STUDIES ON ISOLEUCINE, VALINE AND LEUCINE AUXOTROPHS OF *Sinorhizobium meliloti*

A THESIS

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By

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CANDIDATE'S DECLARATION
(To be included in the thesis)

I hereby certify that the work which is being presented in the thesis entitled "STUDIES ON ISOLEUCINE, VALINE AND LEUCINE AUXOTROPHS OF *Sinorhizobium meliloti*", in fulfilment of the requirement for the award of the Degree of Doctor of Philosophy and submitted in the Department of BIOSCIENCES AND BIOTECHNOLOGY of the University is an authentic record of my own work carried out during a period from August, 1997 to March, 2001 under the supervision of Dr. (Prof.) G.S. Randhawa.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University.

Date : Mar. 12, 2001

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This is to certify that the above statement made by the candidate is correct to the best of my (our) knowledge.

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ABSTRACT

The present work was undertaken to study the symbiotic role of isoleucine, valine, and leucine biosynthetic pathways of *Sinorhizobium meliloti*. Random transposon Tn5 mutagenesis was employed to generate isoleucine, valine, and leucine auxotrophs of *S. meliloti* strain Rmd201 which is a streptomycin resistant derivative of strain AK631. Matings between the donor strain *E. coli* WA803 carrying suicide plasmid pGS9 and recipient strain *S. meliloti* Rmd201 yielded 5,450 kanamycin resistant transconjugants from 122 crosses. Thirty transconjugants out of these were found to be auxotrophs. Based on the growth patterns of these auxotrophs on nutritional pools, four isoleucine + valine and one leucine auxotrophs were identified. Six isoleucine + valine and two leucine auxotrophs isolated by other researchers in this lab were also included in this study.

Intermediate feeding, intermediate accumulation and cross-feeding studies were done to identify the mutated gene in each auxotroph. On the basis of these studies, isoleucine + valine auxotrophs were classified into following categories:

Category I: *ilvB / ilvG* mutant (VK4) which grew on Rhizobium minimal medium (RMM) supplemented with α-acetolactate and isoleucine, and accumulated pyruvic acid.

Category II: *ilvC* mutants (RH1, RH30, VK5, NV29, SY2 and PS7) which did not grow on RMM supplemented with α-acetolactate and isoleucine, and were cross-fed by RH3, RH18 and VK44.

Category III: *ilvD* mutants (RH3, RH18 and VK44) which grew on RMM supplemented with α-keto-β-methylvalerate and valine and were not cross-fed by any other isoleucine + valine auxotroph.

The leucine auxotrophs were placed in the following categories:

Category I: *leuC / leuD* **mutant (RS3) which did not grow on RMM supplemented with α-isopropylmalate and was cross-fed by RH14 and SY1 leucine auxotrophs.**

Category II: *leuB* **mutants (RH14 and SY1) which grew on RMM supplemented with α-ketoisocaproate and cross-fed RS3 leucine auxotroph.**

The isoleucine + valine, and leucine auxotrophs were similar to the parental strain w.r.t. cell surface molecules (lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-glucans), and utilization of dicarboxylic acids and sugars indicating that the symbiotic defects of these auxotrophs were not caused by a change in any of the above characteristics.

For confirming the linkage of Tn5 insertion to auxotrophy in each auxotroph, the plasmid pJB3JI was used to mobilize the Tn5 containing chromosomal segment into *S. meliloti* recipient strain ZB555 (Cys^-, Phe^-, Rf^r, Sm^r). Kanamycin resistance transconjugants were selected and tested for the presence of the donor's auxotrophic marker(s). All kanamycin resistant transconjugants showed donor's auxotrophy. This result confirmed 100% linkage of auxotrophy to Tn5 insertion as well as ruled out the possibility of occurrence of other independent Tn5 insertions in each mutant.

Plasmid-mediated mapping method was used to locate the position of transposon Tn5 in each isoleucine + valine auxotroph. The donor strain of each auxotroph, constructed by the introduction of plasmid pJB3JI, was mated with four *S. meliloti* recipient strains ZB555, ZB556, ZB557 and ZB205, the markers of which cover most of the chromosome. In all crosses, selection was made for the transfer of kanamycin resistance marker which is encoded by Tn5. The co-transfer percentages of the

kanamycin resistance marker with the unselected markers were determined by streaking the transconjugants on appropriate plates. Kanamycin resistance showed 24, 9 and 5% co-transfer values with *ade-15* marker in isoleucine + valine auxotrophs RH18, RH1 and VK4, respectively when *S. meliloti* ZB205 strain was used as a recipient.

Symbiotic properties of isoleucine + valine, and leucine auxotrophs were determined by inoculating them on alfalfa (*Medicago sativa* cv. T9) seedlings grown aseptically on nitrogen-free agar slants. The *ilvB / ilvG* and *ilvC* mutants did not induce nodules and the Nod^+ phenotype was not restored by the supplementation of plant nutrient medium with isoleucine and valine, or α-keto-β-methylvalerate and valine. All *ilvD* mutants induced nitrogen fixing nodules on alfalfa plants and nitrogen fixing efficiencies of these mutants were similar to the nitrogen fixing efficiency of the parental strain Rmd201. All leucine auxotrophs of *S. meliloti* were Nod^+ Fix^- and the symbiotic defect was not restored by supplementation of the plant nutrient medium with leucine or α-ketoisocaproate. The *ilvB / ilvG* auxotroph VK4 did not induce root hair curling and infection thread formation. The *ilvC* auxotrophs (RH1, RH30, VK5, NV29, SY2 and PS7) were capable of curling root hair but no infection threads in root hairs were found. The *ilvD* auxotrophs (RH3, RH18 and VK44) and all leucine auxotrophs resulted in root hair curling and infection thread formation.

Histological studies revealed that the nodules induced by the *leuB* mutants were structurally more advanced than the *leuC / leuD* mutant induced nodules. In the former case rhizobial bacteria were released from the infection thread into plant cells whereas in the latter case the rhizobia did not come out of the infection threads. Histological

structure of the nodule induced by *leuB* mutant RH14 was improved to some extent by the supplementation of the plant nutrient medium with leucine.

The above results indicated that the expression of *ilvB*, *ilvG* and *ilvC* genes of *S. meliloti* is required for induction of nodules on alfalfa plants. The genes *leuC* / *leuD* and *leuB* of *S. meliloti* appear to be required for normal nodule development. The *leuC* / *leuD* gene may be involved in the release of rhizobia from infection threads.

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LIST OF ABBREVIATIONS USED

ade	=	Adenine
ALC	=	α-acetolactate
Am	=	Amyloplast
B	=	Bacteroid
Cm	=	Cloramphenicol
ct	=	Central tissue
cw	=	Cell wall
cys	=	Cysteine
DOC	=	Sodium deoxycholate
ed	=	Electron dense cytoplasm
EPS	=	Exopolysaccharide
ER	=	Endoplasmic reticulum
et	=	Electron transparent cytoplasm
exo	=	Exopolysaccharide
Fix	=	Nitrogen fixation
g	=	Gram
gly	=	Glycine
hc	=	Heterogeneous cytoplasm
hrs	=	Hours
hsn	=	Host specific nodulation
I	=	Infected nodule cell
If	=	Infection zone
ILE	=	Isoleucine
IPM	=	α-isopropylmalate
it	=	Infection thread
Iz	=	Interzone
kb	=	Kilo base
KBU	=	α-ketobutyrate
KIC	=	α-ketoisocaproate
KIV	=	α-ketoisovalerate
Km	=	Kanamycin
KMV	=	α-keto-β-methylvalerate
LB	=	Luria Bertani
LEU	=	Leucine
LPS	=	Lipopolysaccharide
M	=	Molar
m	=	Mitochondria
Met	=	Methionine

mg	=	Milligram
min	=	Minute
ml	=	Millilitre
Mr	=	Meristematic zone
MSY	=	Mannitol salt yeast extract
N	=	Normal
n	=	Nucleus
Ndv	=	Nodule development
Nf	=	Nitrogen fixation zone
Nif	=	Structural genes for nitrogenase enzyme
Nm	=	Neomycin
nm	=	Nanometer
Nod	=	Nodulation
nol	=	---do---
Ori	=	Origin of transfer
pbm	=	Peribacteroid membrane
phb	=	Poly-β-hydroxybutyrate
phe	=	Phenylalanine
pt	=	Peripheral tissue
rb	=	Rhizobial bacteria
Rf	=	Rifampicin
rh	=	Root hair
RMM	=	Rhizobial minimal medium
S	=	Senescence zone
sc	=	Shepherd's crook
sec	=	Second
sg	=	Starch granule
Tc	=	Tetracycline
Thi	=	Thiamine
Tra	=	Transfer
TY	=	Tryptone yeast extract
UI	=	Uninfected nodule cell
v	=	Vacuole
VAL	=	Valine
VB	=	Vascular bundle
µg	=	Microgram
µm	=	Micrometer

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Chapter 1

INTRODUCTION

Plants and animals cannot convert gaseous nitrogen (N_2), which is abundantly available in the atmosphere, into a biologically useful form. Some prokaryotic organisms possess the ability to utilize gaseous nitrogen. These organisms, often called diazotrophs, reduce nitrogen to ammonia with the help of the enzyme complex nitrogenase. The process of nitrogen reduction by diazotrophs is called biological nitrogen fixation. Chemical nitrogen fixation is costly and has lead to land-pollution and eventually made the soil infertile in long run. Biological nitrogen fixation on the other hand is cheap, pollution free and reliable. Any basic research in this area should to be encouraged since nitrogen availability is the factor limiting the fertility of most soils. An understanding of biological nitrogen fixation is directly related to increasing the world's food supply. The nitrogen fixing bacteria, on the basis of extent of their association with plants, have been classified into three groups (i) free living (*Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Rhodospirillum rubrum*, etc.), (ii) associative (*Azospirillum* spp.) and (iii) symbiotic (*Rhizobium, Frankia*, etc.). The last group is of special significance to agriculture since as much as 25% of the terrestrial nitrogen is fixed by rhizobial-legume symbiosis.

The bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium* and *Sinorhizobium* are collectively called rhizobia. These bacteria enter into a symbiotic association with leguminous plants and fix nitrogen in specialized structures called nodules which are induced on the roots of legume plants. The symbiotic association is a multistage process involving recognition and infection of root hairs, development and differentiation of root nodules, multiplication of bacteria and their conversion into bacteroids within the plant cells and finally reduction of molecular nitrogen to ammonia. Symbiosis also involves several other processes which include

biochemical reactions for utilization of fixed nitrogen by plants, uptake and assimilation of the energy rich carbon compounds by bacteroids, osmoregulatory functions, etc. (Fischer, 1994). The identification and characterization of the genes and the gene products involved in symbiosis will help in obtaining improved symbiotic combinations having improved ability of nitrogen fixation.

Several symbiotic genes of bacterial and plant partners have been identified. Rhizobial genes involved in nodule formation (*nod, nol*) (Banfalvi *et al.*, 1981; Kondorosi *et al.*, 1984; Fisher and Long, 1992; Freiberg *et al.*, 1997), nodule invasion (*exo, pss, ndv, lps*) (Borthakur and Johnston, 1987; Priefer, 1989; Charles *et al.*, 1991; Noel, 1992; Leigh and Walker, 1994; Król *et al.*, 1998; Pollock *et al.*, 1998) and nitrogen fixation (*nif, fix*) (Batut *et al.*, 1985; Putnoky *et al.*, 1988; Beringer *et al.*, 1990; Kündig *et al.*, 1993) have been characterized. Some primary biosynthetic pathways of rhizobia also appear to have a role in the symbiotic process. Rhizobial auxotrophs of some amino acids (leucine, tryptophan, etc.), nucleotide bases and vitamins have been found to be defective in the symbiotic process (Schwinghamer, 1970; Pankhurst *et al.*, 1972; Kerppola and Kahn, 1988b; Noel *et al.*, 1988; Barsomian *et al.*, 1992; Newman *et al.*, 1994; Taté *et al.*, 1999b; 1999c). The defective symbiosis is not due to the unavailability of the end products only. The intermediate(s) / enzyme(s) of the particular pathway appear to contribute to symbiosis. The present work was undertaken to understand the symbiotic role of the pathways for the synthesis of isoleucine, valine and leucine in *S. meliloti* which makes symbiotic association with alfalfa (*Medicago sativa*) plants.

This work was started with the following objectives:

i) to generate isoleucine, valine, and leucine auxotrophs of *S. meliloti* by transposon Tn5 mutagenesis.

ii) to carry out genetic and biochemical characterization of the auxotrophs obtained.

iii) to study symbiotic properties of these auxotrophs.

iv) to study histology of the nodules induced by auxotrophs.

Chapter 2

LITERATURE REVIEW

The relevant literature has been presented below under suitable headings:

2.1 Taxonomy of rhizobia

Beijerinck (1888) was first to isolate and cultivate a microorganism from the legume nodules. This microorganism was named as *Bacillus radicicola* by him. Later Frank (1889) renamed this microorganism as *Rhizobium*. Rhizobial bacteria, on the basis of the growth rate, were classified into slow-growers (with generation time more than six hours) and fast-growers (with the generation time less than six hours). The slow-growers nodulate soybean and cowpea plants whereas the fast-growers form nodules on alfalfa, clover, bean and pea plants. The slow-growing rhizobia were placed under the genus *Bradyrhizobium* (Jordan, 1982; 1984). According to the Bergey's Manual of Systematic Bacteriology, the family *Rhizobiaceae* consists of four genera, *Rhizobium*, *Bradyrhizobium*, *Agrobacterium* and *Phyllobacterium*, out of which only first two are nitrogen fixing bacteria (Jordan, 1984).

A new genus, *Azorhizobium*, was recognized for the rhizobial bacteria which can utilize gaseous nitrogen as a sole nitrogen source during growth in free living condition. Later by using the modern methods of bacterial systematics two new genera, *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Lindström *et al.*, 1995) and *Mezorhizobium* (Jarvis *et al.*, 1997) were recognized for rhizobial bacteria. On the basis of the analysis of the 23S rDNA sequences, Pulawska *et al.* (2000) found that the strains of *Agrobacterium* and *Rhizobium* are closely related to each other. These workers suggested that the taxonomic definition of *Agrobacterium* and *Rhizobium* should be considered for revision. The latest taxonomic classification of rhizobia has been presented in Table 1.

Table 1: Taxonomic classification of rhizobia*

Genus	Species	Reference	Host plant (s)
Rhizobium			
	R. leguminosarum	Jordan (1984)	*Vicia, Pisum, Trifolium, Phaseolus, Lens* spp.
	R. galegae	Lindström (1989)	*Galega officinalis*
	R. tropici	Martínez-Romero *et al.* (1991)	*Leucaena* spp., *Macroptilium* spp., *Phaseolus vulgaris*
	R. etli	Segovia *et al.* (1993)	*Phaseolus vulgaris*
	R. gallicum	Amarger *et al.* (1997)	*Phaseolus vulgaris*
	R. giardinii	Amarger *et al.* (1997)	*Phaseolus vulgaris*
	R. hainanense	Chen *et al.* (1997)	Tropical legumes
	R. mongolense	van Berkum *et al.* (1998)	*Medicago ruthenica*
	R. huautlense	Wang *et al.* (1998)	*Sesbania herbacea*
Bradyrhizobium			
	B. japonicum	Jordan (1984)	*Glycine max, G. soja,* etc.
	B. elkanii	Kuykendall *et al.* (1992)	*Glycine max, G. soja,* etc.
	B. liaoningense	Xu *et al.* (1995)	*Glycine soja, G. max*
Azorhizobium			
	A. caulinodans	Dreyfus *et al.* (1988)	*Sesbania rostrata*
Sinorhizobium			
	S. meliloti	Jarvis *et al.* (1982)	*Medicago* spp., *Melilotus* spp., *Trigonella* spp.
	S. fredii	Scholla and Elkan (1984)	*Glycine max, G. soja* etc.
	S. medicae	Eardly *et al.* (1990)	*Medicago* spp.
	S. saheli	de Lajudie *et al.* (1994)	*Sesbania* spp.
	S. teranga	de Lajudie *et al.* (1994)	*Sesbania* spp.
	S. arboris	Nick *et al.* (1999)	*Acacia senegal, Prosopis Chilensis,* etc.
	S. kostiense	Nick *et al.* (1999)	*Acacia senegal, Prosopis Chilensis,* etc.
Mesorhizobium			
	M. loti	Jarvis *et al.* (1982)	*Lotus* spp.
	M. huakuii	Chen *et al.* (1991)	*Astragalus sinicus*
	M. ciceri	Nour *et al.* (1994)	*Cicer arietinum*
	M. tianshanense	Chen *et al.* (1995)	*Glycyrrhiza, Sophora* spp.
	M. mediterraneum	Nour *et al.* (1995)	*Cicer arietinum*
	M. plurifarium	de Lajudie *et al.* (1998)	*Acacia* spp., *Leucaena* spp.

* Reproduced from Hussein (2000).

2.2 Nodule formation

2.2.1. Signals between plant and rhizobia

Rhizobial bacteria are attracted to plant roots by chemical substances called attractants present in plant root exudates. Some of these attractants, like sugars, amino acids, phenolic compounds and carboxylic acids, have nutritional value whereas some others like flavonoids and chalcones are inducers or repressors of nodulation genes (Rolfe, 1988; Gottfert, 1993). Flavonoids bind to rhizobial NodD protein which binds to the promoters of other *nod* genes to induce the expression of these *nod* genes (Fig. 1)(Schlaman *et al.*, 1989; Goethals *et al.*, 1992). The *nod* gene products synthesize Nod factors which result in the deformation and curling of root hairs of the host plant (Schlaman *et al.*, 1989; Journet *et al.*, 1994). These Nod factors also result in the division of cortical cells in the roots of the leguminous plants. Heterologous rhizobial Nod factors have been found to induce cortical cell divisions in red clover root, transformed with the pea lectin gene (Díaz *et al.*, 2000).

NodD proteins from different rhizobia require specific flavonoids (Horvath *et al.*, 1987; McIver *et al.*, 1989). *Rhizobium* spp. NGR234 strain synthesizes 18 different Nod factors (Price *et al.*, 1992). Three different NodD proteins are synthesized by *S. meliloti*; each of these proteins binds to a different flavonoid (Györgypal *et al.*, 1988). Nod factors are lipo-chitooligosaccharides (LCOs) containing a backbone of 4-5 N-acetylglucosamine residues and a fatty acid at the non-reducing terminal sugar residue. The substitutions at the terminal sugar residue determine the host specificity. The main host determinant in *S. meliloti* is a sulphate group at the reducing sugar residue (Peters *et al.*, 1986; Lerouge *et al.*, 1990; Fisher and Long, 1992; Goethals *et al.*, 1992; Journet *et al.*, 1994; Pawlowski

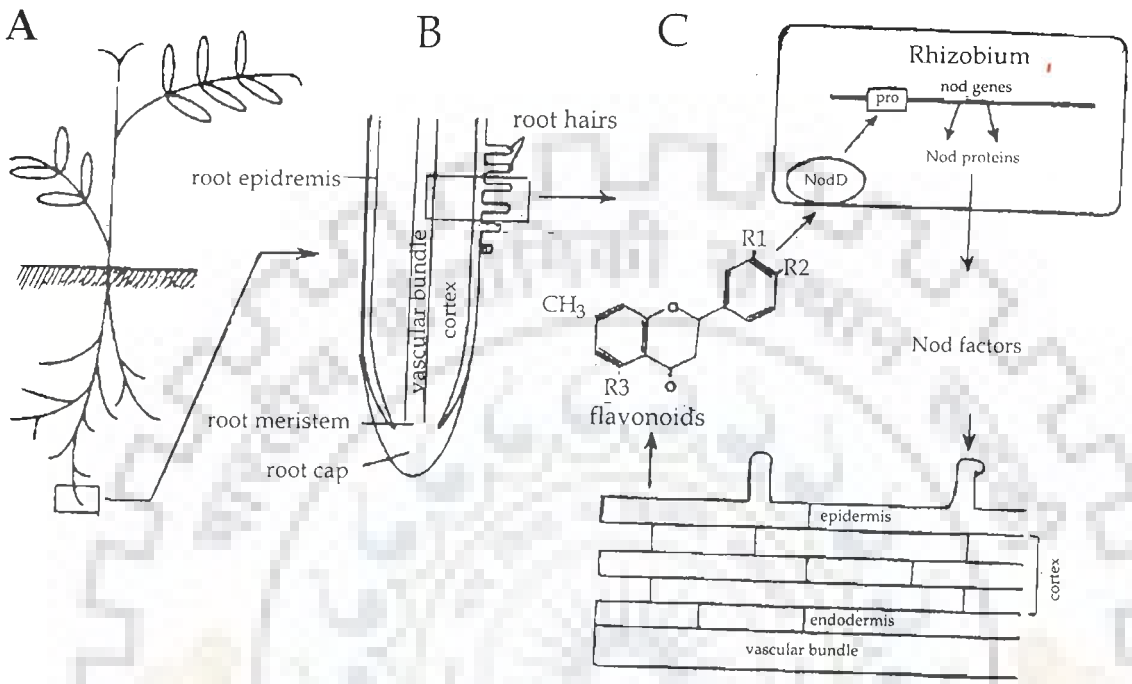


Fig. 1: Schematic representation of signal exchange during legume nodule induction, A. legume plant, B. root tip, C. interaction between legume roots and rhizobia (Pawlowski *et al.*, 1996).

et al., 1996). Zuanazzi *et al.* (1998) isolated *nod* gene inducers from alfalfa roots and found that all compounds were flavonoids. These workers also observed that columestrol and medicarpin, present in the root exudates possess *nod* gene repressing activity. Two partially purified compounds from *S. meliloti* 1021 have been found to increase root respiration at very low concentrations. These compounds are different from lipo-chitooligosacharides (LCOs) (Volpin and Phillips, 1998).

