other reported collections. Since information concerning the question of whether the mutants of strains Rm1021 and Rm41 are unambiguously caused by Tn5-insertion mutants is incomplete, one cannot extend the comparison further. The Tn5-insertion mutants of strain JJ1C10 reported here also include Asparagine⁻ mutants that have not been reported so far.

Since Tn5 was found not to yield certain auxotrophic mutants in R. meliloti (e.g. Thr⁻, Pro⁻, Asp⁻), it was concluded that the use of Tn5 alone would not saturate the genome. Therefore, we began isolating Tn9-insertion mutants of JJ1C10 to compare the range and distribution of different auxotrophs. Of the 560 independent derivatives

screened, 26 were auxotrophic. The specific nutritional deficiency was determined for 13 of them. The mutants are listed in Table IV-2. An extensive comparison to the Tn5-insertion mutants is not possible until a larger number of Tn9-insertion mutants are isolated. A comparison of the available mutants suggests that mutants not found among those isolated by Tn5-mutagenesis are present in the Tn9-induced collection (e.g. a mutant growing on plates supplemented with alanine or a mixture of vitamins). These results are encouraging, since they suggest that with the use of this transposon, it might be possible to isolate certain symbiotic mutants that cannot be isolated with the use of Tn5 alone.

The mutants affected in sulphur metabolism are of some importance. In E. coli, certain Heme⁻ mutants are phenotypically similar to the sulphur-requiring mutants listed in Table IV-1 and IV-2 (McConville and Charles, 1979). Rhizobium cells contribute the heme component of the nodule-specific, symbiotically important protein leghemoglobin (Dilworth and Appleby, 1979). The investigation of the genetics of heme biosynthesis in R. meliloti has just begun (Leong et al. 1982), and the sulphur⁻ mutants reported in this Chapter should provide a useful handle in isolating additional mutants affected in heme biosynthesis. Preliminary studies suggest that some of the sulphur⁻ mutants induce white Fix⁻ nodules (I.K. Hooper, personal communication).

Auxotrophic mutants of strain JJ1C10 induced by Tn9

Strain	Phenotype ^a	Total number of mutants with stated phenotype ^b
WS 208	Ade ⁻	1
WS212	Aro ⁻	1
WS 206	Asn ⁻	1
WS 211	Biotin ⁻	1
WS 203-205	Gln ⁻	3
WS 200, 207	His ⁻	2
WS 201, 202, 209	Sulphur ^{-d}	3
WS 210	Vitamins ⁻ /Ala ^{-c}	1

^aThe abbreviations have been explained in the List of Abbreviations (p xi).

^bA total of 588 independent Tn9-insertion derivatives were screened. In addition to these 13 auxotrophs, there were 13 mutants with known nutritional deficiency.

^cGrowth facilitated by supplementing medium with a mixture of biotin, pantothenate, nicotinic acid, pyridoxine HCl, or alanine.

^dAs described in Table IV-1

Symbiotic mutants

The identification, classification and description of Sym⁻ mutants are included in this section. With reference to the classification, the terms Nod⁻ and Fix⁻ are defined as follows. A Nod⁻ mutant is one that does not induce any nodule-like structures, and a Fix⁻ mutant is one that does not support the growth of host plants in a nitrogen-free plant growth medium despite the induction of nodules or any nodule-like structures. The number of nodules and their morphology were also variable among Fix⁻ mutants (described below) necessitating further classification. Thus the Fix⁻ mutants could be sub-divided into the following categories based on the number of nodules: "sparse nodulation" type, inciting fewer nodules than the parental Fix⁺ strain; "profuse-nodulation" type, inducing a larger number of nodules relative to the parental strain, and the "intermediate" type. Independent of these categories, the Fix⁻ mutants could also be classified on the basis of nodule morphology (described later). However, there was no indication that these two phenotypes of nodule numbers and nodule morphology could be correlated to one another.

Among 8318 random prototrophic Tn5-insertions that were tested 255 potential Fix⁻ and 5 Nod⁻ mutants were identified. Among 155 randomly chosen potential Fix⁻ mutants, 9 were Cm^r, 8 were partially auxotrophic, 33 proved to be Fix⁺ and 20 were partially defective in

fixation as indicated by the appearance of inoculated plants (see Experimental procedures for details) The remainder were confirmed to be prototrophic Fix mutants (Table IV-3). Although, in the plant assays we had attempted to omit the Cm-resistant and auxotrophic clones, further tests showed that some had been accidentally included.

The 83 Fix⁻ Tn5-induced mutants were classified according to the morphology of nodules induced by them in quadruplicate plant assays (Table IV-3). From such a primary screening, randomly chosen mutants were re-tested on 4 or 20 plants. Some strains were found to yield different types of nodules other than, or including the ones produced in the primary screening. In such cases, the mutants were placed in a separate category, based on later tests. These mutants are identified in Table IV-3. Perhaps such variability is due to persisting genetic differences in the individual plants of the given cultivar of alfalfa. The polyploid genome of alfalfa can also contribute to this variability. Since the differences in the nodule morphology induced by some Tn5-insertion derivatives are consistent (see Table IV-3), they can be attributed to mutations in the bacterial strain, presumably induced by Tn5.

Among the Tn5-induced Sym mutants 5 were Nod⁻. Two of these (strains 4D3 and 4F9) did not induce any visible changes in alfalfa roots. These two mutants are similar

^aThese are from a collection of 255 potential Fix⁻ mutants of which 155 were re-screened in quadruplicate plant assays. The strains that were auxotrophic or resistant to Cm are excluded.

^bThe underlined strains produced 10 nodules per plant; others induced from 11 to 35 nodules. (see Fig IV-1 for a distribution)

^cThe morphology was reproducible when tested on 20 plants

^dThe nodule morphology was different in the primary screening. The assignment of category is based on a latter screening on 4 plants

^eAs above, the latter screening involved 40 plants.

^fIn quadruplicate assays, there was heterogeneity in a single plant or among individual plants

^gnodule morphology not recorded

Table IV-3

Fix⁻ mutants of strain JJ1C10 isolated by
insertion mutagenesis with Tn5^a

Strain ^b	Characteristics of the nodules
S8 U2	Normal (Type A)
<u>N1^c</u> , N9, N16 R15, R23, S13, S14, S19	Small, round (Type B)
N4, <u>N23</u>	
<u>R4</u> , <u>R11^c</u> , R14 S16, S19 U1	Small "cauliflower-like" (Type C)
N11, N5, N27, N31 R2, R5 S3, S15, S25 U1, U4 ^d , U11, U17	Elongated (Type D)
<u>N3^e</u> , N6, <u>N8^c</u> , N17, N20, N22 Q1 ^c , Q2, Q4, Q8, Q9 ^c , Q10, Q12-Q15, Q18 R6, R9, R12, R16, R17, R20 ^c , R21 S1, S7, S9, S11, S20, S23, S24, S26, S31-S33, S43 U2, U3, U5, U10, U12, U28	Mixed type ^f (A + B + C + D); variable combinations
R24 S6, S21	Unassigned ^g

to the non-reactive mutants of R. meliloti strains Rml021 (Hirsch et al. 1982) and Rm41 (Forrai et al. 1983).

Strain 4D3 was resistant to Cm, indicating the presence of some sequences of the suicide vehicle, pGS9 in addition to transposon Tn5. Mutants R3, R8 and Q22 induced green spots that at best developed into green or brown lumps rather than nodules. Occassionally parts of the infected roots appeared to be swollen. These mutants are probably similar to the "reactive" Nod⁻ mutants of strain Rml021 described by Hirsch et al. (1982). Since a preliminary screening of the Fix⁻ mutants on alfalfa plants indicated variation in the number of nodules among plants infected with different Fix⁻ mutants, an attempt was made to classify the mutants based on the total number of nodules per plant. As shown in Fig. IV-1, Fix⁻ mutants, as a group, displayed a larger variation in nodule numbers in comparison to the parental strain. The majority of the mutants induced more nodules than strain JJ1C10. Fix⁻ mutants that nodulated even more sparsely than the wild-type seemed to be a distinct category. To obtain an assurance that this category is real, in a separate experiment each representative strain of this category was inoculated on 40 plants; as the controls, Fix⁻ mutants randomly chosen from the "profuse nodulation" category and strain JJ1C10 were used. The results shown in Table IV-4 indicate that the Fix⁻ mutants N1, N8 and R11 induce fewer nodules than other Fix⁻ mutants, and strain JJ1C10.