2.2.2 Nodulation genes

Rhizobial nodulation genes are required for host-recognition and plant invasion. These genes, called as *nod*, *nol* and *noe* genes, have been divided into common *nod* genes and host-specific nodulation (*hsn*) genes. The common nodulation genes (*nodABC*) are required for root hair curling and nodule induction. These genes are highly conserved among all rhizobial species (Long *et al.*, 1982; Horvath *et al*, 1986; Schultze and Kondorosi, 1998). The rhizobia, with mutated *nodABC* genes, cannot elicit symbiotic reaction like induction of root hair curling, root hair infection, thick and short root hair and nodule formation, in the plant (Long *et al.*, 1982; Long, 1989). The enzymes encoded by *nodABC* genes synthesize the core Nod factor (Fisher and Long, 1992). The expression of *nod* genes is activated by three allelic *nodD* genes in presence of plant flavonoids (Mulligan and Long, 1985; Peters *et al.*, 1986). Host specificity is determined by the host specific nodulation (*hsn*) genes which include *nodH*, *nodFEG* and *nodQP* genes. A mutated *hsn* gene results in altered infection and nodulation phenotype. In *S. meliloti*, *hsn* gene cluster is at a distance of 6kb from common *nod* genes (Long *et al.*, 1982; Kondorosi *et al.*, 1984; Horvath *et al.*, 1986). In *R. tropici* CIAT899 *nodHPQ*

genes are organized as an operon that is transcribed in a *nodD*-independent manner and is not regulated by flavonoids (Folch-Mallol *et al.*, 1998).

2.2.3 Nod signal recognition by host plant

Nod signal recognition has been suggested to occur at two levels. Low affinity binding takes place even to unmodified LCOs for bacterial entry into root hair cells and transient expression of *ENOD40* whereas the high affinity recognize only modified LCOs to give constant expression of *ENOD40* triggering the symbiotic responses of plant (Ardourel *et al.*, 1994; Minami *et al.*, 1996). Plant lectins and chitinases have been considered to be possible Nod signal receptors. de Carvalho Niebel *et al.* (1998) have identified a new Nod factor induced gene from *Medicago truncatula*. This gene, designated *MtAnn1*, encodes a protein homologous to the annexin family of calcium and phospholipid-binding proteins.

2.2.4 Role of rhizobial cell surface polysaccharides in early infection events

Certain rhizobial cell surface polysaccharides like cyclic β-(1→2)-glucans, lipopolysaccharides (LPS I, II), capsular polysaccharides (CPS) and extra-cellular exopolysaccharides (EPS I, II) have been found to be involved in early infection events. Cyclic oligosaccharide β-(1→2)-glucan mutants of *S. meliloti* were severely impaired in both attachment and infection thread initiation on alfalfa; the bacteroids produced by these mutants were morphologically abnormal (Dylan *et al.*, 1990; Ielpi *et al.*, 1990). In *R. leguminosarum* bv. *viciae* 3841 the wild type LPS layer has been found to be essential for nitrogen fixation (Poole *et al.*, 1994). Reuhs *et al.* (1998) observed that structurally conserved LPS of *S. fredii* and *S. meliloti* lack necessary information to influence host specificity. The studies of Niehaus *et al.* (1998) revealed that the LPS I and LPS II

fractions of LPS mutant Rm6963 of *S. meliloti* were shifted to lower molecular weights. This mutant had normal symbiosis with *Medicago sativa* but induced ineffective nodules on *M. truncatula.*

Kozak *et al.* (1997) hypothesized that capsular polysaccharides (CPSs or K-antigens) of *S. meliloti* may play a role in the early recognition of rhizobia by the leaf cells. It has been shown that the variable K-antigens of *S. fredii* and *S. meliloti* may affect the interaction of these rhizobia with their specific hosts (Reuhs *et al.*, 1998). EPS and CPS-deficient mutants of *B. japonicum* 2143 exhibited delayed nodulation on soybean and significantly reduced acetylene reduction activities (Eggleston *et al.*, 1996). Reuber and Walker (1993) have reported that the acetyl substituent of succinoglycan (EPS I) is not necessary for alfalfa nodule invasion by *S. meliloti* Rm1021. It has been suggested that a low molecular weight EPS II may act as a symbiotic signal during infection (González *et al.*, 1996). *R. leguminosarum* bv. *trifolii exoB* mutant produced altered acidic exopolysaccharide; this mutant induced the development of abnormal root nodules and was almost completely unable to invade plant cells (Sanchez-Andujar *et al.*, 1997). Krishnan and Pueppke (1998) characterized *exo* mutant of *S. fredii* strain USDA208 and found that the symbiotic function of EPS in *S. fredii* appears to differ fundamentally from that in *S. meliloti*. Cheng and Walker (1998) examined the nature of symbiotic deficiency of *S. meliloti* Rm1021 *exo* mutants and on the basis of their findings proposed that *S. meliloti* succinoglycan is required for both the initiation and elongation of infection threads during nodule invasion and that excess production of succinoglycan interferes with ability of the rhizobia to colonize curled root hairs.

Symbiotic phenotype of the EPS mutant *pssD133* of *R. leguminosarum* bv. *trifolii* suggested a correlation between this mutation and defective endocytosis of bacteria into nodule cells (Król *et al.*, 1998). Wang *et al.* (1999) found that the three size classes of low-molecular-weight (LMW) succinoglycan species are monomers, dimers, and trimers of the repeating unit and that the trimer is the species active in promoting nodule invasion. The EPS II produced by *S. meliloti* in low-phosphate conditions does not allow the invasion of alfalfa nodules (Mendrygal and González, 2000). These researchers proposed that this invasion phenotype is due to the lack of the active molecular weight fraction of EPS II required for nodule invasion. Pellock *et al.* (2000) found that succinoglycan is highly efficient in mediating both infection thread initiation and extension; however, EPS II is significantly less efficient than succinoglycan at mediating both invasion steps, and K-antigen is significantly less efficient than succinoglycan at mediating infection thread extension.

2.2.5 Root hair deformation

Rhizobial infection has been found to occur through two ways, (i) through root hairs, and (ii) via so-called crack entry, where the bacteria enter the plant root through gaps in the epidermis (Chandler *et al.*, 1982) or enter between intact epidermal cells (De Faria *et al.*, 1988). During root hair infection, deformation and curling of root hairs takes place (Fahraeus, 1957). Heidstra *et al.* (1994) have shown that root hair deformation is due to a new induction of root hair tip growth by the Nod factors. This process is accompanied by the induction of several plant genes like *Mtrip1*, encoding a peroxidase (Cook *et al.*, 1995) and the early nodulin genes *ENOD5* and *ENOD12* that encode proline rich polypeptides (Scheres *et al.*, 1990a; 1990b). The expression of *ENOD12* and *Mtrip1*

is induced in all epidermal cells of a zone of the root, starting above the root tip even before root hairs have emerged, and extending to the region containing mature root hairs (Pichon *et al.*, 1992; Cook *et al.*, 1995).

2.2.6 Infection thread formation

Rhizobia induce root hair curling. The rhizobial bacteria entrapped in a curl induce the formation of an infection thread in the crook of curled root hair, beginning with the local hydrolysis of the plant cell wall (Callaham and Torrey, 1981). The infection thread wall appears to be of plant origin, and has an ultrastructure similar to that of the plant cell wall (Rae *et al.*, 1992). The infection thread penetrates root cortical cells and grows towards the nodule primordium; rhizobial bacteria are released into plant cells in the nodule primordium and get transformed into their symbiotic form, the bacteroids (Newcomb, 1981).

2.2.7 Nodule development

Along with the infection of root hairs, root cortical cells start dividing. Many nodulin genes like *ENOD12* (Scheres *et al.*, 1990a), *ENOD40* (Yang *et al.*, 1993; Matvienko *et al.*, 1994) and *GmN93* (Kouchi and Hata, 1993) are expressed in the dividing cortical cells. In temperate legumes (pea, vetch and alfalfa), inner cortical cells divide to form the nodule primordium (Newcomb, 1981), whereas in tropical legumes (soybean) the nodule primordium is formed by the division of the outer cortical cells. In the former case the nodules have an indeterminate development like lateral roots, and are called indeterminate nodules; the nodules of the latter kind have a determinate growth pattern and are called determinate nodules. During their release from infection threads, the bacteria are surrounded by a plant-derived membrane called the peribacteroidal

membrane (PBM). The bacteria, together with the space within the PBM and the PBM, form a functional structure called symbiosome (Roth and Stacey, 1989). The bacteria, for sometime after release, continue to divide and therefore multiple bacteria bounded by a single PBM are also seen in the nodule. The bacteria undergo certain transformations and are known as bacteroids (Hennecke, 1990). Kardailsky *et al.* (1996) identified a new pea gene, *PsNlc1*, encoding a lectin-like glycoprotein in the symbiosomes of root nodules. The development of host invaded cells of the nodules induced by *R. etli* on the roots of *Phaseolus vulgaris* has been observed to be synchronous (Cermola *et al.*, 2000). These workers suggested that multiple-occupancy symbiosomes also arise by fusion of single-occupancy symbiosomes.

2.2.8 Legume nodule structure

The legume nodule consists of a central tissue, containing rhizobial bacteria, surrounded by several peripheral tissues. The peripheral tissues of the nodule are the nodule cortex, the endodermis and the nodule parenchyma (van de Wiel *et al.*, 1990). Vascular bundles are present in the nodule parenchyma. The central tissue consists of infected and uninfected plant cells. A large number of bacteria are present in the infected cells. The central tissue is separated from the nodule parenchyma by a few layers of uninfected cells (Franssen *et al.*, 1992). In indeterminate nodules, the central tissue can be divided into several zones as shown in Fig. 2. The meristem at the distal end of the nodule is called zone I. This consists of uninfected, small cells which contain dense cytoplasm. The zone II, next to meristem, is the prefixation zone. The distal part of this zone shows the penetration of meristematic cells by infection threads and the release of rhizobia into plant cytoplasm. The elongation of plant cells and proliferation of

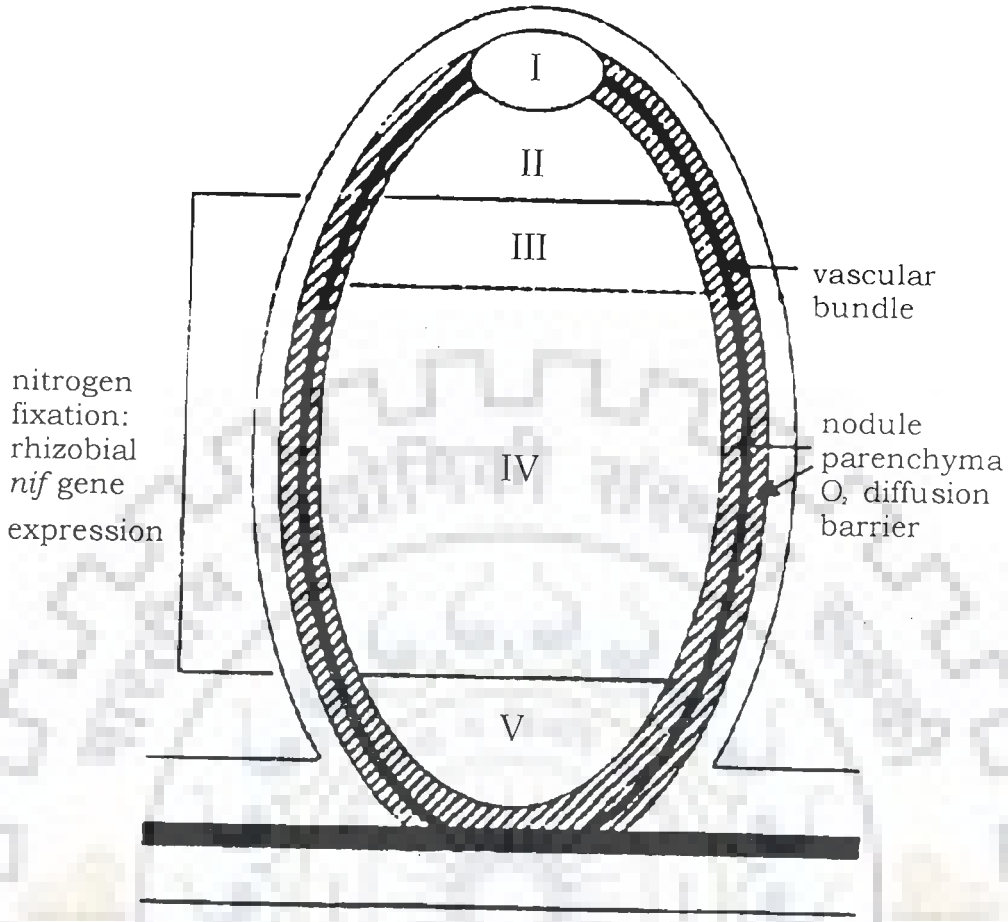


Fig. 2: Schematic representation of root nodule structure; Zonation: I, apical meristematic zone; II, infection zone; III, interzone; IV, nitrogen fixation zone; V, senescence zone (Pawlowski *et al.*, 1996).

symbiosomes can be observed in the proximal part of zone II. The interzone between zone II and zone IV shows the beginning of starch accumulation in infected cells; differentiated bacteroids are also seen in the cells of interzone (Vasse *et al.*, 1990). The zone IV is the nitrogen fixation zone in which large plant cells containing nitrogen-fixing bacteroids are seen. The older nodules have zone V which is called senescent zone; degenerating rhizobia are present in this zone. Thiol proteases have been found to be active in senescent nodules (Pladys and Vance, 1993).

2.3 Nitrogen fixation genes

In biological nitrogen fixation gaseous nitrogen is converted to ammonia and hydrogen in the presence of nitrogenase enzyme. The stoichiometry of the overall reaction is as follows.

$$N_2 + 8H^+ + 8e^- \xrightarrow{16Mg\ ATP \longrightarrow 16Mg\ ADP + 16Pi\ /\ nitrogenase} 2NH_3 + H_2 \uparrow$$

Nitrogen fixation genes are designated as *nif*/*fix* genes. These genes were initially characterized in *Klebsiella pneumoniae* in which 20 adjacent *nif* genes are organized in eight operons in a 24 kb region of DNA. Rhizobial *fix* genes, which are equivalent to *K. pneumoniae nif* genes, are assigned the same names (Beringer *et al.*, 1980; Arnold *et al.*, 1988). The structural genes that code for nitrogenase enzyme are *nifHDKE*. The names and proposed functions of *nif* and *fix* genes of rhizobia are given in Table 2.

The *nod*, *nif* and *fix* genes are located on symbiotic plasmids in *S. meliloti*, *R. leguminosarum* and *Rhizobium* spp. NGR234 whereas these genes are present on chromosome in *R. loti*, *Bradyrhizobium* spp. and *Azorhizobium* spp. *S. meliloti* has two megaplasmids of about 1400 kb (pSym-a or megaplasmid 1) and 1700 kb (pSym-b or

Table 2: Functions of *nif* and *fix* genes in *Sinorhizobium meliloti, Bradyrhizobium japonicum* or *Azorhizobium caulinodans* (Reproduced from Vij, 2000)

Gene	Proposed function and/ or product
***nif* genes**	
nifA	Positive regulator of *nif, fix* and additional genes
nifH	Fe protein of nitrogenase
nifD	α subunit of MoFe protein of nitrogenase
nifK	α subunit of MoFe protein of nitrogenase
nifB	Involved in FeMo cofactor biosynthesis
nifE	Involved in FeMo cofactor biosynthesis
nifN	Involved in FeMo cofactor biosynthesis
nifS	Cysteine desulfurase
nifW	Required for full activity of FeMo protein
nifX	Unknown function
***fix* genes**	
fixABCX	Required for nitrogenase activity: *fix X* shows similarity to ferridoxins
fixNOQP	Microaerobically-induced, membrane-bound cytrochrome oxidase
fixGHIS	Redox process-coupled cation pump
fixLJ	Oxygen-responsive two component regulatory system involved in positive control of *fixK* (*S. meliloti*, *B. japonicum* and *A. caulinodans*) and *nifA* (*S. meliloti*)
$fixK/fixK_2$	Positive regulator of *fixNOQP* (*S. meliloti*, *B. japonicum*, *A. caulinodans*), *nifA* (*A. caulinodans*), nitrate respiration (*B. japonicum*) and negative regulator of *nifA* and *fixK* (*S. meliloti*)
fixR	Similar to NAD-dependent hydrogenases
fixU	Unknown
fixW	May participate in a nitrogenase specific e^- transport system

megaplasmid 2)(Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Hynes *et al.*, 1986; Burkhardt *et al.*, 1987; Sobral *et al.*, 1991; Honeycutt *et al.*, 1993).

The cluster I (*nifHDKE*, *nifN*, *fixABCX*, *nifA*, *nifB*) and cluster II (*fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) of nitrogen fixation genes are located on megaplasmid 1(Batut *et al.*, 1985; David *et al.*, 1987). *nifH*, *nifD* and *nifK* genes code for the nitrogenase polypeptides (Ruvkun and Ausubel, 1980; Rosenberg *et al.*, 1981). These genes, like their counterparts in *K. pneumoniae*, are located in a single operon (*nifHDK*) in *S. meliloti* (Ruvkun *et al.*, 1982). In *B. japonicum* 110, *nifH* and *nifDK* are located on separate operons (Kaluza *et al.*, 1983).

The *fixA*, *fixB*, *fixC*, and *fixX* genes required for nitrogen fixation in nodules have been identified in *S. meliloti* (Ruvkun *et al.*, 1982; Corbin *et al.*, 1983; Pühler *et al.*, 1984; Earl *et al.*, 1987), *B. japonicum* (Fuhrmann *et al.*, 1985; Gubler and Hennecke, 1986), *A. caulinodans* (Kaminski *et al.*, 1988; Arigoni *et al.*, 1991), *R. leguminosarum* bv. *viciae* (Grönger *et al.*, 1987) and *R. leguminosarum* bv. *trifolii* (Iismaa *et al.*, 1989). These genes are organized in a single operon in all species except *B. japonicum*.

Aguilar *et al.* (1985) characterized a nitrogenase fixation gene, *fixF*, located near the common nodulation genes in *S. meliloti* and found that this gene was transcribed in the symbiotic state. The optical and electron microscopic examinations of the structure of nodules induced by *fixAB* and *nifAB* mutants of the wild type strain 1021 in alfalfa root systems revealed that *nifA* may regulate some as yet undiscovered symbiotic genes for products required for the final steps of bacteroid differentiation (Hirsch and Smith, 1987). The predicated amino acid sequences of *fixNOQP* proteins indicate that these genes encode membrane-bound cytochrome *c*-containing heme/copper cytochrome oxidase

(Mandon *et al.*, 1994). Expression from the promoters of *S. meliloti* regulatory genes, *nifA* and *fixK* and structural genes, *nifH* and *fixA*, in other fast-growing rhizobia was measured. Both *nifH* and *fixA* promoters showed heterologous activation; *nifA* and *fixK* promoters were activated only in *S. tropici* (Cebolla *et al.*, 1994). *S. meliloti* strains harboring IncQ and IncP multicopy vectors containing constitutively expressed *K. pneumoniae nifA* were reported to exhibit an increased nodulation competitiveness on alfalfa (Sanjuan and Olivares, 1991). It has been found later that the observed enhancement in nodulation competitiveness by IncQ derivatives carrying *K. pneumoniae nifA* was not dependent on the plasmid-borne *nifA* activity but on the sensitivity of nonresistant strains to the streptomycin carried over from growth cultures (van Dillewijn *et al.*, 1998).

2.4 Mutagenesis in rhizobia

Both chemical and transposon mutagenesis techniques have been used to identify the rhizobial genes of symbiotic importance. Chemical mutagens like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethyl methane sulphonate (EMS) and nitrous acid, were used to isolate rhizobial mutants defective in metabolic pathways and symbiosis (Scherrer and Dénarié, 1971; Fedorov and Zaretskaya, 1977; Pain, 1979; Singh *et al.*, 1984; Noel *et al.*, 1988; McIver *et al.*, 1989; Agarwal, 1994).