Fig. IV-1:

Variation in the number of nodules induced by the Fix⁻ mutants of *R. meliloti*, JJ1C10.

A total of 77 Fix⁻ mutants chosen at random were inoculated on alfalfa seedlings placed within tubes containing N-free Thornton agar. Each mutant was assayed on 4 seedlings (2 seedlings/tube). The nodule counts were recorded after 4 weeks of incubation under plant growth conditions. In the case of strain JJ1C10, 100 individual colonies were assayed as described above.

Fig. IV-1

Variation in the number of nodules induced by the Fix⁻ mutants of *R. meliloti* JJ1010.

A total of 77 Fix⁻ mutants chosen at random were inoculated on alfalfa seedlings, placed within tubes containing N-free Thornton agar. Each mutant was assayed on 4 seedlings (2 seedlings/tube). The nodule counts were recorded after 4 weeks of incubation under plant growth conditions. In the case of strain JJ1010, 100 individual colonies were assayed as described above.

The mean number of nodules incited by them is significantly different from that in the case of the controls. An intermediate type of mutant (strain N3) was also identified (see Table IV-4).

Twenty-six potential Fix^- mutants were identified among 500 prototrophic Tn9-insertion derivatives of strain JJ1C10. Nod^- mutants were not found. These Fix^- mutants have not been compared to the mutants described above. In summary, using the suicide vehicles pGS9 (Tn5) and pGS27 (Tn9), we have isolated 5 Nod^- mutants, 102 Fix^- mutants and 126 potential Fix^- mutants. In addition 20 Fix^+ mutants were also identified. The Fix^- and Fix^+ phenotype of these mutants need to be tested by acetylene reduction assays.

Spontaneous auxotrophic and Sym^- mutants of JJ1C10

The proportion of auxotrophic and Sym^- mutants among Tn5-insertion derivatives of strain JJ1C10 used in this study was 2-2.5%, in contrast to a lower yield of such mutants (0.3-0.4%) in the case of both strains Rm1021 (Meade et al., 1982) and Rm41 (Forrai et al., 1983). This might be due to the efficiency of the suicide vehicles used in this study, and/or a reflection of strain specific differences. We wished to examine an alternative possibility, whether the strain JJ1C10 harbours an indigenous Insertion Sequences element that induces spontaneous mutations in the strain. 1000 colonies of

Table IV-4

Number of nodules induced by some Fix⁻ mutants of JJ1C10^a

Strain	Number of nodules (mean $\pm$ SE) ^b	95% confidence limits of the average nodule number ^c	Comments ^d
JJ1C10	9 $\pm$ 1	8 - 11	"wild-type" strain
Q1	24 $\pm$ 1 ^f	21 - 26	"Profuse nodulation"
R20	20 $\pm$ 2 ^f	15 - 24	as above
Q9	23 $\pm$ 2 ^f	19 - 27	as above
R11	1 ^e	-	Nod ⁻ ?
N1	5 $\pm$ 1 ^f	4 - 5	"sparse nodulation"
N8	1 ^f	1 - 2	as above
N3	12 $\pm$ 1 ^g	9 - 15	"Intermediate"
R8	21 $\pm$ 2 ^f	16 - 26	"Profuse nodulation"

^aBased on nodule counts from 40 plants; 2 plants were grown per tube containing N-free Thornton medium. Nodule counts were recorded after 4 weeks

^bPer 2 plants; SE less than 1 not indicated

^cBased on Students's t-distribution

^dComments in relation to the parental strain, JJ1C10

^e3/20 plants showed nodule-like structures

^fSignificantly different from JJ1C10-inoculated plants at $P < 0.001$

^gSignificantly different from JJ1C10-inoculated plants at $P < 0.025$

JJ1C10 were tested for spontaneous auxotrophy and 900 prototrophic colonies were assayed for the Sym phenotype. One Phe⁻, 1 Sulphur⁻, and 2 Fix⁻ mutants were obtained. No Nod⁻ mutants were obtained (Table IV-5). The frequent incidence of these mutations (about 10⁻³) leads to the suspicion that strain JJ1C10 carries some IS element(s) that induce these mutations. However, these presumptive IS element(s) alone cannot account for the large number of Sym⁻ and auxotrophic mutants among the Tn5- or Tn9-mutagenized derivatives, since they together represent about 4-5% of the total number of insertion derivatives.