Random mutagenesis of genomic DNA with transposon Tn5 involves the introduction of Tn5 "suicide" plasmid from *E. coli* into rhizobia by conjugation (Fig.3 & 4). Such plasmids cannot be stably maintained in rhizobia and selection for neomycin-resistant transconjugants leads directly to the isolation of random Tn5 insertion mutants

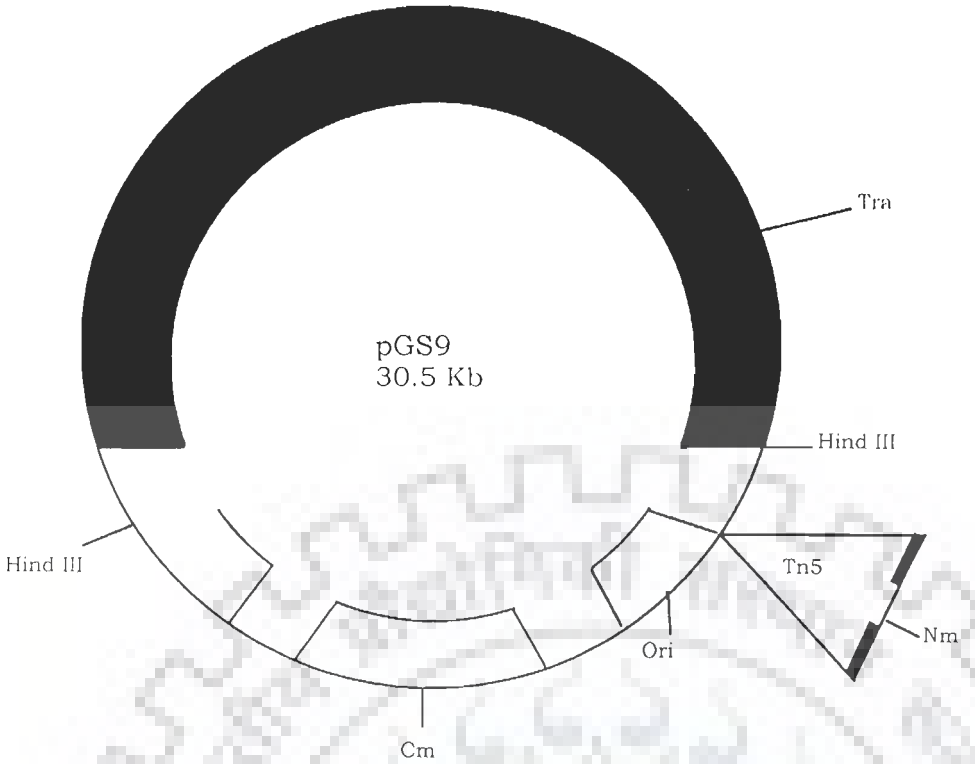


Fig. 3: Suicide vector pGS9 carrying Tn5 (based on Selvaraj and Iyer, 1983)

Abbreviations: Cm, chloramphenicol; Nm, neomycin; Ori, origin of replication

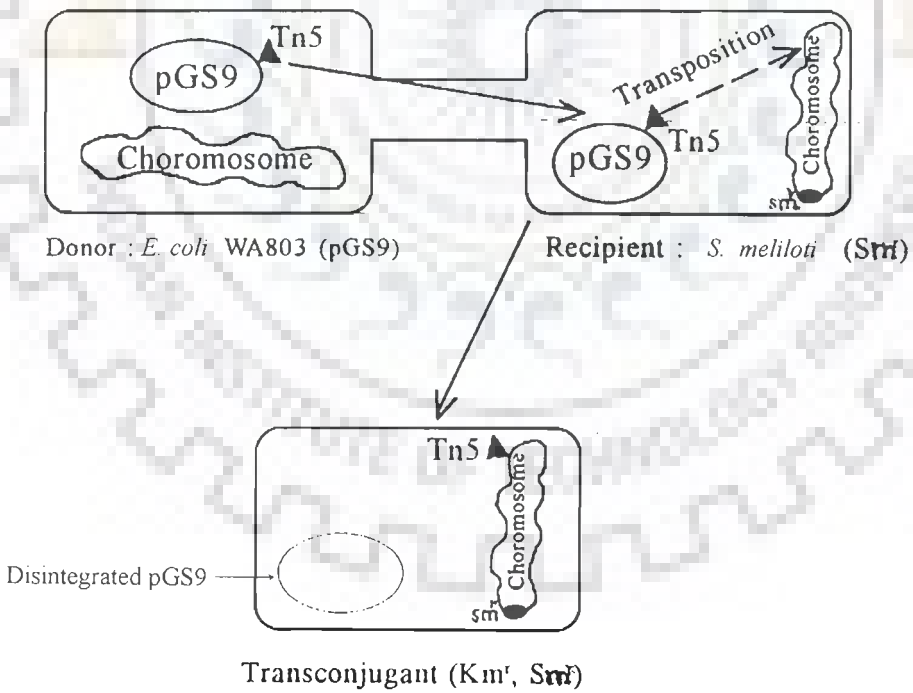


Fig. 4: Diagrammatic representation of transposon Tn5 insertion (based on Selvaraj and Iyer, 1983)

(Beringer *et al.*, 1978; Selvaraj and Iyer, 1983; Simon *et al.*, 1983). This method has been used by several researchers to isolate transposon Tn5-induced mutants of several rhizobial species (Meade *et al.*, 1982; Forrai *et al.*, 1983; Kerppola and Kahn, 1988b; Khanuja and Kumar, 1989; Barsomian *et al.*, 1992; Ortuño-Olea and Durán-Vargas, 2000; Prasad *et al.*, 2000).

Site-directed mutagenesis has proved very useful in the genetic analysis of rhizobia. Ruvkun and Ausubel (1981) described for prokaryotes site-directed mutagenesis method which is called as homogenization, marker exchange, and gene replacement method. In this method a cloned DNA fragment is mutagenized with Tn5 in *E. coli* and subsequently inserted into the genome of the original host by recombination. Several rhizobial genes have been characterized by site-directed mutagenesis method (Ruvkun and Ausubel, 1981; Ausubel, 1982; Ruvkun *et al.*, 1982; Corbin *et al.*, 1983; Watson and Rastogi, 1993; Kereszt *et al.*, 1998). Goryshin and Reznikoff (1998) have developed *in vitro* Tn5 transposition system for site-directed mutagenesis.

2.5 Studies on the mapping of rhizobial genome

Doctor and Modi (1976) mapped leucine and isoleucine-valine loci in *B. japonicum* D-211[s] by transformation analysis. The results suggested a common pathway for isoleucine-valine biosynthesis. Three-point reciprocal crosses indicated that only three of the *leu* mutational sites, i.e., F18, F6, and F99 are linked; F98 and F90 did not show any linkage. IncP1 group plasmid R68.45 (Haas and Holloway, 1976) was used to construct circular linkage maps of the chromosome of *S. meliloti* Rm41 (Kondorosi *et al.*, 1977), *R. leguminosarum* 300 (Beringer *et al.*, 1978) and *R. leguminosarum* bv. *trifolii*

(Megias *et al.*, 1982). Plasmid RP4 (Datta *et al.*, 1971) helped in the construction of circular chromosomal maps of *S. meliloti* Rm2011. Twenty-five mutants unable to utilize nitrate as sole nitrogen source were isolated from *S. meliloti* Rm41. These mutations mapped at four different sites, *narA*, *narB*, *narC* and *narD*; *narB*, *C* and *D* were located between trp-*15* and ade-*15* on the chromosome (Kiss *et al.*, 1979). Kondorosi *et al.* (1980) compared circular linkage maps of *S. meliloti* 2011, *S. meliloti* Rm41 (Fig.5) and *R. leguminosarum* 300 by suppression tests of mutations. Chromosomal locations of mutations in 1 Nod$^-$ and 11 Fix$^-$ derivatives of *S. meliloti* strain 41 were ascertained by transferring the chromosome by plasmid R68.45, in eight fragments, into symbiotically effective recipients and testing the recombinants for symbiotic phenotype. In five mutants, the *fix* alleles were localized on different chromosomal regions (Forrai *et al.*, 1983).

Ziegler *et al.* (1986) constructed a genetic map of the *fla-che* region of the *S. meliloti* SU47 chromosome using co-transduction with bacteriophage ΦM12. Symbiotically important genes *hemA* and *ntrA* were mapped on the chromosome of *Rhizobium* sp. NGR234 (Østerås *et al.*, 1989). Klein *et al.* (1992) developed a system for mapping of the *S. meliloti* chromosome. This system utilizes transposon Tn5-Mob which carries the mobilization site of IncP plasmid RP4 (Simon, 1984). Insertions of Tn5-Mob that were located at particular sites on the *S. meliloti* chromosome were isolated and served as origins of high-frequency chromosomal transfer when IncP *tra* functions were provided in *trans*. Honeycutt *et al.* (1993) reported the physical map of the genome of *S. meliloti* 1021. The usefulness of the map was demonstrated by localizing a total of 12 previously unmapped transposon insertions in the genome. Correlated physical and

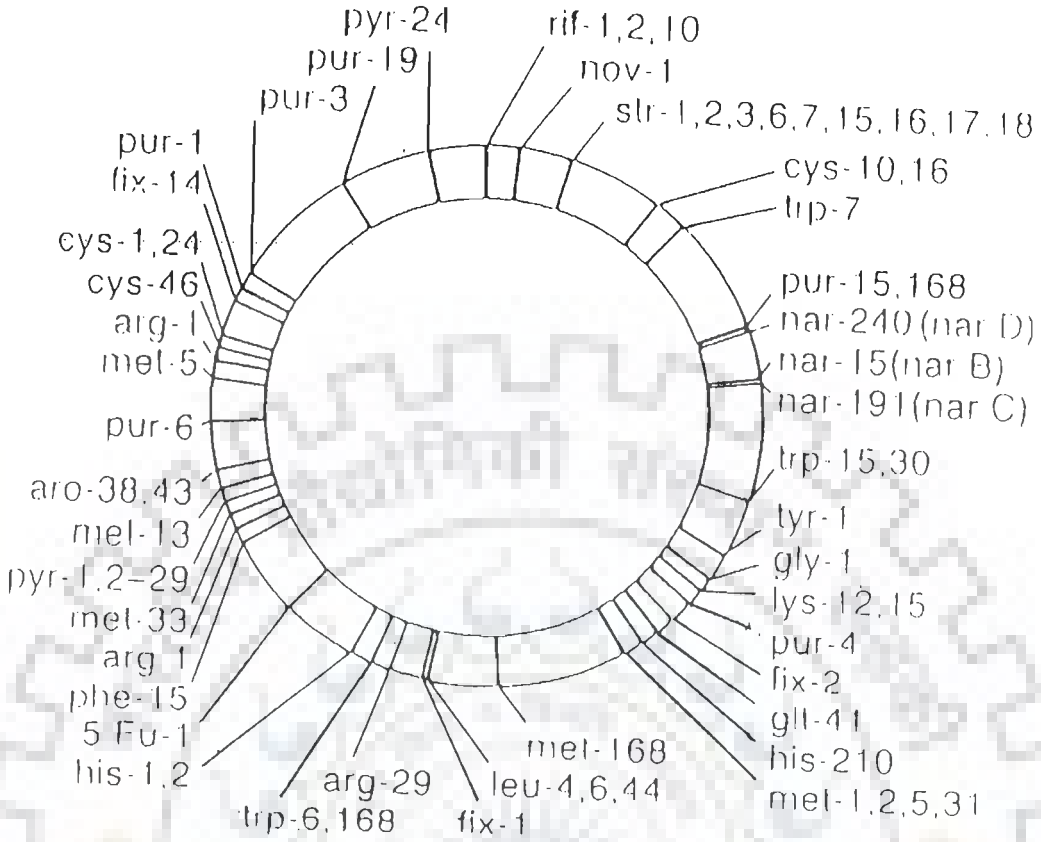


Fig. 5: Chromosomal map of *Sinorhizobium meliloti* Rm41 (based on Kondorsi *et al.*, 1980)

genetic map of the *B. japonicum* 110 genome indicated that this organism has a single, circular chromosome that is approximately 8,700 kb long. The genes directly concerned with nodulation and symbiotic nitrogen fixation are clustered in a chromosomal section that is about 380 kb long (Kündig *et al.*, 1993). Poole *et al.* (1994) identified chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *R. leguminosarum* bv. *viciae* 3841. The NAD (+)-dependent malic enzyme (*dme*) gene was mapped by conjugation between the *cys-11* and *leu-53* markers on the *S. meliloti* chromosome (Driscoll and Finan, 1997). The genes for capsular polysaccharides, *rkpA - J* genes, of *S. meliloti* Rm41 have been located in the chromosomal *rkp-1* region (Petrovics *et al.*, 1993; Kiss *et al.*, 1997). A high-density physical map of *S. meliloti* 1021 chromosome derived from bacterial artificial chromosome library has been reported (Capela *et al.*, 1999). Cai *et al.* (2000) have identified two *S. meliloti* Rm1021 chromosomal loci affecting the poly-3-hydroxybutyrate degradation pathway.

Several symbiotic genes of rhizobia are located on megaplasmids. Genetic and physical maps of megaplasmids of various rhizobial strains have been prepared. In *S. meliloti*, genes involved in nodule induction (*nod*), nitrogenase activity (*nif*) and other important function of nodulation have been located on the megaplasmid pSm-a (Fig.6), and genes required for exopolysaccharide synthesis (*exo*, *exp*), thiamine synthesis (*thi*) and dicarboxylic acid transport (*dct*), as well as two fix genes have been mapped on pSym-b (Fig.7) (Banfalvi *et al.*, 1981; Julliot *et al.*, 1984; Kondorosi *et al.*, 1984; Batut *et al.*, 1985; Glazebrook and Walker, 1989; 1991; Charles and Finan, 1990).

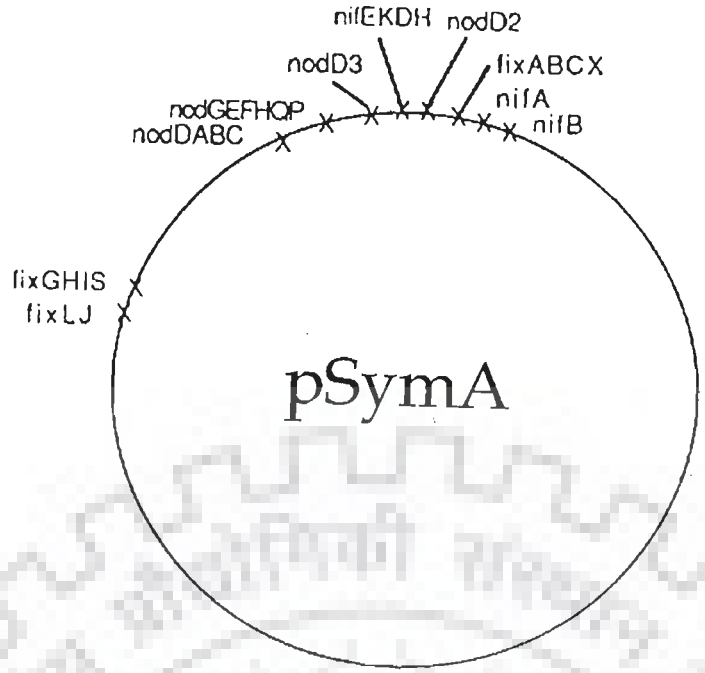


Fig. 6: Genetic map of plasmid pSymA of *Sinorhizobium meliloti* (Glazebrook and Walker, 1991)

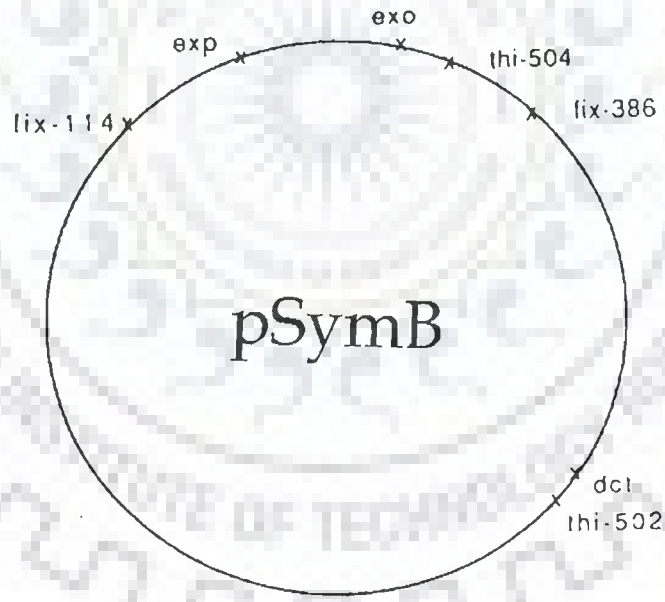


Fig. 7: Genetic map of plasmid pSymB of *Sinorhizobium meliloti* (Glazebrook and Walker, 1991)

Genetic map of *S. meliloti* megaplasmid pRme SU47b was constructed and the positions of previously identified loci for nitrogen fixation, exopolysaccharide synthesis, thiamine synthesis, and C_4-dicarboxylate transport on the megaplasmid map were determined (Charles and Finan, 1990). Perret *et al.* (1991) prepared the physical map of symbiotic plasmid of *Rhizobium* sp. NGR234. van Rhijn *et al.* (1996) described the isolation and characterization of a pSym locus of *Rhizobium* sp. BR816, an isolate of *Leucaena leucocephala*. Miranda-Ríos *et al.* (1997) reported the cloning and sequence analysis of four genes, located on plasmid pb, which are involved in the synthesis of thiamine in *R. etli* (*thiC*, *thiO*, *thiG*, and *thiE*). Freiberg *et al.* (1997) presented the complete nucleotide sequence and gene complement of the plasmid from *Rhizobium* sp. NGR234 that endows the bacterium with the ability to associate symbiotically with leguminous plants. A new 1047 bp long insertion sequence, located in the intergenic region between *nodJ* and *nodQ* of *S. meliloti* has been reported (Biondi *et al.*, 1999). To facilitate sequencing of the *S. meliloti* 1021 pSm-a megaplasmid, a high-resolution map was constructed by ordering 113 overlapping bacterial artificial chromosome clones with 192 markers (Barloy-Hubler *et al.*, 2000).

2.6 Role of bacteroidal metabolism in symbiosis

2.6.1 C_4-dicarboxylate transport and utilization

In nodules, rhizobia utilize C_4-dicarboxylates from the plant host for their carbon and energy requirements. Finan *et al.* (1983) determined the symbiotic properties of two transposon (Tn5)-mediated C_4-dicarboxylate transport (*dct*) mutant strains GF31 and GF252 of *R. leguminosarum* strain GF160. Strain GF31 formed ineffective nodules, and

bacteroids from these nodules showed no succinate transport activity. Strain GF252 formed partially effective nodules, and bacteroids from these nodules showed about 50% of the succinate transport activity of the parent strain. These data indicated that the presence of a functional C_4-dicarboxylic acid transport system is essential for N_2 fixation to occur in pea nodules.

A recombinant plasmid, pRK290:4:46, encoding *S. meliloti* sequences involved in dicarboxylic acid transport, was used for analysis of the relationship between dicarboxylic acid transport and nitrogen-fixation in *B. japonicum* CJ1. Expression of the *dct* sequences on this plasmid in *B. japonicum* gave increased growth rates in culture media containing dicarboxylic acids as the sole C-source. Under free-living nitrogen-fixing conditions, strain CJ1 (pRK290:4:46) exhibited higher nitrogenase (EC-1.18.2.1) activity compared with that of the wild type strain (Birkenhead *et al.*, 1988).

S. meliloti strains mutated in the *ntrA* gene are incapable of dicarboxylate transport (Ronson *et al.*, 1987; Watson, 1990). Aspartate, like succinate, fumarate, and malate, has been shown to require the dicarboxylate transport system for transport into *S. meliloti* for use as a carbon source; *dct* and *ntrA* mutants cannot utilize aspartate (Watson *et al.*, 1988; Watson, 1990). Rastogi and Watson (1991) demonstrated that *S. meliloti* contains an aspartate aminotransferase activity required for symbiotic nitrogen fixation and aspartate is an essential substrate for bacteria in the nodule.

The *S. meliloti* C_4-dicarboxylate transport gene *dctA* is essential for symbiotic nitrogen fixation. Under free-living conditions, the expression of *dctA* is fully dependent on the cognate regulatory genes *dctBD*. Gene fusions studies established that the DctBD-dependent *dctA* expression was occurring throughout the whole nodule (Boesten *et al.*,

1998). Yurgel *et al.* (2000) found that the permease component of the Dct system, DctA, of *S. meliloti* Rm8002 could transport orotate, a monocarboxylic acid. The evaluation of 17 compounds as inducers and inhibitors of transport suggested that substrates recognized by *S. meliloti* Rm8002 DctA must have appropriately spaced carbonyl groups and an extended conformation, while good inducers are more likely to have a curved conformation.

2.6.2 Role of biosynthetic pathways of amino acids, nucleotide bases and vitamins in symbiosis

Rhizobial bacteria require the primary building blocks, *viz.*, amino acids, nucleotide bases and vitamins, during colonization of plant roots and all stages of the symbiotic process. These bacteria have the ability to synthesize the primary building blocks from simple nutrient materials. These building blocks and/or their intermediates may also be obtained from the host plant. A few genes involved in the biosynthesis of some of the primary building blocks have been found to play a direct role in symbiosis. The possible roles of specific intermediates at various stages of symbiotic interactions have been determined by isolating auxotrophic mutants of rhizobia and studying their symbiotic properties with legume plants.

2.6.2.1 Biosynthesis of amino acids and symbiosis

2.6.2.1.1 Arginine

The mutants of *S. meliloti* blocked in the latter part of the arginine biosynthetic pathway, in either ornithine transcarbamylase or argininosuccinate synthetase, were effective (Dénarié *et al.*, 1976; Fedorov and Zaretskaya, 1977; Kerppola and Kahn, 1988b). The mutants of *S. meliloti* 104A14 strain blocked in the early part of the arginine

biosynthetic pathway that leads to ornithine were ineffective (Kerppola and Kahn, 1988b). These results indicated that the symbiotic defect of the ornithine auxotrophs was probably not related to the role of ornithine as an arginine precursor.

2.6.2.1.2 Aspartate

Kim *et al.* (1988) isolated by transposon Tn5 mutagenesis two aspartate mutants of *S. fredii* HH303. These mutants induced nodules on soybean but the nodules lacked the characteristic pink color inside and were unable to fix nitrogen. Addition of aspartic acid to the plant growth medium did not restore the symbiotic effectiveness of the mutants.

2.6.2.1.3 Asparagine

Twenty-one asparagine auxotrophs of *S. meliloti* 104A14 were isolated by Kerppola and Kahn (1988b) using nitrous acid mutagenesis followed by penicillin enrichment. Seventeen auxotrophs formed nodules on alfalfa plants but these nodules were unable to fix nitrogen. Symbiotic properties of the remaining asparagine auxotrophs could not be checked due to the problem of reversion to prototrophy.

2.6.2.1.4 Cysteine

Cysteine-dependent mutants of *S. meliloti* 2011, obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and penicillin treatment, have been found to be as effective as the parental type strain (Scherrer and Dénarié, 1971). Two transposon Tn5-induced cysteine auxotrophs of *S. fredii* HH303 also formed symbiotically effective nodules on soybean (Kim *et al.*, 1988). By its inability to grow on sulfate as the sole sulfur source a mutant strain (CTNUX8) of *R. etli* carrying Tn5 was isolated and characterized by Taté *et al.* (1997). Sequence analysis showed that Tn5 was inserted into a *cysG* (siroheme synthetase)-homologous gene. *cysG*-like gene had a basal level expression in thiosulfate-

or cysteine- grown cells and was induced when sulfate or methionine was used. The strain CTNUX8 induced pink, effective (N_2 fixing) nodules on the roots of *Phaseolus vulgaris* plants. These results indicated that sulfate (or sulfite) is the sulfur source of *R. etli* in the rhizosphere, while cysteine, methionine, or glutathione is supplied by the root cells to bacteria growing inside the plant.