Insertion mutants of strain JJ1

A small number of Tn5-induced derivatives of strain JJ1 were isolated. Of a total of 650 prototrophic Tn5-insertion derivatives screened on alfalfa, only one was Sym⁻. This mutant induced Fix⁻ nodules. This frequency of Sym⁻ mutants is much lower than that induced in JJ1C10. However, auxotrophs were found among the Tn5-insertion derivatives at a frequency of 3%, which is comparable to that obtained with JJ1C10. The only known difference between JJ1 and JJ1C10 is the loss of a 140 Mdal plasmid in the latter. The reason for this difference in the mutability of the two strains is unknown. As one possible reason, one might speculate that some regions specifying Sym functions (other than the nif HDK) are reiterated on the 140 Mdal plasmid. (the 140

Table IV-5

Spontaneous auxotrophic mutants of derivatives of
R. meliloti strain JJ1^a

Parental strain ^b	Phenotype ^c		
	Ade ⁻	Sulphur ⁻	Phe ⁻
JJ1	MW101, 102, and MW 103	MW104 ⁻	N.D
JJ1C10	N.D	MW105	MW106
KN1007	MW107	N.D	N.D

N.D = Not detected

^a 1000 colonies of each strain were screened.

^b JJ1 carries two indigenous plasmids, pKN1 (350 Mdal) and pTA2 (140 Mdal), from which JJ1C10 was derived by curing pTA2. pKN1007 is a derivative of JJ1C10 into which pTA2 has been re-introduced (Adachi *et al.*, 1983).

^c Mutants with phenotypes not listed here were not found.

Mdal does not hybridize to a Kp nifHDK probe (Adachi et al., 1983)). Puhler et al. (1982) have presented evidence for the presence of Kp nifHDK-like sequences in two different genetic elements within some R. meliloti strains. There is also some evidence for a reiteration of nifHDK genes in R. phaseoli (Quinto et al., 1982). Alternatively, the insertion specificity of Tn5 could be different in strain JJ1, or there might be some unknown differences between JJ1 and JJ1C10, other than the loss of an indigenous plasmid in the latter.

An examination of the Tn5-induced nutritional deficiency among 28 auxotrophic mutants of JJ1 revealed that the mutant pattern is completely different to that of JJ1C10 (Table IV-6). Thus the proportion of Ade⁻ auxotrophs was 8% for JJ1C10 and 71% for JJ1. We suspect that the latter mutants were not induced by Tn5 itself, since all of 11 independent Ade⁻ mutants screened gave rise to spontaneous revertants that remained resistant to Nm and Sm. In contrast, other randomly chosen mutants of JJ1 and JJ1C10, including one Ade⁻ mutant of JJ1C10 gave rise to spontaneous Nm^S Sm^S prototrophs. A screening for the presence of spontaneous auxotrophs among JJ1 colonies showed that 3 were Ade⁻ and one was Sulphur⁻. Thus some of the Ade⁻ mutants, initially construed to be Tn5-induced mutants, can be accounted for by assuming the presence of an IS element in strain JJ1 that induces Ade⁻ mutations. However, their frequency (0.3%) does not explain the

Table IV-6

Auxotrophic mutants of JJ1 induced by Tn5^c

Strain	Phenotype ^a	Total number of mutants with stated phenotype ^b
WS1001, 1004, 1006, 1007, 1010, 1012-1019, 1022- 1027	Ade ⁻	20
WS1003	Aro ⁻	1
WS1020, 1021	Gln ⁻	2
WS1005	Gly ⁻	1
WS1008, 1011	Ilv ⁻	2
WS1002	Met ⁻	1
WS1028	Ser ⁻	1

a, b

see respective footnotes in Table IV-2

c

28 auxotrophs were found among 1000 Tn5-insertion derivatives
of the strain.

occurrence of Ade⁻ mutants at a frequency of 2% in a Tn5-mutagenized population of JJ1.