2.6.2.1.5 Glycine

Scherrer and Dénarié (1971) isolated 3 glycine-dependent auxotrophic mutants of *S. meliloti* 2011 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and penicillin treatment. These auxotrophs were more effective in nitrogen fixation than the parental strain.

2.6.2.1.6 Histidine

Histidine auxotrophs of *B. japonicum* have been reported to be Nod^-; the external supplementation of histidine restored nodulation in these mutants (Sadowsky *et al.*, 1986; So *et al.*, 1987; Yadav *et al.*, 1998). Two Tn5-induced histidine auxotrophs of cowpea rhizobia strain IRC256 have been reported to be Nod^+ Fix^+ (McLaughlin *et al.*, 1987).

2.6.2.1.7 Isoleucine and Valine

Two isoleucine and valine auxotrophs of *S. fredii* HH303 were obtained by transposon Tn5 mutagenesis. These auxotrophs formed ineffective nodules on soybean, and addition of isoleucine and valine to the plant nutrient medium did not restore the symbiotic effectiveness of the auxotrophic mutants (Kim *et al.*, 1988). Aguilar and Grasso (1991) found that a Tn5-induced isoleucine and valine auxotroph (1028) of *S. meliloti* 1021 failed to induce nodules on alfalfa. The addition of α-ketoisovalerate and isoleucine to the plant medium did not restore the symbiotic defect in this mutant. The

Tn5 insertion was located in the *ilvC* gene which codes for isomeroreductase, the second enzyme in the parallel pathways for the biosynthesis of isoleucine and valine. In this mutant the common nodulation genes *nodABC* were not activated by the inducer luteolin. These results indicated that an active isomeroreductase enzyme is required for successful nodulation of alfalfa. It was proposed that the enzyme isomeroreductase in *S. meliloti* 1021 is able to recognize substrates other than those in isoleucine-valine pathway and that the product of its conversion is required for the expression of nodulation genes.

2.6.2.1.8 Leucine

Inoculation of lucerne seedlings with four leucine-requiring (Leu^-) mutants of *S. meliloti* strain L5-30 resulted in the formation of ineffective nodules. In these nodules, bacteria were not released from the infection threads into the host cytoplasm. Supplying the defective plant-bacterial system with L-leucine or one of its precursors, α-ketoisovalerate or α-ketoisocaproate, caused the release of rhizobia into the plant cytoplasm and a restoration of nitrogen fixation (Truchet *et al.*, 1980). Leucine mutants of *S. meliloti* strain 104A14 formed nodules on alfalfa but these nodules were unable to fix nitrogen. Addition of leucine to the plant growth medium did not restore effectiveness to any of these mutants (Kerppola and Kahn, 1988b).

Nichik *et al.* (1995) obtained by transposon Tn5 mutagenesis a mutant of *S. meliloti* SAM1 having a block in leucine biosynthetic pathway. Addition of leucine to lucerne plants inoculated by the leucine auxotroph did not cause the restoration of symbiosis. The mutant nodule bacteria penetrating in root hair formed infection threads. However, rhizobia were not released from infection threads and perished within them.

2.6.2.1.9 Methionine

Two methionine-dependent mutants of *S. meliloti* 2011 strain were obtained after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and penicillin treatment. These mutants induced nodule formation on lucerne and were effective, like the parental strain, in nitrogen fixation (Scherrer and Dénarié, 1971). Kerppola and Kahn (1988b) obtained four methionine auxotrophs of *S. meliloti* 104A14 strain by nitrous acid mutagenesis followed by penicillin enrichment. These mutants induced nodules on alfalfa plants but in contrast to the above report the nodules were ineffective in nitrogen fixation. The difference may be due to the step affected by the mutations, or it may be a cause of strain-specific requirements (Kerppola and Kahn, 1988b).

On the basis of the characterization of a Tn5 induced mutant strain (CTNUX23) of *R. etli* CE3, Taté *et al.* (1999c) concluded that *metZ* gene, required for the biosynthesis of methionine, is essential for nodulation of *Phaseolus vulgaris*. According to these workers cysteine or glutathione, but not methionine, is supplied by the root cells to bacteria growing inside the plant.

2.6.2.1.10 Proline

Proline auxotrophic, catabolic and overproducing mutants of *R. leguminosarum* bv. *viciae* strain C1204b were generated and used as inoculants of pea (*Pisum sativum* L. cv Alaska). Cytosolic and bacteroid levels of proline determined for the nodular tissue did not support the role of proline as a key metabolite supplied by the host (Chien *et al.*, 1991).

2.6.2.1.11 Phenylalanine

Prasad *et al.* (2000) obtained a Tn5 induced phenylalanine auxotrophic mutant (RH38) of *S. meliloti* Rmd201. This mutant formed nodules on alfalfa plants but the nodules were completely ineffective in nitrogen fixation. Results suggested that the alfalfa plant host does not provide phenyalanine to bacteroids in nodules.

2.6.2.1.12 Tryptophan

Wells and Kuykendall (1983) isolated 11 tryptophan-requiring mutants of *B. japonicum* strain USDA I-110 ARS by nitrous acid mutagenesis. Only three auxotrophs with defects specifically in tryptophan synthetase were found to be capable of forming nodules. Kummer and Kuykendall (1989) reported that except tryptophan synthase mutants all other tryptophan auxotrophs of *B. japonicum* strain USDA I-110 did not induce nodules on soybean host plants. It was suggested that indole glycerol phosphate, an intermediate of tryptophan biosynthetic pathway, is necessary for nodulation in *B. japonicum*. The *trpE* mutants of *S. meliloti* Rm1021 have been reported to be Nod^+ but the nodules induced by these mutants revealed significant differences from the nodule structure of parental strain and fixed comparatively less nitrogen (Barsomian *et al.*, 1992; Prasad *et al.*, 2000). These reports indicated that alfalfa plant host provides tryptophan to bacteroids in nodules.

Barsomian *et al.* (1992) hypothesized on the basis of the reports of Rioux *et al.* (1986a; 1986b) that anthranilic acid, synthesized by the expression of *trpE* gene, acts as an *in planta* siderophore helping iron uptake for bacterial development. Normal flow of metabolites through the biosynthetic pathways of aromatic amino acids has been found to be essential for the bacteroid development (Jelesko *et al.*, 1993). Recently Taté *et al.*

(1999b) have reported that the *trpB* gene of *R. etli* strain CTNUX4 is essential for effective symbiotic interaction.

2.6.2.1.13 Tyrosine

Two tyrosine mutants of *S. meliloti* 104A14, obtained by nitrous acid mutagenesis, were reported to induce ineffective nodules on alfalfa plants (Kerppola and Kahn, 1988b). Prasad *et al.*, (2000) found normal symbiotic activity (Nod^+ Fix^+) for a Tn5-induced tyrosine auxotroph of *S. meliloti* Rmd201.

2.6.2.2 Biosynthesis of purines and symbiosis

Several workers have reported defective symbiosis for purine auxotrophs of *S. meliloti* (Scherrer and Dénarié, 1971; Malek and Kowalski, 1983; Kerppola and Kahn, 1988b; Swamynathan and Singh, 1992; 1995; Gupta, 1996), *R. leguminosarum* (Pankhurst and Schwinghamer, 1974; Pain, 1979), *S. fredii* HH303 (Kim *et al.*, 1988), *Rhizobium* strain NGR234 (Chen *et al.*, 1985) and *R. etli* (Noel *et al.*, 1988). The purine auxotrophs induce pseudonodules that lack bacterial content, show centrally located vascular tissue, and fail to reduce atmospheric nitrogen.

Biochemical block in symbiotically defective purine auxotrophs of *R. leguminosarum* was before 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Pankhurst and Schwinghamer, 1974). Symbiotic defectiveness of two adenine auxotrophs of *Rhizobium* strain NGR234 appeared to be due to EPS overproduction (Chen *et al.*, 1985). Purine auxotrophs of *R. etli* induced pseudonodules on bean plants. These auxotrophs induced root hair curling and cortical cell division but did not elicit infection thread formation. No bacteria were isolated from the pseudonodules (Noel *et al.*, 1988). Addition of adenosine to the plant growth medium had no effect on nodule

phenotype but the supplementation of 5-aminoimidazole-4-carboxamide riboside (AICA-riboside), the unphosphorylated form of the purine precursor 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) significantly enhanced root nodule development (Noel *et al.*, 1988; Newman *et al.*, 1992).

Purine auxotrophs of *S. fredii* HH303 and *R. leguminosarum* bv. *viciae* 128C56 having biochemical block before AICAR induced poorly developed nodules which did not contain rhizobia. Addition of AICA-riboside to the plant nutrient medium enhanced nodulation and promoted nitrogen fixation (Newman *et al.*, 1994).

The transposon Tn5-induced purine auxotrophs of *Rhizobium* strain NGR234 (ANU2861 and ANU2866) were found to be defective for nodule formation on a wide variety of legumes and the non-legume *Parasponia*. The strains ANU2861 and ANU2866 produce less acidic exopolysaccharides and cyclic β-(1→2)-glucans per gram of cells than does the parent strain (Batley *et al.*, 1987) although they produce colony types that appear excessively mucoid on complex agar medium (Chen *et al.*, 1985).

On *Macroptilium atropurpureum* (siratro) ANU2861 rapidly induced a reaction analogous to a hypersensitive response at the site of infection (Djordjevic *et al.*, 1988). When the plant growth medium was supplemented with AICA-riboside, strains AUN2861 and ANU2866 induced the formation on siratro of root outgrowths that resembled Fix$^-$ nodules. The strain ANU2861 was found to possess a defective lipopolysaccharide that was corrected by the addition of AICA-riboside to the growth medium. These results indicated that metabolic flow through this pathway is essential for nodule morphogenesis (Djordjevic *et al.*, 1996). Stevens *et al.* (2000) have shown that *purM* and *purN*, which specify early steps in purine biosynthesis, are needed for

nodulation of peas by *R. leguminosarum* bv. *viciae*. These genes were found to express at relatively high levels even when the metabolic products of the pathway were present.

2.6.2.3 Biosynthesis of pyrimidines and symbiosis

Scherrer and Dénarié (1971), by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and penicillin treatment, isolated a pyrimidine auxotroph of *S. meliloti* 2011 strain. This auxotroph induced ineffective nodules on alfalfa plants. Two out of 15 pyrimidine auxotrophs of *R. leguminosarum* 3000 were defective in symbiosis (Pain, 1979). Supplementation of uridine to the plant growth medium did not result in the suppression of Ndv^- (nodule development defective) phenotype of pyrimidine auxotroph of *R. leguminosarum* bv. *phaseoli* strain CFN42 (Noel *et al.*, 1988). Kerppola and Kahn (1985; 1988a; 1988b) reported that mutations in carbamoyl phosphate synthetase gene and the genes controlling the steps of pyrimidine biosynthesis before orotic acid resulted in ineffective symbiosis in *S. meliloti* strain 104A14.

Kim *et al.* (1988) obtained two uracil-requiring mutants of *S. fredii* HH303 by transposon Tn5 mutagenesis. These mutants induced ineffective nodules (Nod^+ Fix^-) on soybean plants. Addition of uracil to the plant growth medium did not restore the symbiotic effectiveness to these auxotrophs.

Vineetha *et al.* (2001) isolated 23 pyrimidine auxotrophs of *S. meliloti* Rmd201 by transposon Tn5 mutagenesis. These auxotrophs, classified into *car*, *pyrC* and *pyrE*/*pyrF* categories, induced white nodules which were ineffective in nitrogen fixation. Light and electron microscopic studies revealed that the nodules induced by *pyrC* mutants were more developed than the nodules of *car* mutants. Similarly the nodules induced by *pyrE*/*pyrF* mutants had more advanced structural features than the nodules of

pyrC mutants. These results indicated that some of the intermediates and/or enzymes of pyrimidine biosynthetic pathway of *S. meliloti* play a role in bacteroidal transformation and nodule development.

2.6.2.4 Biosynthesis of vitamins and symbiosis

2.6.2.4.1 Biotin

The studies of Streit *et al.* (1996) done with *S. meliloti* 1021 showed that both synthesis and uptake of biotin promote colonization of alfalfa roots by these bacteria. Two lines of evidence indicated that plant-derived biotin normally promotes root colonization (i) adding avidin, a biotin binding reagent, significantly reduced rhizosphere growth of *S. meliloti* 1021, and (ii) growth of Tn5-induced biotin auxotrophs still increased 10-fold in the rhizosphere. Synthesis, however, was found to be more important source of biotin for *S. meliloti* 1021 because in root colonization tests biotin auxotrophs competed very poorly with the parent strain.

2.6.2.4.2 Nicotinic acid

A Tn5-induced nicotinic acid auxotrophic mutant of *S. fredii* HH303 was isolated by Kim *et al.* (1988). These workers observed that this mutant produced mature nodules like those of the parental strain, but the nodules lacked the characteristic pink color inside and were unable to fix nitrogen. Addition of nicotinic acid to the plant growth medium did not restore the symbiotic effectiveness of the mutant. These results indicated that nicotinic acid is not supplied by the legume host and that synthesis of nicotinic acid is important for effective nodule formation.

2.6.2.4.3 Riboflavin

Schwinghamer (1970) and Pankhurst *et al.* (1972) showed that riboflavin required for growth *in vitro* by a mutant of *R. leguminosarum* bv. *trifolii*, was also required by this mutant for the conversion of vegetative cells into functional N_2-fixing bacteroids within root nodules on *Trifolium pratense*. The demand for riboflavin shown by the mutant appeared to be greater for bacteroid formation than for vegetative growth. Riboflavin has been found to be key growth factor for alfalfa root colonization by *S. meliloti* 1021 (Streit *et al.*, 1996).

2.6.2.4.4 Thiamine

Thiamine has been shown to be a key growth factor for alfalfa root colonization by *S. meliloti* 1021 (Streit *et al.*, 1996).

2.6.2.4.5 Pantothenic acid

A Tn5 induced pantothenic acid auxotroph of *S. fredii* HH303 has been reported to form effective nodules similar to the parental strain on soybean plants (Kim *et al.*, 1988).

2.7 Other genes involved in symbiosis

Some genes other than those mentioned above are also involved in the process of symbiosis. Two H_2 uptake-negative (Hup^-) *B. japonicum* mutants were obtained that also lacked symbiotic N_2 fixation (acetylene reduction) activity (Moshiri *et al.*, 1983). A novel gene, *slp*, required for nodulation competition on the common bean, has been isolated from *R. etli* strain TAL182 (You *et al.*, 1998).

The cytochrome production pattern of a *R. etli* strain CE3 *ntrC* mutant (CFN2012) was studied and the results suggested that the overproduction of cbb_3 terminal oxidase correlates with an enhancement in symbiotic nitrogen fixation (Soberón *et al.*, 1999). Aspartate aminotransferase (AAT), which plays a critical role in the assimilation of symbiotically fixed nitrogen into aspartate and asparagine in legume root nodules, occurs as a cytosolic form (AAT1) and a plastid form (AAT2) in alfalfa nodules. Studies on the distribution pattern of gene transcripts suggested that AAT1 has a role in maintenance of the O_2 diffusion barrier in nodules and that AAT_2 plays a major role in assimilation of recently fixed nitrogen (Yoshioka *et al.*, 1999). Taté *et al.* (1999a) have reported that the ectopic expression of the *R. etli* strain CE3 *amtB* (ammonium / methylammonium transport B) gene in bacteroids alters the ability of bacteria to invade the host cells and the symbiosome differentiation process.

RosR regulon of *R. etli* has been reported to influence expression of diverse genes, some of which affect the cell surface and nodulation competitiveness (Bittinger and Handelsman, 2000). Summers *et al.* (2000) isolated a transposon mutant of *S. meliloti* that constitutively expressed higher levels of acid and alkaline phosphatase enzymes. Sequence analysis of the transposon insertion site revealed that the affected gene has a high homology to Lon proteases from a number of organisms. Further studies on this mutant suggested that the *S. meliloti* Lon protease is involved in regulating exopolysaccharide synthesis and is required for nodulation of alfalfa.

The above review of literature shows that several genes having a role in rhizobial-legume symbiosis have been identified and characterized. The roles of some primary biosynthetic pathways specifically those of isoleucine, valine and leucine, of rhizobial

bacteria have not been fully explored. There is a need to identify if any isoleucine, valine, and leucine biosynthetic intermediate(s) / enzyme(s) is (are) directly involved in symbiotic process. Histological studies of the nodules induced by leucine auxotrophs, having mutations in different biosynthetic genes, of rhizobia are required to determine the role of the genes of these biosynthetic pathways in symbiosis.

Chapter 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Bacterial strains and plasmids

The characteristics and sources of bacterial strains and plasmids used/constructed in this study are listed in Table 3.

3.1.2 Alfalfa cultivar

Alfalfa (*Medicago sativa*) cultivar T9 was used for plant inoculation tests. The seeds of this cultivar were obtained from National Seeds Corporation, New Delhi, India.

3.1.3 Composition of growth media used

3.1.3.1 Growth media for *Sinorhizobium meliloti*

3.1.3.1.1 Complete media

3.1.3.1.1.1 Tryptone yeast extract (TY) medium (Khanuja and Kumar, 1988)

Constituent	Amount/ litre
Tryptone	5g
Yeast extract	3g
$CaCl_2.2H_2O$	0.12g
Distilled water	to make volume 1 litre

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution. To make solid medium 16g agar (HiMedia) was added to the liquid medium before autoclaving.

3.1.3.1.1.2 Tryptone yeast extract (TY) swarm plates

For these plates TY medium contained 3g/litre agar.

Table 3: Bacterial strains and plasmids used / constructed in this study

Strains / Plasmids	Relevant characteristics	Source/Reference
Sinorhizobium meliloti		
AK631	Nod$^+$, Fix$^+$, compact colony variant of wild type strain Rm41	Adam Kondorosi
Rmd 201	Spontaneous Sm^r derivative of AK631 (Nod$^+$ Fix$^+$)	Khanuja and Kumar (1988)
PP631	AK631(pJB3JI)	Peter Putnoky
ZB555	Rm41 *cys46 phe15 rfl sm1*	Z. Banfalvi
ZB556	Rm41 *gly1 met2 ade4 rfl sm1*	--- do---
ZB557	Rm41 *phe15 leu4 rfl sm1*	--- do---
ZB205	Rm41 *ade15 narB rfl 5fu'*	--- do---
VK4	Rmd201 *ilvB*::Tn5	This Lab
RH1, RH30	Rmd201 *ilvC*::Tn5	This Study
VK5, NV29, SY2, PS7	Rmd201 *ilvC*::Tn5	This Lab
RH3, RH18	Rmd201 *ilvD*::Tn5	This Study
VK44	Rmd201 *ilvD*::Tn5	This Lab
RS3	Rmd201 *leuC*::Tn5	This Lab
RH14	Rmd201 *leuB*::Tn5	This Study
SY1	Rmd201 *leuB*::Tn5	This Lab
VK41	VK4 (pJB3JI)	This Study
RH11	RH1(pJB3JI)	---do---
RH301	RH30 (pJB3JI)	---do---
VK51	VK5 (pJB3JI)	---do---
NV291	NV29 (pJB3JI)	---do---
SY21	SY2 (pJB3JI)	---do---
PS71	PS7 (pJB3JI)	---do---
RH31	RH3 (pJB3JI)	---do---

Contd...

RH181	RH18 (pJB3JI)	This Study
VK441	VK44 (pJB3JI)	---do---
RS31	RS3 (pJB3JI)	---do---
RH141	RH14 (pJB3JI)	---do---
SY11	SY1 (pJB3JI)	---do---
Escherichia coli WA803(pGS9)	Met⁻ Thi⁻ Cm^r Km^r	Selvaraj and Iyer (1983)
Plasmids pGS9	IncN repP15A Cm^r Km^r	Selvaraj and Iyer (1983)
pJB3JI	Km^r derivative of pR68.45 capable of mobilizing genomic segments of its host, Tc^r Cb^r Nal^r	Brewin *et al.* (1980)

3.1.3.1.1.3 Mannitol salt yeast extract (MSY) medium (Khanuja and Kumar, 1989)

Constituent	Amount/ litre
Mannitol	10g
Yeast extract	1g
$K_2HPO_4.3H_20$	0.2g
KH_2PO_4	0.2g
$MgSO_4.7H_2O$	0.1g
$CaCl_2.2H_2O$	0.05g
Distilled water	to make volume 1 litre

The pH of the medium was adjusted to 6.8 with 0.1N NaOH solution. To make solid medium 16g agar was added before autoclaving.

3.1.3.1.2 Minimal medium

3.1.3.1.2.1 Rhizobium minimal medium (RMM) (Singh *et al.*, 1984).

Solutions A and B were prepared as follows:

Solution A:

Constituent	Amount/ litre
$Na_2HPO_4.12H_2O$	0.45g
$(NH_4)_2SO_4$	2.0g
$FeCl_3$	2.0mg
$MgSO_4.7H_2O$	0.1g
$CaCl_2.2H_2O$	0.04g
Distilled water	to make volume 990 ml

This solution, after adjusting its pH to 7.0 with 0.1N NaOH, was autoclaved.

Solution B:

This solution, containing glucose (20%) in distilled water, was filter sterilized.

To prepare 1 litre of RMM, 10 ml of solution B was added to 990 ml solution A.

To make solid RMM, 16g agar was added to solution A before autoclaving.

3.1.3.2 Luria Bertani (LB) medium for *Escherichia coli* (Sambrook *et al.*, 1989)

Constituent	Amount/ litre
Tryptone	10g
Yeast extract	5g
Sodium chloride	10g
Distilled water	to make volume 1 litre

The pH of the medium was adjusted to 7.0 with 0.1N NaOH solution. For making solid medium 16g agar was added before autoclaving.

3.1.3.3 Nitrogen free plant nutrient medium for *Medicago sativa*

For plant assays, nitrogen free plant nutrient medium developed by Engelke *et al.* (1987) was used. Twelve stock solutions (A to L) of following concentrations were prepared.