Interestingly, spontaneous Ade⁻ mutants do not occur in JJ1C10 (<0.1%), but occur at a frequency of 0.1% in a population of strain KN1007 (Adachi et al. 1983), which is a JJ1C10--derivative into which the 140 Mdal plasmid has been re-introduced (see Table IV-5). This circumstantial evidence leads to a suspicion that the 140 Mdal plasmid of strain JJ1 carries an IS element that inserts preferentially into ade gene(s). The reasons for the augmentation of Ade⁻ mutants among Tn5-insertion derivatives of JJ1 are not clear. We initially suspected enhancement of the insertion efficiency of the presumptive IS element of JJ1. This can be ruled out since a collection of Tn5-induced auxotrophs of strain KN1007 contained only 1 Ade⁻ mutant among 10 auxotrophs. Thus for reasons that are not clear, insertion mutagenesis of strain JJ1 with Tn5 yields a limited range of auxotrophs and fewer Sym⁻ mutants. Since Tn9-induced auxotrophs of JJ1 (6 in total) contain only 1 Ade⁻ mutant and the remainder have different nutritional deficiencies (see Table IV-7), whatever the explanation for the occurrence of a large number of Ade⁻ mutants among Tn5-insertion derivatives, it must take into consideration that the observation is specific to Tn5-mutagenesis of strain JJ1. Further experiments are needed to clarify these observations.

Table IV-7

Auxotrophic mutants of JJ1 induced by Tn9

Strain	Phenotype ^a	Total number of mutants with stated phenotype ^b
WS301	Ade ⁻	1
WS300	Gln ⁻ /Asn ^{-c}	1
WS302-304	Sulphur ^{-d}	3
WS305	His ⁻	1

^aSee respective footnotes in Table IV-2

^b839 independent Tn9-insertions were screened; in addition to these auxotrophs, there were 13 mutants with unknown nutritional deficiency.

^{c,d}See respective footnotes in Table IV-1

Discussion

We have described the isolation and assembly of a large number of insertion mutants of strain JJ1C10, that should facilitate further studies on the organization of sym genes, and the involvement of vegetative genes in symbiosis. The Sym mutants form the largest collection of random insertion mutants of R. meliloti. The scant representation of Nod mutants among this large collection of Sym mutants- 0.06% of the total Tn5-insertion derivatives, in contrast to 2-2.5% of Fix⁻ mutants suggests that the number of genes involved in the early steps of symbiosis might be small. Meade et al. (1982) and Forrai et al. (1983) have reported similar results. Recently, Downie et al. (1983a, b) have shown that a part of a 10 Kb region of the Sym plasmid of a R. leguminosarum strain contains the genetic determinants for nodulation and host-specificity. Assuming that there are no chromosomal nod genes, these results suggest that a smaller number of genes are involved in nodulation, than in fixation. This might also be true for R. meliloti.

The large number of random Fix⁻ mutants described in this Chapter should facilitate identification of new fix genes. Corbin et al. (1983) have identified four fix gene clusters in R. meliloti strain Rm102F34. These are located in the vicinity of nifHDK region. In addition, there is genetic evidence for the presence of at least 3 chromosomal fix genes in strain Rm41 (Forrai et al. 1983).

It will be informative to identify the location of Tn5-insertions in our Sym⁻ mutants.

The curling of root hairs is a typical early response of infected roots in the case of certain Rhizobium-legume symbiosis, including the R. meliloti-alfalfa system (Dart, 1977). The physical analysis of Tn5-insertions in two Hac⁻ (deficient at root hair curling) mutants of R. meliloti strain 1021^r has indicated the location of the insertion at about 20 Kb from the nifHDK region of the megaplasmid (Long et al., 1982). Such an analysis of the two non-reactive mutants (4D3 and 4F9) described in this Chapter should indicate whether or not the mutations in these derivatives are in a similar location. The location of mutations in the case of Nod⁻ mutants that give Hac⁺ phenotype is not known for other Nod⁻ mutants described in the literature and in this study. Recently Banfalvi et al. (1983) have reported that R-primes carrying large segments (100-200 Kb) of the megaplasmid of R. meliloti strain Rm41 can complement both Nod⁻Hac⁻ and Nod⁻Hac⁺ mutants. Since these R-primes carry nifHDK genes, it can be concluded that some nod genes which upon mutagenesis retain the Hac⁺ phenotype are also located on the megaplasmid (in strain Rm41).

Paau et al. (1981) have demonstrated the presence of a protein (agglutinin) in alfalfa seeds that agglutinates R. meliloti, but not other Rhizobium strains. They also proposed that agglutinin is responsible for the specific

recognition and binding between R. meliloti and alfalfa roots. The studies on early phases of R. meliloti-alfalfa symbiosis suggest that the root hair curling ensues the specific binding of the bacteria to the roots (see Dart, 1977). Paau et al. (1981) examined the Tn5-induced Nod^- mutants of strain Rml021 (Meade et al. 1982) for agglutination. The mutants that reacted with the agglutinin were later shown to be Hac^- , and the ones that failed to react were Hac^+ (Hirsch et al. 1982). This paradox could perhaps be resolved by examining a larger number of independently derived Nod^- mutants that are Hac^+ or Hac^- . The Nod^- mutants described in this study should be useful for this purpose. The observation that some Fix^- mutants induce morphologically distinct nodules (Table IV-3) is interesting. It is believed that the nodule shape is characteristic of the host plant and is determined by the meristematic activity in the host tissue (Dart, 1977). A cytological analysis of the different types of nodules and the genetic and physical analysis of the respective Fix^- mutants should provide additional insights into nodule development. Such studies would lead to the correlation of bacterial mutations and host response with respect to nodule morphogenesis. The recent cytological studies of Hirsch et al. (1983) on nodules incited by Tn5-insertion mutants of Rml021 (NifH^- , D^- , and K^-) have shown that mutations in the nitrogenase structural genes also affect nodule morphogenesis and

organization.

The different categories of Fix^- mutants, classified on the basis of the total number of nodules induced per host plant, are of some interest. The majority of Fix^- mutants belong to the "profuse-nodulation" category. Some natural Fix^- strains (referred to as "ineffective") also induce profuse nodulation (D.A. Phillips, personal communication, Werner et al., 1980). One possible explanation for this observation is that when nodules are initiated with Fix^- mutants, the host plant senses and responds to this situation by allowing an increase in the number of nodules and therefore the number of colonizing endosymbionts. This implies that the host plant can participate actively in determining the number of nodules. Perhaps such a control involves nodule-specific proteins. Legocki and Verma (1980) have shown that there are changes in such proteins ("nodulins") in the nodules of soybean induced by Fix^+ and Fix^- strains of R. japonicum. An alternative possibility is that the increase in nodule numbers is solely a consequence of mutation in one of several (but not all) bacterial fix genes. However, this seems unlikely, since the co-infection of a plant with a "profuse-nodulation" strain and the wild-type strain does not result in enhanced nodulation (Selvaraj and Iyer, preliminary observations). Hirsch et al (1982) have also suggested that certain Sym^- mutants might exhibit a parasitic or pathogenic, rather than symbiotic response

which is not exhibited by the wild-type strains.

The mutants reported in this study form the largest collection of Tn5-induced auxotrophs of R. meliloti, and therefore enables a comparison of the pattern of auxotrophy among E. coli mutants, the only other species for which a large collection of Tn5-insertion mutants is available (Shaw and Berg, 1979). Assuming that the different delivery vehicles used in generating mutants in the two species do not influence the pattern of phenotypic classes, it can be concluded that Tn5 exhibits a host-independent insertion specificity. Preliminary evidence from the studies of Acetobacter also support this conclusion (Singer and Finnerty, 1983).

The frequent emergence of spontaneous auxotrophs and Fix mutants among cultures of R. meliloti strains JJ1 and JJ1C10 suggests the presence in them of IS element(s). Further studies are needed to prove these mutations are caused by IS elements. ISRm1 is the only insertion sequence described in R. meliloti (Ruvkun et al. 1980, 1982). It inserts specifically into sym genes, at a frequency of about 10^{-2} . An IS element found in R. lupini inserts into plasmid RP4 at a frequency of 10^{-2} to 10^{-1} . Neither of these elements is known to induce any auxotrophic mutations. It will be informative to study indigenous IS elements of R. meliloti, since IS elements play a major role in the evolution of genes and genomes.