Solution	Constituent	Amount of constituent (g)	Distilled water (ml)
A	K_2HPO_4	2.090	10
B	KH_2PO_4	0.544	10
C	$CaCl_2$	7.351	10
D	$C_6H_5O_7Fe.3H_2O$	0.335	10
E	$MgSO_4$	6.162	10
F	K_2SO_4	4.356	40

Contd...

G	$MnSO_4$	0.034	20
H	H_3BO_3	0.026	20
I	$ZnSO_4$	0.030	20
J	$CuSO_4$	0.002	20
K	$CaSO_4$	0.006	20
L	Na_2MoO_4	0.006	20

All these stock solutions were autoclaved separately. To make 1 litre of plant nutrient medium, 10g agar was added to 985 ml distilled water and the resulting water agar medium was autoclaved. Four ml of stock solution F and one ml of remaining stock solutions were added to autoclaved agar solution and pH of the medium was adjusted to 6.8 with 0.1N NaOH or 0.1N HCl. This medium was then poured into glass tubes to prepare slants for growing alfalfa plants. All autoclavings were done at 15 psi for 20 min.

3.1.4 Diluent used

Saline i.e. 0.9% (w/v) NaCl (HiMedia) was used for carrying out serial dilutions and to make cell suspensions.

3.1.5 Supplements to media

3.1.5.1 Antibiotics

All antibiotics used were purchased from HiMedia Laboratories. Stock solutions of streptomycin sulphate and kanamycin acid sulphate were prepared in sterile distilled water, and 50% ethanol was used as a solvent to prepare stock solutions of tetracycline hydrochloride and chloramphenicol. Rifampicin was dissolved in dimethylsulphoxide. The final concentrations of different antibiotics used in different

media were as follows: streptomycin sulphate (100μg/ml); kanamycin acid sulphate (40μg/ml for *E. coli* and 400μg/ml for *S. meliloti*), rifampicin (40μg/ml), chloramphenicol (40μg/ml) and tetracycline hydrochloride (15μg/ml). Stock solutions of antibiotics were filter-sterilized and then added to autoclaved media.

3.1.5.2 Amino acids, nitrogenous bases and vitamins

The stock solutions of amino acids, nitrogenous bases and vitamins were prepared in distilled water and autoclaved as described earlier. Required volumes from stock solutions were added to autoclaved media to make final concentrations of 50, 30 and 10μg/ml for amino acids, nitrogenous bases and vitamins, respectively. The auxotrophy of each strain was determined on Holliday pools (Holliday, 1956) which were slightly modified. The composition of modified Holliday pools used in the study was as follows:

Pool 1: Adenine, histidine, phenylalanine, glutamine, thymine and pantothenic acid.

Pool 2: Guanine, leucine, tyrosine, asparagine, serine and riboflavin.

Pool 3: Cysteine, isoleucine, tryptophan, uracil, glutamate and biotin.

Pool 4: Methionine, lysine, threonine, aspartic acid, alanine and cobalamine.

Pool 5: Thiamine, valine, proline, arginine and glycine.

Pool 6: Adenine, guanine, cysteine, methionine and thiamine.

Pool 7: Histidine, leucine, isoleucine, lysine and valine.

Pool 8: Phenylalanine, tyrosine, tryptophan, threonine and proline.

Pool 9: Glutamine, asparagine, uracil, aspartic acid and arginine.

Pool 10: Thymine, serine, glutamate, alanine and glycine.

Pool 11: Pantothenic acid, riboflavin, biotin and cobalamin.

3.1.5.3 Intermediates of isoleucine, valine and leucine

α-ketobutyrate, α-keto-β-methylvalerate, α-ketoisovalerate and α-ketoisocaproate were purchased from Sigma Chemical Company, U.S.A. 2-acetoxy-2-methyl-3-oxobutyric acid ethyl ester (precursor of α-acetolactate) and 2-isopropylmalate were obtained from Aldrich Chemical Company, U.S.A. Distilled sterile water was used as a solvent for preparing stock solutions of the above intermediates. Each intermediate was added at a final concentration of 50μg/ml to the RMM medium.

3.1.5.4 Sugars and dicarboxylic acids

Sugars (glucose, arabinose, lactose, galactose, maltose, xylose, mannose, mannitol and sucrose) and dicarboxylic acids (malate, aspartate and succinate) were purchased from HiMedia. Each sugar/ dicarboxylic acid was added at a final concentration of 2g/ litre to the RMM medium as sole carbon source before autoclaving.

3.1.5.5 Sodium deoxycholate (DOC)

DOC (HiMedia) was added to MSY medium @ 1 mg/ ml before autoclaving.

3.1.5.6 Dyes

Aniline blue (HiMedia), calcofluor white (Sigma) and congo red (HiMedia) were added to MSY medium @ 0.02% (w/v) each before autoclaving. Methylene blue (0.01%) (w/v) and toluidine blue (1%) (w/v) from HiMedia were used to stain infection threads and semithin sections of nodules, respectively. Toluidine blue was prepared in 1% (w/v) borax (Sigma).

3.1.6 Reagents for pyruvic acid accumulation studies

3.1.6.1 Pyruvic acid

Pyruvic acid sodium salt (LOBA Chemie) solution 100mg/ml was prepared as a standard sample.

3.1.6.2 Chromatogram development solvent

To prepare this reagent *s*-butanol (S.d. fine- chem. ltd.) was mixed with propoinic acid (LOBA Chemie) in a ratio 95:5 (v/v) and then saturated with water (Umbarger and Mueller, 1950).

3.1.6.3 Detection reagent

This reagent was prepared by dissolving 2 gm *o*-phenylenediamine (LOBA Chemie) in 100ml of 70% (v/v) ethanol (LOBA Chemie).

3.1.7 Preparation of α-acetolactate

Stock solution of α-acetolactate was prepared from 2-acetoxy-2-methyl-3-oxobutyric acid ethyl ester (Aldrich) by suspending 1.032g (5.0 mmol) to a final volume of 10ml with 0.975 M NaOH and mixing vigorously until a neutral solution was obtained (Aulabaugh and Schloss, 1990).

3.1.8 Composition of solutions for light and electron microscopy of sections of alfalfa root nodules

3.1.8.1 Requirements for preparation of blocks

(a) 0.2M phosphate buffer

Na_2HPO_4	6.41g
$Na_2HPO_4.7H_2O$	41.3g
Double distilled water	1000 ml

(b) Fixatives

(i.) Primary fixative i.e. Karnovsky fixative (Karnovsky, 1965)

12.5ml	8% (w/v) paraformaldehyde (Sigma) (Added 2g of paraformaldehyde in 25ml distilled water and heated the solution to 60-70°C followed by the addition of few drops of 1N NaOH)
5.0ml	12.5% (v/v) glutaraldehyde (Sigma)
25.0ml	phosphate buffer
7.5ml	double distilled water

(ii.) Secondary fixative or post fixative i.e. 2% (w/v) OsO_4

Stock solution of OsO_4 (2% w/v) was made by dissolving 1g of OsO_4 in 50ml of distilled water. This stock solution was stored at 4°C in a tightly stoppered brown coloured bottle.

(c) Acetone series

Acetone solutions (30, 50, 70, 80, 90 and 95%) (v/v) were prepared separately and stored at 4°C.

(d) Araldite CY212 (resin) embedding medium

Araldite CY212	10 ml
Hardner (Dodecenyl succinic anhydride, DDSA; HY 964)	10 ml
Accelerator (Tridimethyl aminomethyl phenol, DMP30; DYO64)	0.4 ml
Plasticizer (Dibutylphthalate)	1.0 ml

All ingredients were mixed and vigorously stirred.

3.1.8.2 Solutions for staining ultrathin sections for electron microscopy

3.1.8.2.1 Uranyl acetate solution

A saturated solution of uranyl acetate was prepared by adding excess of uranyl acetate to 10ml of 50% (v/v) ethanol in a 15ml centrifuge tube. Centrifugation was done at 5000rpm for 2min and the supernatant was transferred to another tube. This tube was tightly stoppered and stored at 4°C in a refrigerator.

3.1.8.2.2 Lead citrate solution

One and half pellets of sodium hydroxide were added to 12ml of filtered 50% (v/v) ethanol in a 15ml centrifuge tube. After dissolution of NaOH, 50mg of lead citrate was added and the contents were mixed by shaking. Centrifugation was done at 5000rpm for 2min and the supernatant stored at 4°C in refrigerator.

3.2 METHODS

3.2.1 General bacteriological procedures

3.2.1.1 Maintenance of bacterial cultures

The bacterial strains were maintained in glycerol (15% final concentration) at -20°C. The strains of *S. meliloti* and *E. coli* were also maintained at 4°C on TY and LB agar medium slants, respectively.

3.2.1.2 Culturing of bacteria

Single colonies were obtained by plating or streaking cells of strains on complete or minimal medium. Incubation of 2-3 days was required at 28°C for *S. meliloti* and 37°C for *E. coli* for the colonies to appear on complete medium. In case of *S. meliloti*

incubation of about one week was required for colonies to appear on minimal medium. A fully grown *S. meliloti* colony usually contained about 10^7 cells.

Cell suspensions for spot tests for studying growth responses of bacterial strains were prepared by suspending cells from a colony in 0.05ml of 0.9% NaCl. 10^5 to10^6 cells were applied to the surface of agar medium with a loop to make a spot. Log phase *E. coli* cultures were obtained by incubating the inoculated liquid LB medium containing the required antibiotic for 14-16 hrs at 37°C. Log phase culture of a strain of *S. meliloti* was obtained by inoculating a single colony of the strain in liquid TY medium and incubating the inoculated medium for 24-32hrs at 28°C. The tubes or flasks containing bacterial cultures were incubated at a suitable temperature in an orbital shaker (GALLENKAMP) operated at 120 rpm speed of rotation for aeration.

3.2.1.3 Serial dilutions

The bacterial cultures were serially diluted according to the need of the experiment. Serial dilutions were made in saline i.e. 0.9% (w/v) NaCl. For obtaining 10^{-2} dilution, 0.1 ml of a neat culture was added to a screw cap glass tube containing 9.9 ml saline. After proper mixing, 0.1 of bacterial suspension from this tube was added to another tube containing 9.9 ml saline so as to have 10^{-4} dilution. Further dilutions, as per the need of the experiment, were similarly made.

3.2.1.4 Bacterial matings

Bacterial matings were done according to Kondorosi *et al.* (1977). Log phase cultures of recipient (0.05 ml) and donor (0.05 ml) strains were mixed in 1:1 ratio (3:1 ratio for Tn5 mutagenesis) and spread on TY agar plates. These plates were incubated at 28°C for 24 hrs.

3.2.2 Isolation and screening of auxotrophs

3.2.2.1 Random transposon Tn5 mutagenesis

Random Tn5 mutagenesis of *S. meliloti* Rmd201 strain was done by using Tn5 delivery suicide plasmid pGS9 (Selvaraj and Iyer, 1983). Log phase cultures in liquid TY medium of *E. coli* strain WA803(pGS9) as donor and *S. meliloti* strain Rmd201 as recipient were obtained. 0.075 ml of *S. meliloti* and 0.025 ml of *E. coli* cultures were mixed and spread with the help of a sterile glass rod on half of the surface of TY agar medium in a Petri dish. The remaining half of the TY agar surface was further divided into two halves. 0.05 ml of Rmd201 on one half and 0.05 ml of WA803(pGS9) on the other half were spread. Incubation of these plates was done at 28°C for 24 hrs.

Each bacterial growth (donor / recipient / mating mixture) was scrapped and suspended in 2 ml saline. 0.1 ml suspension of the mating mixture was plated on TY agar plates containing streptomycin (100 µg/ ml) and kanamycin (400 µg/ ml). The donor (0.05 ml) and recipient (0.05 ml) suspensions were also plated separately on selective plates as controls. Incubation of these plates was done at 28°C for 5 days. Fifty colonies were purified on TY+Km^{400}+Sm^{100} agar plates. All kanamycin resistant (Km^r) transconjugants were checked for the loss of chloramphenicol marker to confirm the loss of suicide plasmid pGS9. The above cross was repeated 122 times and 50 Km^r transconjugants were selected from each cross.

3.2.2.2 Screening for auxotrophs by replica plating method

A colony of each Km^r transconjugant was streaked with the help of a sterile toothpick on RMM and TY+Km^{400}+Sm^{100} agar plates. Incubation of these plates was done at 28°C for 3 days. During this period the bacterial growth on each streak was

observed after every 12 hrs. The Tn5 derivatives which showed growth on TY+Km^{400}+Sm^{100} but not on RMM agar plates were considered to be auxotrophs. Each of these auxotrophs was purified for a single colony on TY+Km^{400}+Sm^{100} agar plates. The purified auxotrophs were maintained as described before for further studies.

3.2.3 Determination of the nature of auxotrophy

A loopful of each auxotroph was suspended in a drop of saline on a sterile glass slide. This suspension with the help of a sterile toothpick, was streaked on RMM agar medium supplemented with Holliday pools (one pool at a time). Incubation was done at 28°C for 5 days. The nature of auxotrophy was found out on the basis of growth patterns on various Holliday pools. The requirement(s) of each auxotroph was confirmed by streaking it on RMM agar medium supplemented with the suspected nutrient(s).

3.2.4 Reversion analysis of auxotrophs

A sample (10 ml) of late log phase culture in liquid TY medium of the auxotroph was centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet, after washing twice with liquid RMM medium (2 ml each time), was resuspended in a suitable volume of liquid RMM medium so as to obtain about 10^9 cells/ ml. A sample (0.1 ml) of this cell suspension was spread on RMM and TY agar media. Incubation was done at 28°C for 5 days. The numbers of bacterial colonies on RMM and TY media were counted after incubation. The number of colonies on RMM medium represented the number of prototrophic revertants whereas the number of colonies on TY medium indicated the total number of cells spread on each medium. On the basis of these numbers spontaneous reversion frequency for each auxotroph was calculated. The excision of

transposon Tn5 in prototrophic revertant colony was determined by streaking cells from this colony on TY agar medium containing 400μg/ ml kanamycin.

3.2.5 Location of biochemical block in each auxotroph

3.2.5.1 Intermediate feeding studies

The position of biochemical block in each amino acid auxotroph was determined by feeding the intermediates of the amino acid biosynthetic pathway to the auxotroph and subsequently observing the growth. A cell suspension of each isoleucine + valine auxotroph was streaked with a sterile toothpick on RMM medium supplemented with α-ketobutyrate + valine, α-acetolactate + isoleucine and α-keto-β-methylvalerate + valine (one combination at a time). RMM medium supplemented with isoleucine and valine was used as a positive control. Leucine auxotrophs were streaked on RMM medium supplemented with α-ketoisovalerate, α-isopropylmalate and α-ketoisocaproate (one at a time). RMM medium supplemented with leucine was used as a positive control.

The cell suspension of the parental strain was also streaked on various plates as a control. Growth pattern in each plate was observed after incubation at 28°C for 4-5 days.

3.2.5.2 Detection of pyruvic acid accumulation

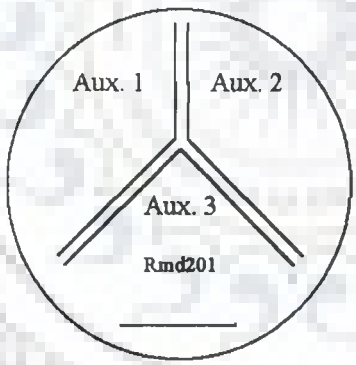
A sample (10 ml) of log phase culture of each isoleucine + valine auxotroph was centrifuged at 5000 rpm for 10 min. The pellet obtained was washed twice with liquid RMM (2 ml each time), and after resuspending it in 10 ml of RMM, incubated at 28°C for 48 hrs on an orbital shaker (speed 120 rpm). The resulting bacterial culture was centrifuged at 10,000 rpm for 10 min and the supernatant was analyzed chromatographically for pyruvic acid. The presence or absence of pyruvic acid in the

supernatant was determined by the method of Umbarger and Mueller (1950) as described in Smith and Pattee (1967) as follows:

A sample (0.5 ml) of the supernatant was spotted on chromatographic paper. A solution of pyruvic acid (100mg/ ml) was used as a standard. The chromatogram was developed (ascending method) for 6.5 hrs in a solvent that consisted of *s*-butanol-propionic acid (95:5, v/v) saturated with water (Umbarger and Mueller, 1950). Pyruvic acid was detected by spraying the chromatogram with *o*-phenylenediamine reagent followed by heating at 100°C for 2 min (Smith and Pattee, 1967).

3.2.5.3 Cross-feeding assays

Cell suspensions (in 0.9% saline) of all isoleucine + valine auxotrophs in combinations of three at a time were streaked adjacent to each other on RMM agar medium containing minimal amounts of isoleucine (2µg/ ml) and valine (2µg/ ml). The streaking pattern of auxotrophs (Aux.) was as follows:



The cell suspension of parental strain was also streaked on one side as a positive control. The leucine auxotrophs were similarly streaked along with the positive control. Incubation was done at 28°C and growth patterns were recorded after 4-5 days of incubation.

3.2.6 Pleiotropic effects of Tn5 insertions in auxotrophs

Possible pleiotropic effects of Tn5 insertions in all isoleucine + valine, and leucine auxotrophs were studied by detecting changes, if any, in auxotrophs in various characteristics like production of cell surface molecules and utilization of sugars and dicarboxylic acids.

3.2.6.1 Production of cell surface molecules

3.2.6.1.1 Test for the production of lipopolysaccharides (LPS)

All auxotrophs and the parental strain were streaked on MSY agar medium containing 1mg/ ml of sodium deoxycholate (DOC). The growth of these strains was recorded after incubation at 28°C for 3 days. The strains which showed growth on MSY + DOC agar medium were considered to be LPS producing (Swamynathan and Singh, 1995).

3.2.6.1.2 Test for the production of cellulose fibrils and succinylated exopolysaccharides (EPS I)

The presence of cellulose fibrils and succinylated exopolysaccharides (EPS I) on the surface of rhizobial cells is indicated by the binding of congo red dye and calcofluor white, respectively. Red colonies on medium containing congo red are produced by the strains producing cellulose fibrils (Kneen and La Rue, 1983). The strains producing EPS I give fluorescence under ultraviolet light (Leigh *et al.*, 1987). MSY agar plates containing 0.02% (w/v) congo red and 0.02% (w/v) calcofluor (one at a time) were prepared. All auxotrophs as well as the parental strain were streaked on these plates. Incubation was done at 28°C for 3 days. Congo red plates were observed for red colonies and calcofluor plates were observed for fluorescence in ultraviolet light.

3.2.6.1.3 Test for the production of β-glucans

3.2.6.1.3.1 Test for the production of β-(1→3) glucans

Blue colonies on medium containing aniline blue are formed by bacterial strains which produce β-(1→3) glucans (Nikanishi *et al.*, 1976). All auxotrophs and the parental strain were streaked on MSY agar medium containing 0.02% (w/v) aniline blue. Incubation was done at 28°C for 3 days. The presence or absence of blue colonies was recorded for each strain.

3.2.6.1.3.2 Test for the production of β-(1→2) glucans

The production of β-(1→2) glucans was assayed on swarm plates (Geremia *et al.*, 1987). A loopful of cell suspension of each auxotroph was spotted on TY swarm plates containing 0.3% (w/v) agar. The cell suspension of the parental strain was also spotted on each of these plates. Incubation was done at 28°C for 3 days and spots were examined for swarming after every 24 hrs.

3.2.6.2 Utilization of sugars and dicarboxylic acids by auxotrophs

Each auxotroph was streaked on RMM agar medium (without glucose) supplemented with the nutritional requirement of the auxotroph and a sugar (arabinose, lactose, galactose, maltose, xylose, mannose, mannitol or sucrose) / dicarboxylic acid (malate, aspartate or succinate); the parental strain, as a positive control, was also streaked, on this medium. All auxotrophs and the parental strain were also streaked on RMM agar medium. Incubation was done at 28°C for 5 days and after 48 hrs of incubation bacterial growth was observed daily.

3.2.7 Construction of donor strains of auxotrophs

Each auxotroph and the *S. meliloti* AK631 (pJB3JI) strain were mated as a recipient and a donor, respectively, on TY agar plate to transfer genome mobilizing plasmid pJB3JI (Tc^r) into the auxotroph. The transconjugants containing plasmid pJB3JI were selected on TY agar plates containing tetracycline (15µg/ ml) and kanamycin (400µg/ ml). Five transconjugants were purified for single colonies on TY medium containing tetracycline (15µg/ ml) and kanamycin (400µg/ ml). These purified transconjugants were used as donors in the subsequent conjugation experiments.

3.2.8 Linkage of Tn5 insertion to auxotrophy

The donor strain (containing plasmid pJB3JI) of each auxotroph was mated with the recipient strain *S. meliloti* ZB555 (rifampicin resistant) on TY agar plates. One hundred kanamycin resistant transconjugants were selected on TY agar medium containing rifampicin (40µg/ ml) and kanamycin (400µg/ ml). These transconjugants were checked for auxotrophy of the donor.

3.2.9 Genetic mapping of Tn5 insertion in isoleucine + valine auxotrophs

The location of transposon Tn5 insertion in each of isoleucine + valine auxotrophs VK4, RH1 and RH18 was determined by plasmid pJB3JI mediated mapping method. The donor strain (containing plasmid pJB3JI) of each of the above auxotrophs was mated separately with four *S. meliloti* mapping strains, *viz.*, ZB555, ZB556, ZB557 and ZB205. Kanamycin resistant transconjugants from each cross were selected on TY agar medium containing rifampicin (40µg/ ml) and kanamycin (400µg/ ml). The co-transfer of kanamycin resistance with each unselected recipient marker was determined by streaking these transconjugants on appropriate selective plates. The co-inheritance frequency or

linkage of selected marker (kanamycin resistance phenotype) with the unselected marker was calculated and the distance between them was determined using the following formula:

$$c = (1-d)^3$$

where

c = linkage frequency of two markers

d = distance between them

3.2.10 Plant inoculation tests

Glass tubes (20 x 2.5 cm) were plugged with cotton and autoclaved. Under sterile conditions twenty-five ml of nitrogen-free plant nutrient medium containing agar at about 45°C was transferred to each tube. The tubes were plugged and immediately placed in slanting position. Filter-sterilized supplements were added to the plant nutrient medium before transfer of this medium to tubes.