APPENDIX IV-A: UV-SENSITIVE MUTANTS OF Rhizobiummeliloti

UV-sensitive mutants of *R. meliloti* strain JJ1

While screening the Tn5-insertion derivatives of JJ1, for auxotrophy, the derivatives were also tested for hypersensitivity to ultraviolet rays, and sensitivity to methylmethane sulphonate (MMS), in an attempt to isolate mutants defective in general recombination (RecA^-). RecA^- mutants of other well-studied bacterial species display sensitivity to both these DNA-damaging agents. Therefore one potential method of identifying RecA^- mutants of *R. meliloti* could be on the basis of their sensitivity to UV and MMS. Of 800 independent Tn5-insertion derivatives screened, (as described in "experimental Procedures"), two were sensitive to UV, and 8 were sensitive to MMS. However, none of these was cross-sensitive in the same test. Subsequent confirmatory tests showed that the MMS-sensitive phenotype noticed in the preliminary screening was not reproducible on a gradient plate containing a range of concentration of MMS. Therefore, further studies were done with only the two UV-sensitive mutants (strain JJ1-162 and JJ1-542).

The UV-sensitivity of the two mutants is shown in Fig. IV-2. They were hypersensitive to UV doses which had relatively little effect on the parental strain JJ1 and a met::Tn5 derivative of JJ1. The latter strain served as a control for the Tn5-specific effect, if any, on UV-sensitivity. The MMS-sensitivity of the UV mutants and the two control strains were similar (50% loss of

Fig. IV-2. UV-sensitivity of the Tn5-insertion mutants of JJ1, JJ1-542 and JJ1-168. Cells were grown to mid-log phase in TY medium, washed with PBS (phosphate-buffered saline), resuspended in PBS and irradiated with different doses of short-wave UV. The viable counts were determined on TY agar. The viable counts in the unirradiated samples were $3-5 \times 10^7$ cells/ml.
 (○—○) JJ1-168; (●—●), JJ1-542; (□—□), JJ1; (■—■), a Met⁻ Tn5-insertion mutant of JJ1 (strain WS 1002).

viability on solid media containing 0.04% V/V of MMS).

Therefore these mutants do not meet the general diagnostic criteria of known RecA⁻ mutants of other bacteria. A preliminary attempt to measure the frequency of general recombination in the strains with the following experimental scheme did not yield conclusive results.

Plasmid pGS12 is a stable cointegrate containing pRK248 and pACYC177 (see Fig. II-2; page 92). From the data of Chang and Cohen (1978), the pACYC177-component was inferred to have about 1 kb of homologous DNA sequence in the region of the origin of replication with the pACYC184-component of plasmid pCU101 (see Fig. II-3).

Since pCU101 is unstable in R. meliloti (Chapter II), an R. meliloti derivative proficient at general recombination and carrying pGS12, should rescue pCU101-associated markers following the introduction. However, this was not the case with all the four strains described above.

Therefore another experimental design, perhaps providing a larger homologous segment of DNA should be used in further experiments.

Recently Better and Helinski (1983) have isolated a Tn5-induced RecA⁻ mutant of R. meliloti strain 102F34. This mutant was more sensitive to UV and MMS than its parental strain. Thus the two UV mutants of strain JJ1 isolated here might not be RecA⁻, since they are not sensitive to MMS. UvrA⁻-like mutants of R. trifolii have been isolated (Walton and Mosely, 1981). The UV^S mutants

of R. meliloti appear to be similar to these mutants.

They could be useful to study DNA metabolism in R.

meliloti. A general property of UvrA⁻ mutants is the

hypermutability of the strains by UV. If the strains

JJ1-542 and JJ1-162 are indeed UvrA⁻, they might also be

useful for the induction of point mutations by UV.