Alfalfa (*Medicago sativa* cv. T9) seeds were soaked in sterile distilled water for 20 min and surface sterilized by treating with 0.1% $HgCl_2$ for 1 min followed by 1 min treatment with absolute alcohol. After giving five washing with sterile distilled water, the seeds were spread on 1% agar plates. These plates were incubated at 25°C in dark. After two days, two seedlings were transferred to each tube.

Alfalfa seedlings were inoculated with the parental strain *S. meliloti* Rmd201 and its auxotrophs. Ten ml of log phase culture of a rhizobial strain in liquid TY medium was centrifuged at 5000 rpm for 10 min. The pellet, after washing twice with sterile distilled water, was suspended in 5 ml of sterile distilled water. Half ml of this cell suspension was added to each tube containing seedlings. Black paper was wrapped on the lower portions

of tubes to protect the plant roots from direct exposure to light. The tubes containing seedlings were placed in a plant growth chamber maintained at 25°C and provided with 2000 lux light. The plants were given 16 and 8 hrs, light and dark periods, respectively. After six weeks the plants were taken out of the tubes and data on nodule characteristics and shoot dry weight were recorded. Shoot dry weights were recorded after drying plants at 85°C for 72 hrs.

3.2.11 Nodule occupancy tests

The nodules induced by rhizobial strains were checked for the occupancy of the inoculated strains. All nodules from a plant were surface-sterilized and crushed in a drop of saline. The resulting cell suspension was diluted in saline to get 10^{-1} and 10^{-2} dilutions. 0.1 ml of neat suspension was spread on a TY agar plate containing 100µg/ ml streptomycin. Both dilutions were spread similarly on the same medium. Three replications were used for the neat suspension and each of the two dilutions. These plates were incubated at 28°C for 3 days. The colonies obtained were streaked on agar plates containing RMM and RMM + Km^{400} + Sm^{100} + auxotrophic requirement. These plates were incubated at 28°C for 3 days. The colonies which showed growth on RMM medium were recorded as prototrophic revertants.

3.2.12 Light microscopy (LM) for observing infection thread formation

After 4 days of inoculation, alfalfa plants were taken out of tubes. The root portion of each plant was washed and cut into 1 cm long pieces. These root pieces were placed in methylene blue solution (0.01% w/v in distilled water) for 15 min and washed twice with sterile water. Each stained root piece was placed on a glass slide, covered with a cover slip, and examined at 40 x magnification under light microscope (Leica *DM* LB).

3.2.13 Microscopic studies of nodule sections

Microscopic studies of the sections of the nodules induced by the parental strain Rmd201 and its leucine auxotrophs RS3 and RH14 were carried out. Ten nodules for each strain were collected from plants after six weeks of inoculation. Each nodule was cut longitudinally into two halves. One half of the nodule was crushed in saline (0.5 ml) to determine nodule occupancy by the inoculated strain. The other half of the nodule was used for microscopic studies.

3.2.13.1 Primary fixation of nodules

The nodules were washed thrice with sterile distilled water and transferred to glass vials (5 ml capacity) containing Karnovsky fixative. To facilitate penetration, acrolein (at 0.1% final concentration) was added to the fixative. The vials were placed in a vacuum chamber to prevent the floating of the nodules on the surface. These were then kept at 4°C for 24 hrs.

3.2.13.2 Preparation of specimen block

3.2.13.2.1 Washing of nodule samples

The nodule samples were taken out of the primary fixative and washed in 0.1M phosphate buffer. These samples were left overnight in the same buffer at 4°C.

3.2.13.2.2 Secondary fixation of nodules

The nodule samples were placed in secondary fixative (1% w/v OsO_4) at 4°C for 2 hrs and subsequently washed with 0.1M phosphate buffer.

3.2.13.2.3 Dehydration of nodules

To remove water the nodule samples were passed through a graded acetone series (30, 50, 70, 80, 90, 95% and dry acetone). A sample was kept in each of the acetone concentrations at 4°C for 15 min. Then two changes of 30 min each in dry acetone were given at room temperature.

3.2.13.2.4 Removal of acetone from nodules

The nodule samples were cleared off acetone by placing in toluene at room temperature for 60 min. Acetone was removed from the samples to facilitate infiltration in the next step.

3.2.13.2.5 Infiltration

The following infiltration mixtures were prepared:

a. 1 part of embedding medium and 3 parts of toluene

b. 2 parts of embedding medium and 2 parts of toluene

c. 3 parts of embedding medium and 1 part of toluene

The nodule samples, which were taken out of toluene, were placed in mixture 'a'. After overnight these were transferred to mixture 'b' for 1 hr and then to mixture 'c' for 1 hr under vacuum. Thereafter two changes of 1 hr each in pure embedding medium at 50°C were given.

3.2.13.2.6 Embedding of nodule

The nodule samples were embedded in araldite embedding medium using gelatin blocks. The nodules were oriented, to facilitate subsequent longitudinal sectioning, before polymerization of the embedding medium. The embedding blocks were kept at 50°C for 24 hr and then the temperature of the oven was raised to 60°C. In 48 hrs complete

polymerization of the embedding medium occurred. These blocks were then trimmed using a trimmer and fitted in a specimen block holder.

3.2.13.3 Preparation of semithin sections

The block holder was attached to Ultracut E Microtome (C. Reichert, Austria OmU3) and semithin sections (0.5 to 2.0 μm thick) were cut using a glass knife. The sections floating in water in the metallic trough fitted with knife were taken out with a thin brush and placed on a clean glass slide. The slide was dried by placing on a hot plate at 80°C for 30 seconds. The sections were stained with 1% (w/v) toluidine blue for 1 min. After staining, the sections were washed with distilled water and observed under light microscope (Leica *DM* LB). The area of interest was photographed.

3.2.13.4 Preparation of ultrathin sections

The area of the section to be examined under TEM was selected and the blocks were further hand trimmed. Ultrathin sections (60-90 nm thick) were cut using Ultracut E Microtome (C. Reichert, Austria OmU3). These sections were lifted onto 200 mesh size copper grids. These grids were coated with 3% formvar (polyvinyl formaldehyde) in ethylene dichloride and carbon by the process of evaporation under vacuum. After double coating, formvar was removed by dissolving in ethylene dichloride or chloroform to leave behind the carbon film. Double staining of sections with uranyl acetate and lead citrate was done to obtain a good contrast. The grids were placed for 15 min in 0.5 ml of uranyl acetate solution on a watch glass. A cardboard cover was placed over the watch glass to provide dark condition for staining. The grids were washed twice with 50% ethanol and twice with double distilled water. After washing the grids were dried on a filter paper and then placed for 10 min in 0.5 ml of lead citrate solution on a clean watch

glass. The grids, after removing from the above solution, were washed with 0.1N NaOH and then twice with double distilled water. After washing, the grids were dried and viewed under transmission electron microscope (PHILIPS EM300). The area of interest was photographed. After viewing, the grids were stored in a grid holder at room temperature.

3.2.14 Statistical analysis

The data on characteristics of plants inoculated with the parental strain Rmd201 and its isoleucine + valine, and leucine auxotrophs were statistically analysed. All values were expressed as mean ± standard error of mean (SEM). Significance between a control value and an experimental value was found out using student ' t ' test.

Chapter 4

RESULTS

Sinorhizobium meliloti strain Rmd201 is a streptomycin resistant derivative of strain AK631, which is a compact colony variant of the wild type strain Rm41. Auxotrophs of *S. meliloti* Rmd201 were generated through random transposon mutagenesis and isoleucine + valine, and leucine auxotrophic mutants were selected for detailed investigations. Isoleucine + valine, and leucine auxotrophs were characterized genetically and biochemically. Symbiotic properties of these auxotrophs were studied by inoculating alfalfa seedlings with these auxotrophs. Nodules induced by leucine auxotrophs were subjected to microscopic studies. The results of these experiments have been presented in this chapter.

4.1 Random transposon Tn5 mutagenesis

From 122 crosses between *E. coli* WA803(pGS9) donor and *S. meliloti* Rmd201 recipient, 5,450 Tn5-induced kanamycin resistant transconjugants were selected on TY agar medium containing kanamycin (400µg/ ml) and streptomycin (100µg/ ml). The frequency of occurrence of Tn5-induced transconjugants was 2×10^{-5} per recipient whereas the spontaneous resistance to 400µg/ ml concentration of kanamycin in *S. meliloti* strain Rmd201 was much lower i.e. $< 10^{-8}$.

All Tn5-induced transconjugants were chloramphenicol-sensitive indicating the absence of vector plasmid pGS9. The Tn5 derivatives were streak-purified on TY agar medium containing kanamycin (400µg/ ml) and streptomycin (100µg/ ml).

4.2 Screening of Tn5 derivatives for auxotrophs

The Tn5 derivatives were screened for auxotrophs by streaking on RMM (Rhizobium minimal medium). Thirty Tn5 derivatives, which did not grow on RMM, were presumed to be auxotrophs and used in further studies.

4.3 Nutritional requirements of auxotrophs

The nutritional requirements of auxotrophic strains were determined on the basis of their growth patterns on modified Holliday pools. The nutritional requirements of these strains were as follows (the names of strains for various auxotrophies are given in brackets): isoleucine + valine (RH1, RH3, RH18 and RH30), arginine + uracil (RH33, RH37 and RH47), adenine + thiamine (RH19 and RH39), tryptophan + tyrosine + phenylalanine (RH5), cysteine (RH2, RH21 and RH27), methionine (RH20, RH31, RH41 and RH45) tryptophan (RH4 and RH6), uracil (RH7, RH9 and RH36), adenine (RH13), leucine (RH14) and phenylalanine (RH38, RH40 and RH42). The requirement(s) of three auxotrophs, *viz.*, RH43, RH46 and RH48 could not be determined.

Isoleucine + valine, and leucine auxotrophs obtained during this study were selected for further investigations. Six isoleucine + valine and two leucine auxotrophs isolated by other researchers in the lab were also included. Table 4 gives a list of the auxotrophs chosen for the present studies.

4.4 Reversion analysis

Spontaneous reversion to prototrophy occurred in all isoleucine + valine, and leucine auxotrophs. Reversion frequencies for the auxotrophs have been presented in

Table 4: List of isoleucine + valine, and leucine auxotrophic strains of *Sinorhizobium meliloti* Rm201 used in this study

S. No.	Name of auxotrophic strain	Auxotrophic requirement(s)
1.	RH1	Isoleucine + Valine
2.	RH3	---do---
3.	RH18	---do---
4.	RH30	---do---
5.	VK4	---do---
6.	VK5	---do---
7.	VK44	---do---
8.	NV29	---do---
9.	SY2	---do---
10.	PS7	---do---
11.	RH14	Leucine
12.	SY1	---do---
13.	RS3	---do---

Table 5. The reversion properties ranged from 1.2 x 10^{-9} in strains RH1 and SY2 to 2.8 x 10^{-9} in strains RH3 and NV29. Most of the prototrophic revertants were kanamycin sensitive.

4.5 Location of biochemical block in isoleucine + valine, and leucine auxotrophs

The biochemical blocks in the auxotrophs were located by intermediate feeding and accumulation, and cross feeding studies.

4.5.1 Studies on feeding of metabolic intermediates

Growth responses of 10 isoleucine + valine auxotrophs of *S. meliloti* Rmd201 strain to the feeding of different intermediates of isoleucine and valine biosynthetic pathways are given in Table 6. All these auxotrophs were unable to grow on RMM supplemented with α-ketobutyrate and valine. These auxotrophs, with the exception of VK4, also did not grow on RMM supplemented with α-acetolactate and isoleucine. All isoleucine + valine auxotrophs showed growth on RMM supplemented with α-keto-β-methylvalerate and valine.

All leucine auxotrophs (RH14, SY1 and RS3) were unable to grow on RMM supplemented with α-ketoisovalerate or α-isopropylmalate (Table 7). Supplementation of RMM with α-ketoisocaproate restored the growth of all leucine auxotrophs.

4.5.2 Pyruvic acid accumulation studies

Out of 10 isoleucine + valine auxotrophs tested, only one (VK4) auxotroph was found to accumulate detectable amount of pyruvic acid in the culture medium.

Table 5: Spontaneous reversion frequencies of isoleucine + valine, and leucine auxotrophic strains of *Sinorhizobium meliloti* Rmd201

S. No.	Name of the auxotrophic strain	Auxotrophic requirement(s)	Spontaneous reversion frequency
1.	RH1	Isoleucine + Valine	1.2×10^{-9}
2.	RH3	---do---	2.8×10^{-9}
3	RH18	---do---	2.3×10^{-9}
4.	RH30	---do---	1.7×10^{-9}
5.	VK4	---do---	1.8×10^{-9}
6.	VK5	---do---	1.6×10^{-9}
7.	VK44	---do---	1.1×10^{-9}
8.	NV29	---do---	2.8×10^{-9}
9.	SY2	---do---	1.2×10^{-9}
10.	PS7	---do---	2.1×10^{-9}
11.	RH14	Leucine	1.5×10^{-9}
12.	SY1	---do---	1.9×10^{-9}
13.	RS3	---do---	1.4×10^{-9}

Table 6: Growth responses of isoleucine + valine auxotrophs of *Sinorhizobium meliloti* strain Rmd201 to different intermediates of isoleucine and valine biosynthetic pathways

S. No.	Name of the strain	RMM supplemented with 50µg/ ml of				RMM
		KBU+VAL	ALC+ILE	KMV+VAL	ILE+VAL	
1.	Rmd201	+	+	+	+	+
2.	RH1	-	-	+	+	-
3.	RH3	-	-	+	+	-
4.	RH18	-	-	+	+	-
5.	RH30	-	-	+	+	-
6.	VK4	-	+	+	+	-
7.	VK5	-	-	+	+	-
8.	VK44	-	-	+	+	-
9.	NV29	-	-	+	+	-
10.	SY2	-	-	+	+	-
11	PS7	-	-	+	+	-

Abbreviations: RMM, Rhizobium minimal medium; KBU, α-ketobutyrate; ALC, α-acetolactate; KMV, α-keto-β-methylvalerate; ILE, isoleucine; VAL, valine; +, growth; -, no growth

Table 7: Growth responses of leucine auxotrophs of *Sinorhizobium meliloti* strain Rmd201 to different intermediates of leucine biosynthetic pathway

S. No.	Name of the strain	RMM supplemented with 50µg/ ml of				RMM
		KIV	IPM	KIC	LEU	
1.	Rmd201	+	+	+	+	+
2.	RH14	-	-	+	+	-
3.	SY1	-	-	+	+	-
4.	RS3	-	-	+	+	-

Abbreviations: RMM, Rhizobium minimal medium; KIV, α-ketoisovalerate; IPM, α-isopropylmalate; KIC, α-ketoisocaproate; LEU, leucine; +, growth; -, no growth

4.5.3 Cross-feeding assays

Isoleucine + valine auxotroph VK4 was cross-fed by all other isoleucine + valine auxotrophs. Six isoleucine + valine auxotrophs, *viz.*, RH1, RH30, VK5, NV29, SY2 and PS7, were cross-fed by three isoleucine + valine auxotrophs, *viz.*, RH3, RH18 and VK44. The leucine auxotroph RS3 was cross-fed by the other two leucine auxotrophs RH14 and SY1.

Based on intermediate feeding, intermediate accumulation and cross-feeding studies the isoleucine + valine auxotrophs were classified into following categories:

Category I: *ilvB / ilvG* mutant (VK4) which grew on RMM medium supplemented with α-acetolactate and isoleucine, and accumulated pyruvic acid.

Category II: *ilvC* mutants (RH1, RH30, VK5, NV29, SY2 and PS7) which did not grow on RMM medium supplemented with α-acetolactate and isoleucine, and were cross-fed by RH3, RH18 and VK44.

Category III: *ilvD* mutants (RH3, RH18 and VK44) which grew on RMM supplemented with α-keto-β-methylvalerate and valine and were not cross-fed by any other isoleucine + valine auxotroph.

The leucine auxotrophs were placed in the following categories:

Category I: *leuC / leuD* mutant (RS3) which did not grow on RMM supplemented with α-isopropylmalate and was cross-fed by RH14 and SY1 leucine auxotrophs.

Category II: *leuB* mutants (RH14 and SY1) which grew on RMM supplemented with α-ketoisocaproate and cross-fed RS3 leucine auxotroph. Fig.8 shows the position of block in each of the isoleucine + valine, and leucine auxotrophs in the biosynthetic pathways of isoleucine and valine, and leucine.

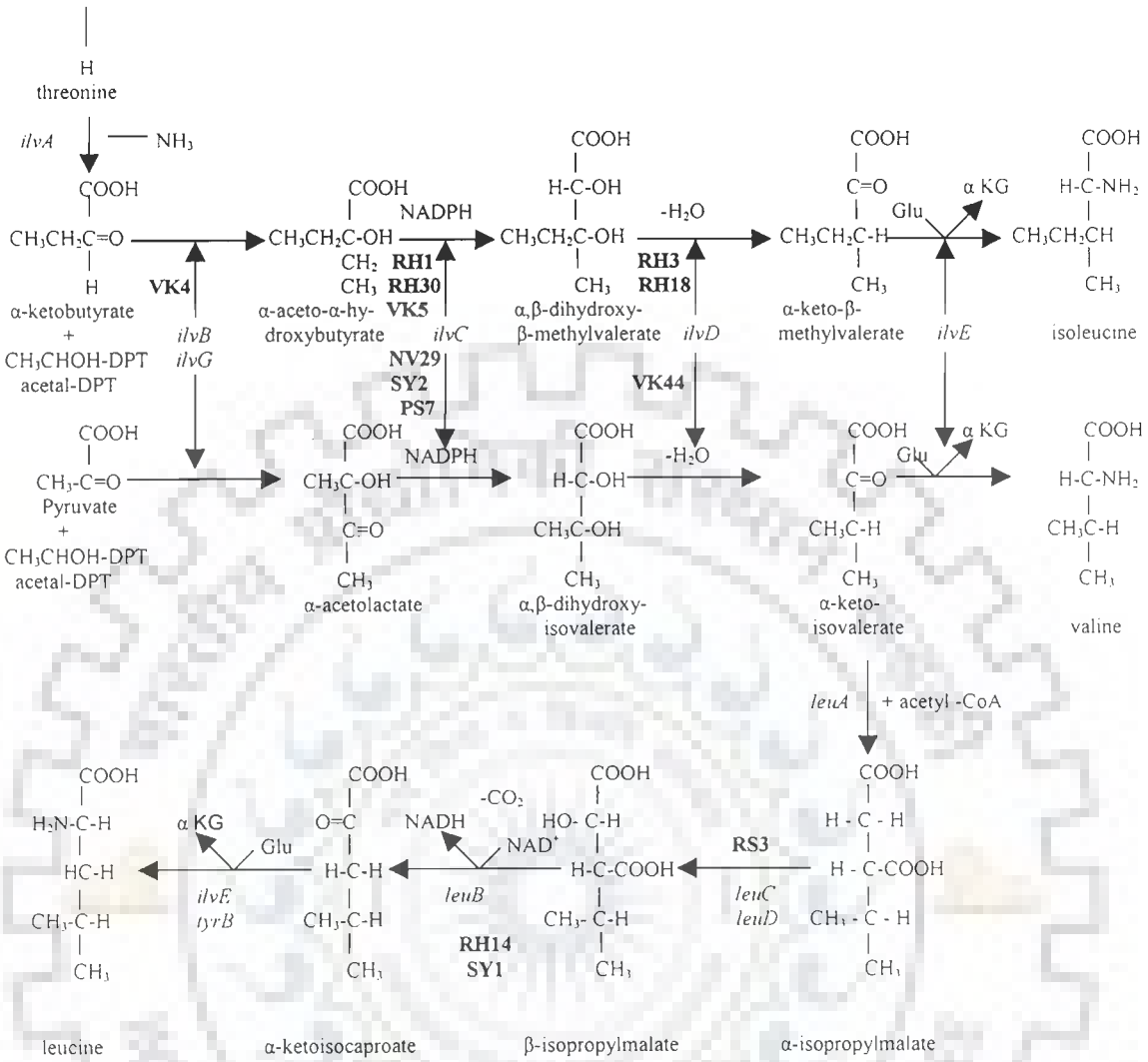


Fig. 8: The pathways of the synthesis of isoleucine and valine, and leucine showing the positions of mutation in the isoleucine + valine, and leucine auxotrophs of *Sinorhizobium meliloti* Rmd201

4.6 Pleiotropic effects of Tn5 insertions in auxotrophs

4.6.1 Production of cell surface molecules

All isoleucine + valine, and leucine auxotrophs, like the parental strain, showed growth in presence of sodium deoxycholate, took up the congo red dye, fluoresced in presence of calcofluor white under UV light and showed swarming on swarm plates indicating the normal production of lipopolysaccharides, cellulose fibrils, succinylated exopolysaccharides and β-(1→2) glucans, respectively, in these auxotrophs like the parental strain Rmd201. These auxotrophs and the parental strain did not show binding with aniline blue dye which indicated that β-(1→3) glucans, like the parental strain Rmd201, were not produced by these auxotrophic strains.

4.6.2 Utilization of sugars and dicarboxylic acids

The growth of all isoleucine + valine, and leucine auxotrophs on RMM supplemented with the respective auxotrophic requirement(s) was similar to that of the parental strain Rmd201 on RMM. These auxotrophs did not show any change in their growth behavior when glucose in RMM medium was replaced by any one of the other sugars (arabinose, lactose, galactose, maltose, xylose, mannose, mannitol and sucrose) or dicarboxylic acids (malate, aspartate and succinate) as a sole carbon source.

4.7 Linkage of Tn5 insertion to auxotrophy

The genome-mobilizing plasmid pJB3JI was introduced into each auxotroph and the resulting strain was mated as a donor with *S. meliloti* strain ZB555 (Cys^-, Phe^-, Rf^r, Sm^r) as a recipient. One hundred kanamycin resistant transconjugants were selected and tested for the presence of the donor's auxotrophic marker(s). In all crosses, all kanamycin

resistant transconjugants showed respective donor's auxotrophic marker(s) confirming 100% linkage of Tn5 insertion to auxotrophy in all isoleucine + valine, and leucine auxotrophs.

4.8 Genetic mapping of Tn5 insertions

Plasmid-mediated mapping method was used to locate the position of transposon Tn5 insertion in each isoleucine + valine auxotroph. The donor strain of each auxotroph, constructed by the introduction of plasmid pJB3JI, was mated with four *S. meliloti* recipient strains, *viz.*, ZB555, ZB556, ZB557 and ZB205, the markers of which cover most of the chromosome. In all crosses selection was made for the transfer of kanamycin resistance marker which is encoded by Tn5. The co-transfer percentages of unselected markers with kanamycin resistance marker were determined by streaking the transconjugants on appropriate selective plates. The kanamycin resistance marker showed 24, 9 and 5% co-transfer values with *ade-15* marker in RH18, RH1 and VK4 isoleucine + valine auxotrophs, respectively. The distances, calculated on the basis of linkage frequencies, are given in Tables 8, 9 and 10, and the possible chromosomal region containing Tn5 insertions of three isoleucine + valine auxotrophs is shown in Fig. 9.

4.9 Symbiotic properties of auxotrophs

Symbiotic properties of all isoleucine + valine, and leucine auxotrophs were determined by inoculating alfalfa seedlings, grown on nitrogen free plant nutrient medium, with these auxotrophs. All *ilvD* mutants (RH3, RH18 and VK44) induced cylindrical, pink nodules. The mean dry shoot weight of the plants inoculated with these

Table 8: Mapping of transposon Tn5 in *Sinorhizobium meliloti* Rmd201 isoleucine + valine auxotrophic strain RH18

Donor strain	Recipient strain	Selected marker	Unselected marker	No. of Km^r transconjugants analysed	No. of Km^r transconjugants showing the transfer of unselected marker	Linkage	Distance
RH181	ZB555	Km^r (*ilv* $^-$)	cys^+	215	0	——	——
			phe^+	215	0	——	——
RH181	ZB556	Km^r (*ilv* $^-$)	gly^+	250	0	——	——
			ade^+	250	0	——	——
			met^+	250	0	——	——
RH181	ZB557	Km^r (*ilv* $^-$)	phe^+	140	0	——	——
			leu^+	140	0	——	——
RH181	ZB205	Km^r (*ilv* $^-$)	ade^+	200	48	0.24	0.38

Abbreviations: Km^r, kanamycin resistance; *ilv* $^-$, isoleucine + valine auxotrophy; cys^+, cysteine prototrophy; phe^+, phenylalanine prototrophy; gly^+, glycine prototrophy; ade^+, adenine prototrophy; met^+, methionine prototrophy; leu^+, leucine prototrophy.

Table 9: Mapping of transposon Tn5 in *Sinorhizobium meliloti* Rmd201 isoleucine + valine auxotrophic strain RH1

Donor strain	Recipient strain	Selected marker	Unselected marker	No. of Km^r transconjugants analysed	No. of Km^r transconjugants showing the transfer of unselected marker	Linkage	Distance
RH11	ZB555	Km^r (*ilv* $^-$)	cys^+	145	0	----	----
			phe^+	145	0	----	----
RH11	ZB556	Km^r (*ilv* $^-$)	gly^+	190	0	----	----
			ade^+	190	0	----	----
			met^+	190	0	----	----
RH11	ZB557	Km^r (*ilv* $^-$)	phe^+	130	0	----	----
			leu^+	130	0	----	----
RH11	ZB205	Km^r (*ilv* $^-$)	ade^+	200	18	0.09	0.55

Abbreviations: Km^r, kanamycin resistance; *ilv* $^-$, isoleucine + valine auxotrophy; cys^+, cysteine prototrophy; phe^+, phenylalanine prototrophy; gly^+, glycine prototrophy; ade^+, adenine prototrophy; met^+, methionine prototrophy; leu^+, leucine prototrophy.

Table 10: Mapping of transposon Tn5 in *Sinorhizobium meliloti* Rmd201 isoleucine + valine auxotrophic strain VK4

Donor strain	Recipient strain	Selected marker	Unselected marker	No. of Km^r transconjugants analysed	No. of Km^r transconjugants showing the transfer of unselected marker	Linkage	Distance
VK41	ZB555	Km^r (*ilv* $^-$)	cys^+	180	0	----	----
			phe^+	180	0	----	----
VK41	ZB556	Km^r (*ilv* $^-$)	gly^+	210	0	----	----
			ade^+	210	0	----	----
			met^+	210	0	----	----
VK41	ZB557	Km^r (*ilv* $^-$)	phe^+	160	0	----	----
			leu^+	160	0	----	----
VK41	ZB205	Km^r (*ilv* $^-$)	ade^+	200	10	0.05	0.63

Abbreviations: Km^r, kanamycin resistance; *ilv* $^-$, isoleucine + valine auxotrophy; cys^+, cysteine prototrophy; phe^+, phenylalanine prototrophy; gly^+, glycine prototrophy; ade^+, adenine prototrophy; met^+, methionine prototrophy; leu^+, leucine prototrophy.

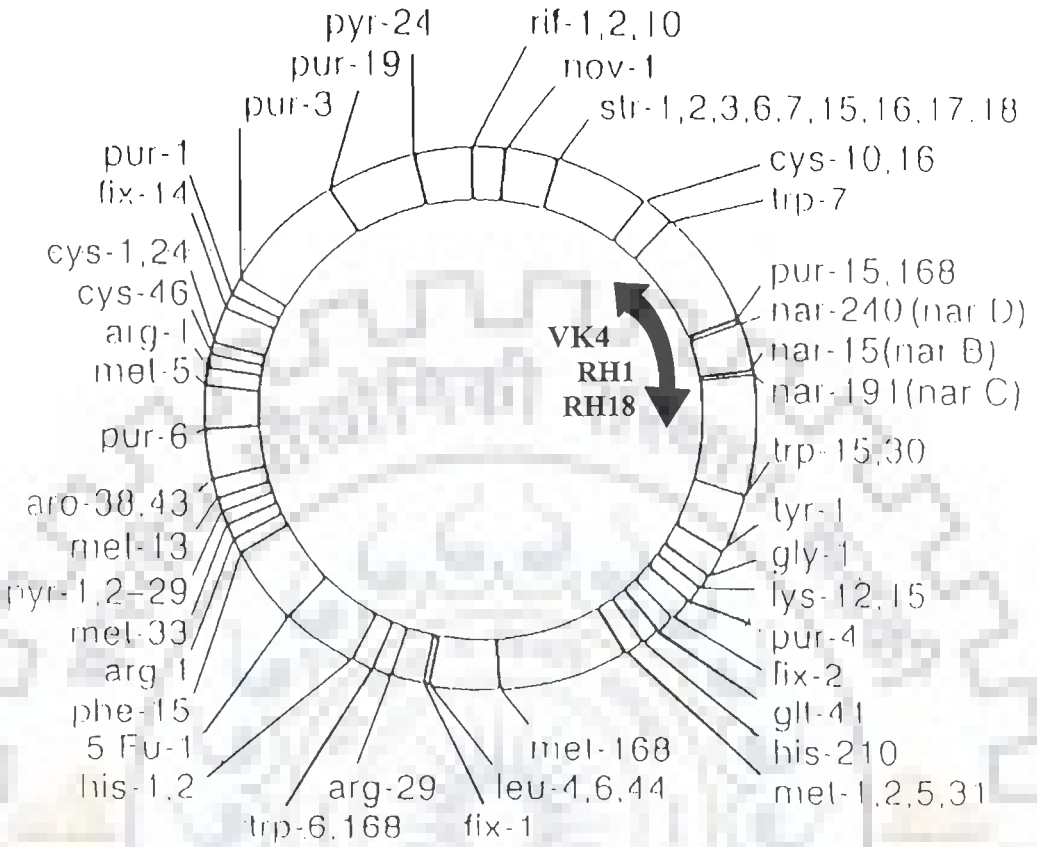


Fig. 9: Chromosomal map of *Sinorhizobium meliloti* Rm41 showing the possible region of Tn5 insertions in VK4, RH1 and RH18 isoleucine + valine auxotrophs

mutants did not differ significantly from that of the plants inoculated with the parental strain Rmd201 indicating that the nitrogen fixing efficiencies of *ilvD* mutants were similar to that of the parental strain. These strains were hence Nod^+ Fix^+. The single *ilvB / ilvG* mutant (VK4) and all *ilvC* mutants (RH1, RH30, VK5, NV29, SY2 and PS7) did not induce nodules (Table 11). These strains were therefore Nod $^-$(Plate 1).

All leucine auxotrophs induced round, white nodules. The mean dry shoot weights of the plants inoculated by these auxotrophs did not differ significantly from that of the uninoculated plants indicating that these auxotrophs did not fix nitrogen (Table 11). The leucine auxotrophs were hence Nod^+ Fix $^-$ (Plate 2).

Some alfalfa plants inoculated with auxotrophs had morphological features like the plants inoculated with the parental strain Rmd201. The nodules on these plants were found to be occupied by the prototrophic revertants. The data on these plants were not included.

4.10 Inoculation experiments using supplemented plant nutrient medium

The symbiotic interactions of isoleucine + valine, and leucine auxotrophs were studied using the supplemented nitrogen free plant nutrient medium. The supplementation with isoleucine and valine, or with α-keto-β-methylvalerate and valine did not restore the symbiotic defect of *ilvB / ilvG* (VK4) and *ilvC* (RH1, RH30, VK5, NV29, SY2 and PS7) mutants. Similarly supplementation with leucine or α-ketoisocaproate could not restore the symbiotic defects of leucine auxotrophs (Table 11).

Table 11: Symbiotic properties of alfalfa plants inoculated with *Sinorhizobium meliloti* Rmd201 and its isoleucine + valine, and leucine auxotrophs

Name of The strain	Auxotrophic requirement(s) / mutated gene	Mean shoot dry weight [a] (mg)	Nodule characteristics	
			Shape	Colour
Uninoculated	-	5.30±0.7	-	-
Rmd201	-	26.9±1.5	Cylindrical	Pink
VK4	Isoleucine + Valine / *ilvB / ilvG*	6.10±0.3*	-	-
RH1	Isoleucine + Valine / *ilvC*	5.50±0.9*	-	-
RH30	---do---	5.70±1.3*	-	-
VK5	---do---	5.90±0.5*	-	-
NV29	---do---	5.40±3.8*	-	-
SY2	---do---	5.80±2.5*	-	-
PS7	---do---	5.40±1.1*	-	-
RH3	Isoleucine + Valine / *ilvD*	24.1±1.8♣	Cylindrical	Pink
RH18	---do---	22.9±2.3♣	---do---	---do---
VK44	---do---	23.1±1.7♣	---do---	---do---
RS3	Leucine / *leuC / leuD*	6.50±1.5*	Round	White
RS3#	---do---	7.20±0.4*	Irregular	Slightly pink
RS3##	---do---	7.90±0.6*	---do---	---do---
RH14	Leucine / *leuB*	6.90±1.7*	Round	White
RH14 #	---do---	8.50±0.9*	Irregular	Slightly pink
RH14##	---do---	8.80±0.5*	---do---	---do---
SY1	---do---	6.70±1.3*	Round	White
SY1#	---do---	8.10±1.5*	Irregular	Slightly pink
SY1##	---do---	8.40±1.7*	---do---	---do---

[a] Each value is mean of ten plants; * Does not differ significantly from the mean shoot dry weight of the uninoculated plants ($P<0.05$); ♣ Does not differ significantly from the mean shoot dry weight of the plants inoculated with the parental strain Rmd201 ($P<0.05$)

Nitrogen free plant medium supplemented with 50µg/ ml α-ketoisocaproate

Nitrogen free plant medium supplemented with 50µg/ ml leucine

With these supplementations, however, the nodules induced by leucine auxotrophs became irregular and slightly pink.

4.11 Nodule occupancy tests

Nodule occupancy tests were conducted on alfalfa plants used to determine symbiotic properties of isoleucine + valine, and leucine auxotrophs. The rhizobial bacteria were isolated from the nodules of each plant and checked for auxotrophic marker(s). The percentages of nodule occupancy of various auxotrophs are given in Table 12. The data shows that percentages of plants showing 100% occupancy of nodules by the auxotrophs varied from 60% for leucine auxotroph SY1 to 92.5 % for isoleucine + valine auxotroph RH18.

4.12 Root hair curling and infection thread formation

The *ilvB / ilvG* mutant VK4 did not induce root hair curling and infection thread formation. The *ilvC* mutants (RH1, RH30, VK5, NV29, SY2 and PS7) were capable of curling the root hair but no infection threads in root hairs were found. The *ilvD* mutants (RH3, RH18 and VK44) and all leucine auxotrophs resulted in root hair curling and infection thread formation (Plate 3).

Plate 1: Morphological features of the representative plants of alfalfa inoculated with *Sinorhizobium meliloti* Rmd201 and its isoleucine + valine auxotrophic mutants.

A. Uninoculated plant

B. Rmd201 inoculated plant

C. VK4 inoculated plant

D. RH1 inoculated plant

E. RH18 inoculated plant

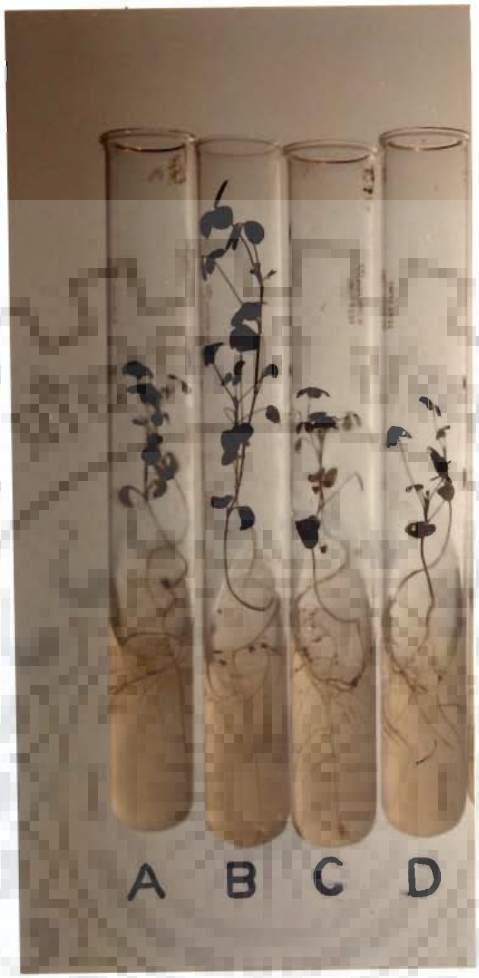


Plate 2: Morphological features of the representative plants of alfalfa inoculated with *Sinorhizobium meliloti* Rmd201 and its leucine auxotrophic mutants.

A. Uninoculated plant

B. Rmd201 inoculated plant

C. RS3 inoculated plant

D. RH14 inoculated plant

Table 12: Nodule occupancies of alfalfa plants with isoleucine + valine, and leucine auxotrophs of *Sinorhizobium meliloti* Rmd201

S. meliloti strain	Auxotrophic requirement(s)	Mutated gene	Symbiotic phenotype	No. of plants showing 100% occupancy of nodules by the auxotroph*	Percentage of plants showing 100% occupancy of nodules by the auxotroph
VK4	Isoleucine + Valine	*ilvB / ilvG*	Nod^-	------	------
RH1	---do---	*ilvC*	---do---	------	------
RH30	---do---	---do---	---do---	------	------
VK5	---do---	---do---	---do---	------	------
NV29	---do---	---do---	---do---	------	------
SY2	---do---	---do---	---do---	------	------
PS7	---do---	---do---	---do---	------	------
RH3	---do---	*ilvD*	$Nod^+ Fix^+$	34	85.0
RH18	---do---	---do---	---do---	37	92.5
VK44	---do---	---do---	---do---	33	82.5
RS3	Leucine	*leuC / leuD*	$Nod^+ Fix^-$	27	67.5
RH14	---do---	*leuB*	---do---	29	72.5
SY1	---do---	---do---	---do---	24	60.0

* Forty alfalfa plants were inoculated with each auxotroph

4.13 Microscopic examination of sections of the nodules induced by the parental strain Rmd201 and its leucine auxotrophs

4.13.1 Rmd201 parental strain

4.13.1.1 Light microscopy

The longitudinal cross section of a nodule induced by the parental strain Rmd201 showed a central tissue, which was surrounded by several peripheral tissues. The peripheral tissues included vascular bundles. The central tissue consisted of five zones, *viz.*, apical meristematic zone, infection zone, interzone between infection and nitrogen fixation zones, nitrogen fixation zone and senescence zone (Plate 4A). Uninfected and constantly dividing nodule cells were present in the meristematic zone (Plate 4B). Intercellular spaces of the nodule cells in the infection zone contained infection threads and a few nodule cells of this zone were found to be infected by rhizobia. Prominent nuclei were seen in a few nodule cells of this zone (Plate 4B). Several starch granules were also visible in the infection zone.

In the interzone, most of the nodule cells towards infection zone were without rhizobia whereas most of the nodule cells towards nitrogen fixation zone were containing rhizobial cells (Plate 4C). Some starch granules were also found in the interzone. In the nitrogen fixation zone, a large number of nodule cells were packed with bacteroids (Plate 5A). A large number of elongated and rodshaped bacteroids were arranged perpendicular to a centrally located vacuole (Plate 5B). In the senescence zone oldest nodule cells were observed (Plate 5B).

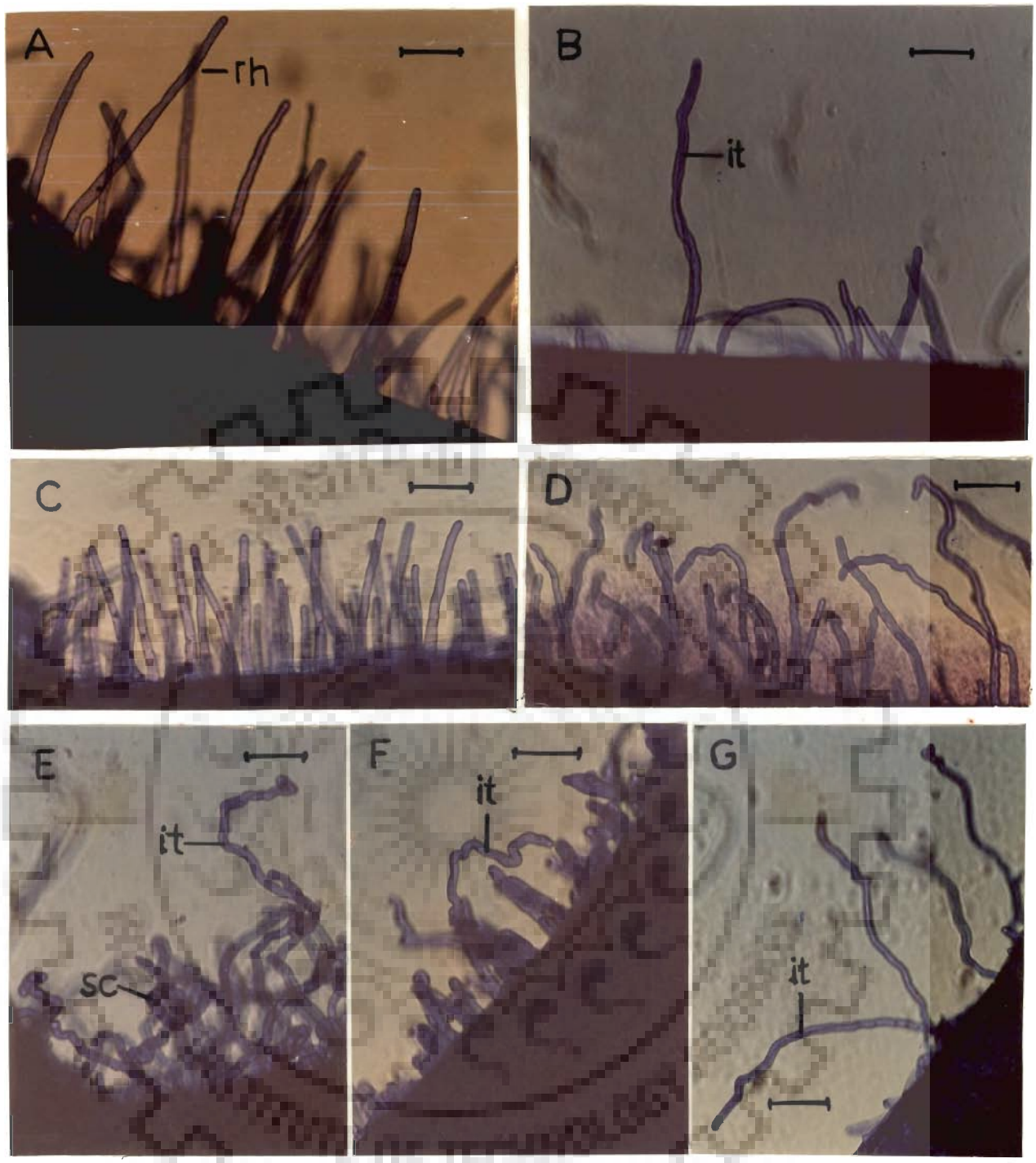


Plate 3: Root hair curling and infection thread formation in root hairs of alfalfa seedlings inoculated with *Sinorhizobium meliloti* Rmd201 and its isoleucine + valine, and leucine auxotrophic mutants, **A.** Uninoculated **B.** Rmd201 inoculated **C.** VK4 inoculated **D.** RH1 inoculated **E.** RH18 inoculated **F.** RS3 inoculated and **G.** RH14 inoculated.