CONCLUSIONS

3 3

OF / DE

Rhizobium meliloti is a Gram-negative bacterium that fixes atmospheric nitrogen in symbiotic association with alfalfa, a forage crop of economic importance. The purpose of this project was to develop methods for the genetic analysis and manipulation of R. meliloti, with the intent of eventually applying these methods to study and manipulate the bacterial component of the R. meliloti-alfalfa symbiosis. The strain IZ450 was chosen based on its sensitivity to a number of antibiotics and its relatively simpler plasmid composition. A spontaneous rifampicin-resistant derivative of it, JJ1, was used until a derivative of it lacking one of its two indigenous plasmids became available. The latter strain, JJ1C10, which remained proficient at symbiotic nitrogen-fixation was used in all subsequent studies. The following methods were developed and used in the genetic analysis of derivatives of R. meliloti strain IZ450.

Plasmid DNA-mediated genetic transformation is one of the commonly used methods in introducing native, and engineered plasmids into bacterial cells. Such a method was developed for a number of R. meliloti isolates of diverse origin. This method is being used in other laboratories for cloning the replication/maintenance regions of indigenous

plasmids of R. meliloti.

Insertion mutants are valuable tools in the genetic analysis and manipulations of an organism. Several attempts were made to obtain bona fide transposon Tn5-insertion mutants of R. meliloti strain JJ1, using published procedures. In these experiments a suicide delivery plasmid pJB4JI, carrying transposon Tn5 and bacteriophage Mu DNA (the latter conferring the suicidal property) was used. Mutants of JJ1 isolated by using pJB4JI were found to be an ambiguous nature; that is, the mutational event could seldom be ascribed to a Tn5 insertion.

Therefore a different type of suicide vehicle, devoid of bacteriophage Mu was constructed. This plasmid, composed of a pl5A replicon, an N group plasmid transfer system and transposon Tn5 was effective in inducing bona fide Tn5-insertion mutants of R. meliloti strain JJ1. Modelled after this plasmid, suicide vehicles carrying transposons Tn1 and Tn9 were also constructed. These suicide vehicles were also effective with a number of other strains of R. meliloti and Rhizobium leguminosarum.

While using the suicide vehicles bearing Tn5 in generating insertion mutants of R. meliloti strain JJ1, it was noticed that all of the putative mutants acquired the streptomycin-resistant (Sm^r) phenotype. This raised a suspicion that Tn5 was inserting into a

specific locus in R. meliloti strain JJ1. The basis of this phenomenon was studied further. These studies showed that Tn5 carries a gene specifying Sm^r-resistance. The Sm-resistance phenotype was not expressed in E. coli. It was also shown that the Tn5-associated Sm-resistance phenotype was useful as a genetic marker, and that it was expressed in a number of diverse strains of R. meliloti, R. leguminosarum and Rhizobium trifolii.

Using the suicide vehicles constructed as a part of this study, a large number (> 9000) of independent insertion derivatives of R. meliloti strain JJ1C10 was isolated. These derivatives were analyzed for auxotrophy, and defective symbiosis (Sym⁻). A number of auxotrophs, and mutants defective in nodulation (Nod⁻) and in nitrogen-fixation (Fix⁻) were identified. Among the auxotrophs, some mutants that have not been described previously (e.g. Asparagine⁻) were found. Two categories of Nod⁻ mutants were found; one that did not incite any nodule and the other that induced some morphogenetic changes in the roots which at best culminated in green calli. The Fix⁻ mutants could be classified based on the number of nodules incited by them; independent of this classification, they could also be grouped according to nodule morphology. Among Tn5-insertion mutants of strain JJ1,

UV-sensitive mutants were identified. Diagnostic assays such as screening for sensitivity to alkylating agents, have indicated that these are not Rec⁻ (recombination-defective), as originally suspected. These different types of mutants of R. meliloti should facilitate further genetic analysis of R. meliloti.

Derivatives of transposons Tn1 and Tn5 carrying the "mob (oriT)" region of a P-group conjugative plasmid were constructed. The "mob (oriT)" region confers upon a genetic element carrying it, the ability to be mobilized by a P-group conjugative plasmid. The modified transposons carrying this region retained their ability to transpose, and therefore they should be useful in further manipulation of R. meliloti. Suicide vehicles, similar to the ones described above, but carrying these engineered transposons were also constructed. These should be useful in further manipulations of R. meliloti.

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