Abbreviations: it, infection thread; rh, root hair and sc, shepherd's crook. Bar: 100μm (x 100).

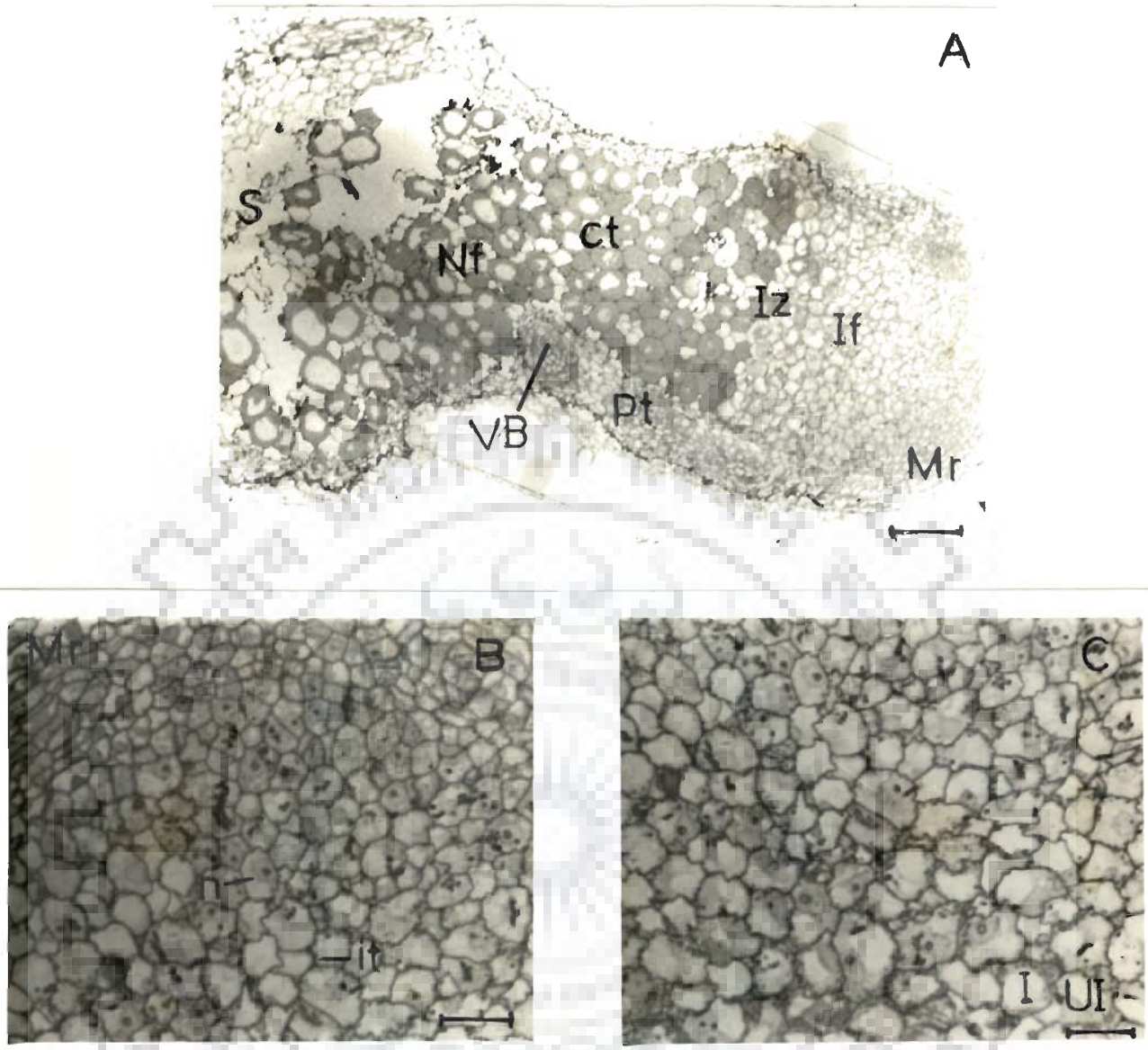


Plate 4: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A.** A nodule section showing distinct peripheral tissue (pt) and central tissue (ct); meristematic zone (Mr), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S) are seen in central tissue, Bar: 100µm (x 100), **B.** Nodule cells of meristematic zone (Mr) and infection zone (If) showing infection threads (it) and prominent nuclei (n), Bar: 25µm (x 400), and **C.** Zone between infection and nitrogen fixation zone showing infected nodule cells (I), and uninfected nodule cells (UI), Bar: 25µm (x 400).

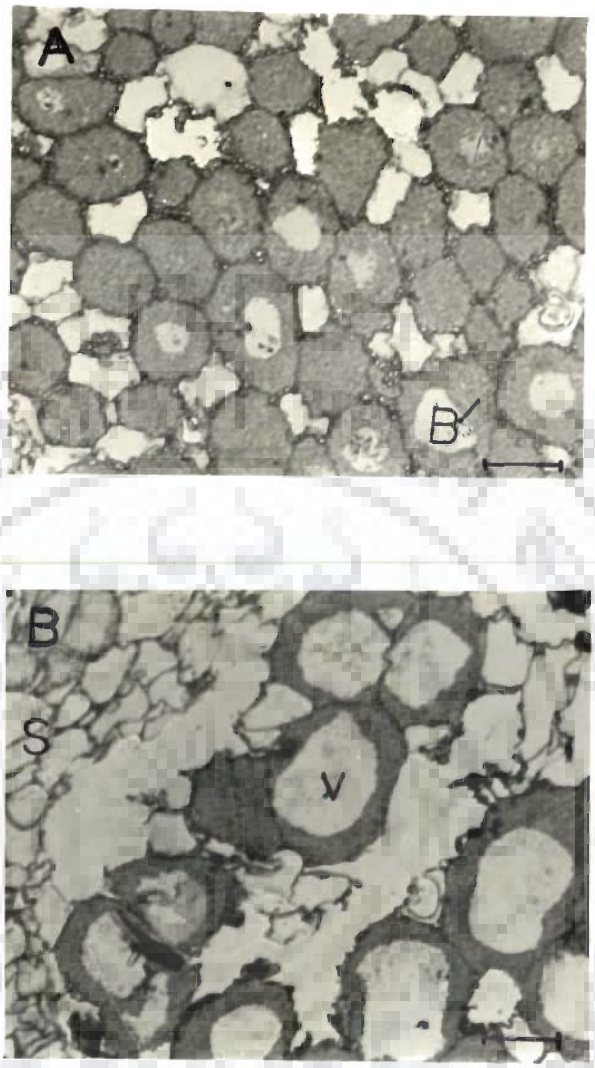


Plate 5: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A.** Nodule cells packed with bacteroids (B) in nitrogen fixation zone, Bar: 25µm (x 400), and **B.** Nitrogen fixation zone showing bacteroids were arranged perpendicularly to a centrally located large vacuole (v); side of senescence zone showing oldest nodule cells, Bar: 25µm (x 400).

4.13.1.2 Electron microscopy

Electron microscopic examination of a nodule section revealed that the released bacteria in the infection zone were electron dense and contained poly-β-hydroxy butyrate (PHB) granules. The freshly released bacterial cells were enclosed in a peribacteroidal membrane (pbm) (Plate 6A). These cells were spherical but some elongated rhizobial cells in the infection region near to nitrogen fixation zone were also seen (Plate 6B).

The bacteroids in the interzone had heterogeneous cytoplasm with electron dense and electron transparent regions. These bacteroids were elongated and did not contain PHB granules (Plate 7A). The bacteroids in the nitrogen fixation zone showed complete heterogeneity of cytoplasm. These bacteroids were of several shapes but most of these were elongated (Plate 7B). The plant cell organelles in the nodule cells filled with bacteroids and in those without bacteroids took the peripheral position near to the cell wall (Plate 7B; 7C). Degenerated bacteroids, which had transparent cytoplasm and broken pbm, were seen in the senescence zone (Plate 8A; B).

4.13.2 *leuC/leuD* mutant RS3

4.13.2.1 Light microscopy

The entire nodule consisted of loosely packed nodule cells. A profuse network of infection threads in the intercellular spaces was seen throughout the nodule (Plate 9A). Most of the plant cells in the centre of the nodule were flooded with amyloplasts (Plate 9A; B). Rhizobial bacteria were not seen in plant cells indicating that their release from infection threads did not occur. Prominent nuclei were rarely observed in most of nodule cells.

4.13.2.2 Electron microscopy

There was no release of rhizobial bacteria from infection threads into nodule cells (Plate 10A). Amyloplasts, mitochondria and endoplasmic reticulum profiles were seen in nodule cells (Plate 10B; C).

4.13.3 *leuB* mutant RH14

4.13.3.1 Light microscopy

The nodule induced by the *leuB* mutant RH14 showed a few advanced features over the *leuC* / *leuD* mutant induced nodule. Out of five distinct zones of a parental strain induced nodule, meristematic and infection zones were clearly seen (Plate 11A). Prominent nuclei and vacuoles were visible in many nodule cells in infection zone (Plate 11 B). The plant cells in the basal half of the nodule contained several amyloplasts (Plate 11C). Released rhizobia were observed in many nodule cells of the infection zone (Plate 11B).

4.13.3.2 Electron microscopy

Electron dense rhizobial bacteria were observed in infection threads. PHB granules were seen in these rhizobial cells (Plate 12A). Normal bacterial release from the infection threads into nodule cells was observed. Some of the released rhizobial cells were elongated. The cytoplasm of the released bacteria showed electron dense and electron transparent regions (Plate 12B). Rhizobial bacteria in lysed condition were seen in several nodule cells (Plate 13A). Rhizobia were not found in some nodule cells (Plate 13B).

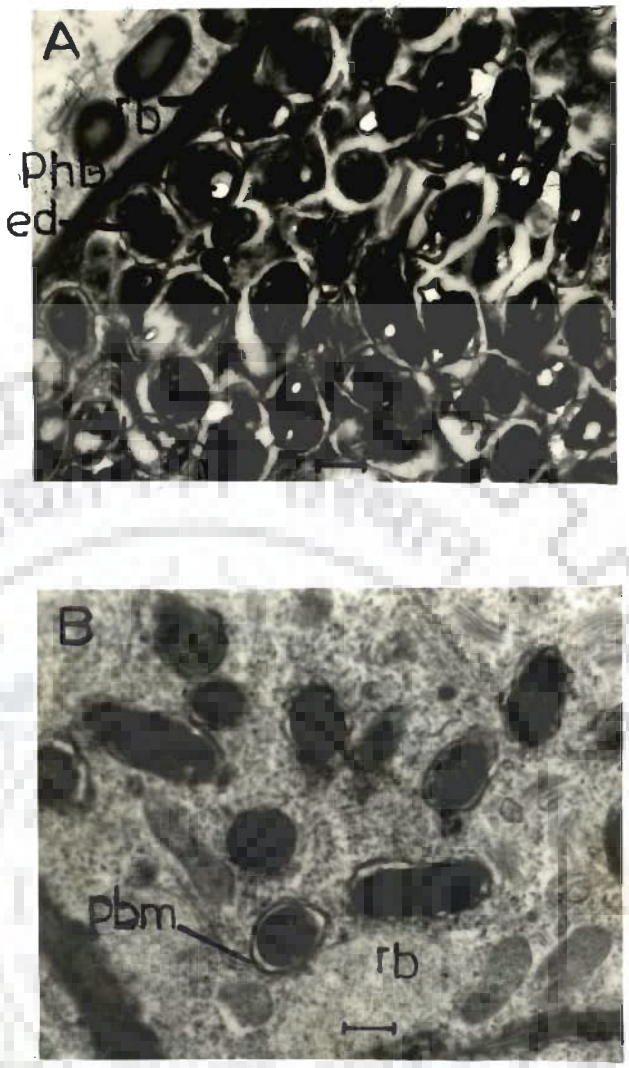
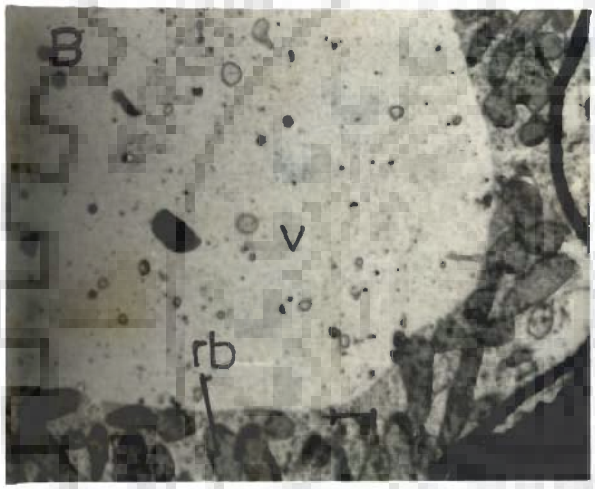


Plate 6: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, A. A part of the nodule cell showing freshly released rhizobial bacteria (rb) in the infection region near to nitrogen fixation zone were electron dense (ed) and containing poly-β-hydroxybutyrate (phb) granules, Bar: 1μm (x 5800), and B. Nodule cell from same zone showing elongated rhizobial bacteria (rb) enclosed by a wavy peribacteroidal membrane (pbm), Bar: 1μm (x 6600).



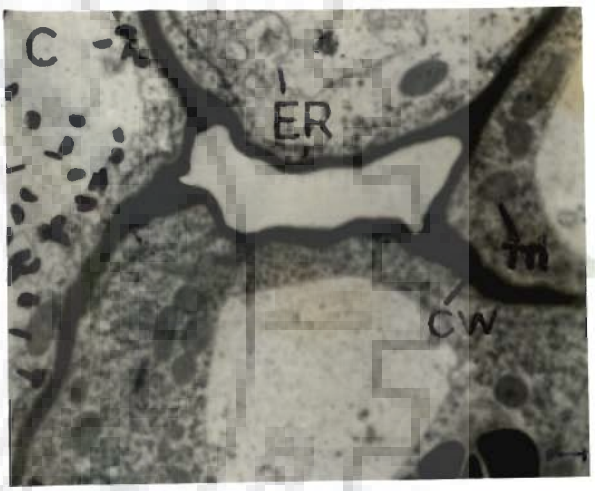


Plate 7: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, **A.** A part of nodule cell of the interzone showing rhizobial bacteria having heterogeneous cytoplasm (hc) and numerous vacuoles (v), Bar: 1μm (x 3900), **B.** A nodule cell of nitrogen fixation zone with large central vacuole showing elongated rhizobial bacteria (rb) with complete heterogeneity of cytoplasm, Bar: 1μm (x 4900), and **C.** An empty cell from the nitrogen fixation zone showing peripherally placed organelles like mitochondria (m) and endoplasmic reticulum (ER). Intercellular spaces and cell wall (cw) are clearly seen, Bar: 1μm (x 4500).

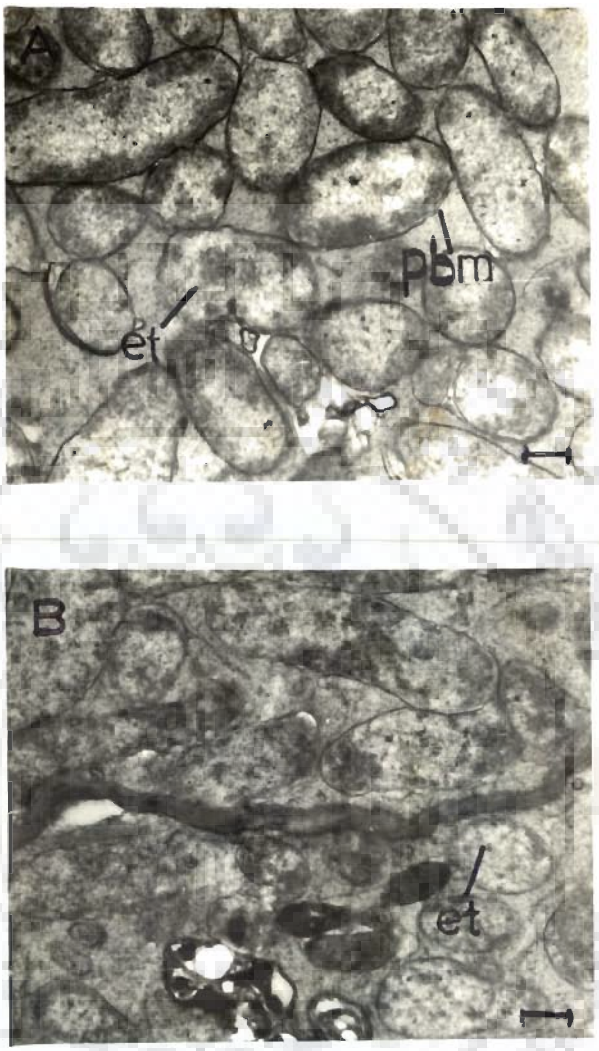


Plate 8: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by *Sinorhizobium meliloti* Rmd201, A. and B. A part of nodule cell (of senescence zone) showing degeneration of rhizobial bacteroids (B); electron transparent cytoplasm (et) and broken peribacteroidal membrane (pbm) is also visible in some bacteroids, Bar: 1μm (x 6100) and 1μm (x 6800) respectively.

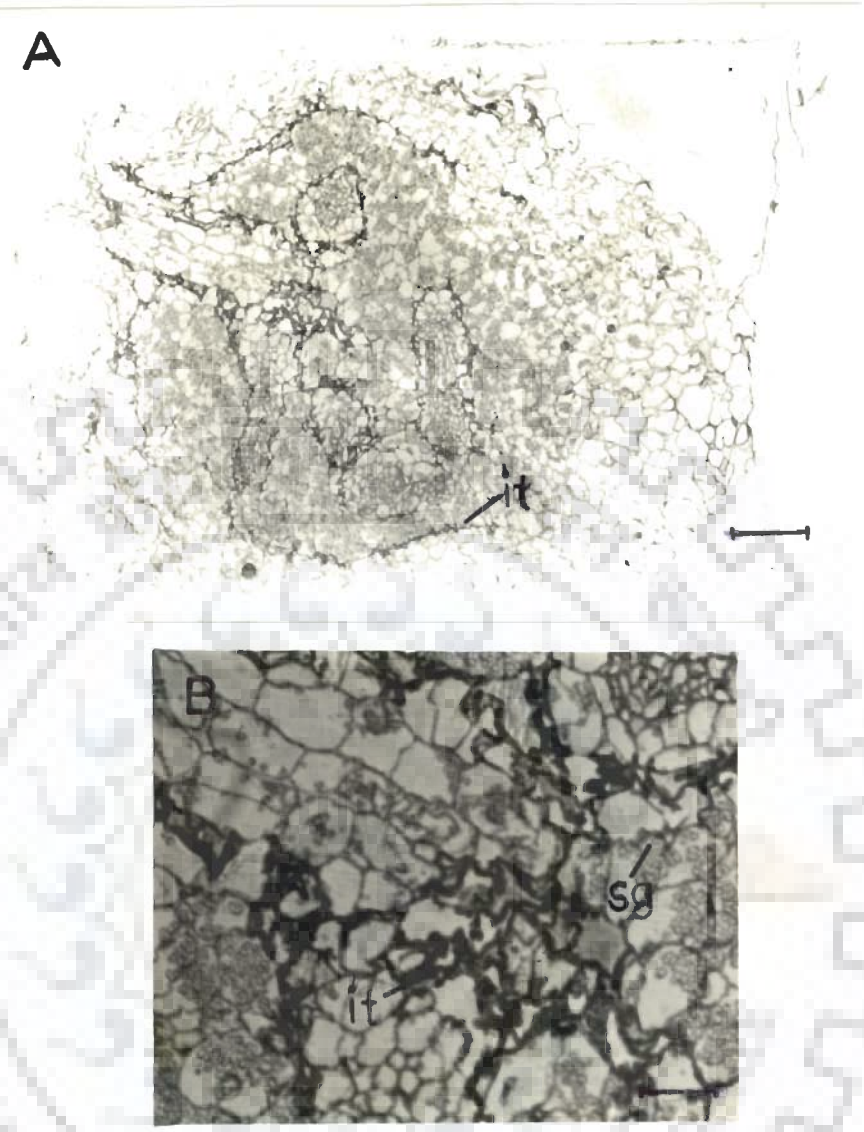


Plate 9: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain RS3, a *leuC / leuD* auxotroph of *Sinorhizobium meliloti* Rmd201, A. Loosely packed nodule cells with profuse network of infection threads (it); different zones are absent in this nodule, Bar: 100µm (x 100), and B. Nodule cells showing no release of rhizobial bacteria and most of these cells are flooded with starch granules (sg), Bar: 25µm (x 400).

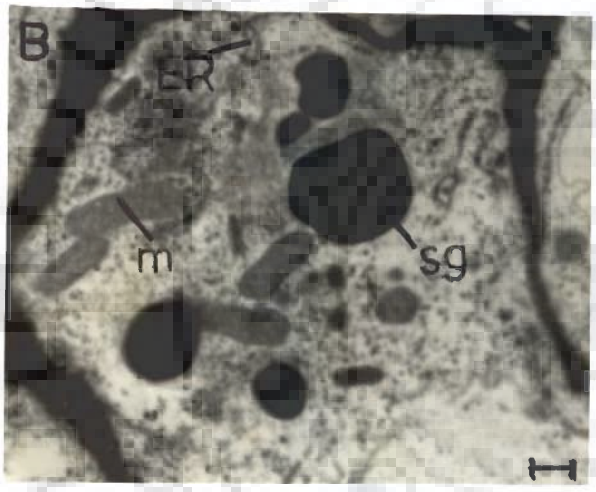


Plate 10: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain RS3, a *leuC / leuD* auxotroph of *Sinorhizobium meliloti* Rmd201, A. A part of nodule cell showing infection thread; no release of rhizobial bacteria is visible, Bar: 1 μm (x 2100), B. and C. A part of nodule cell showing cell organelles like mitochondria (m), endoplasmic reticulum (ER). Starch granules also seen in these cells, Bar: 1 μm (x 5300) and 1 μm (x 4000) respectively.

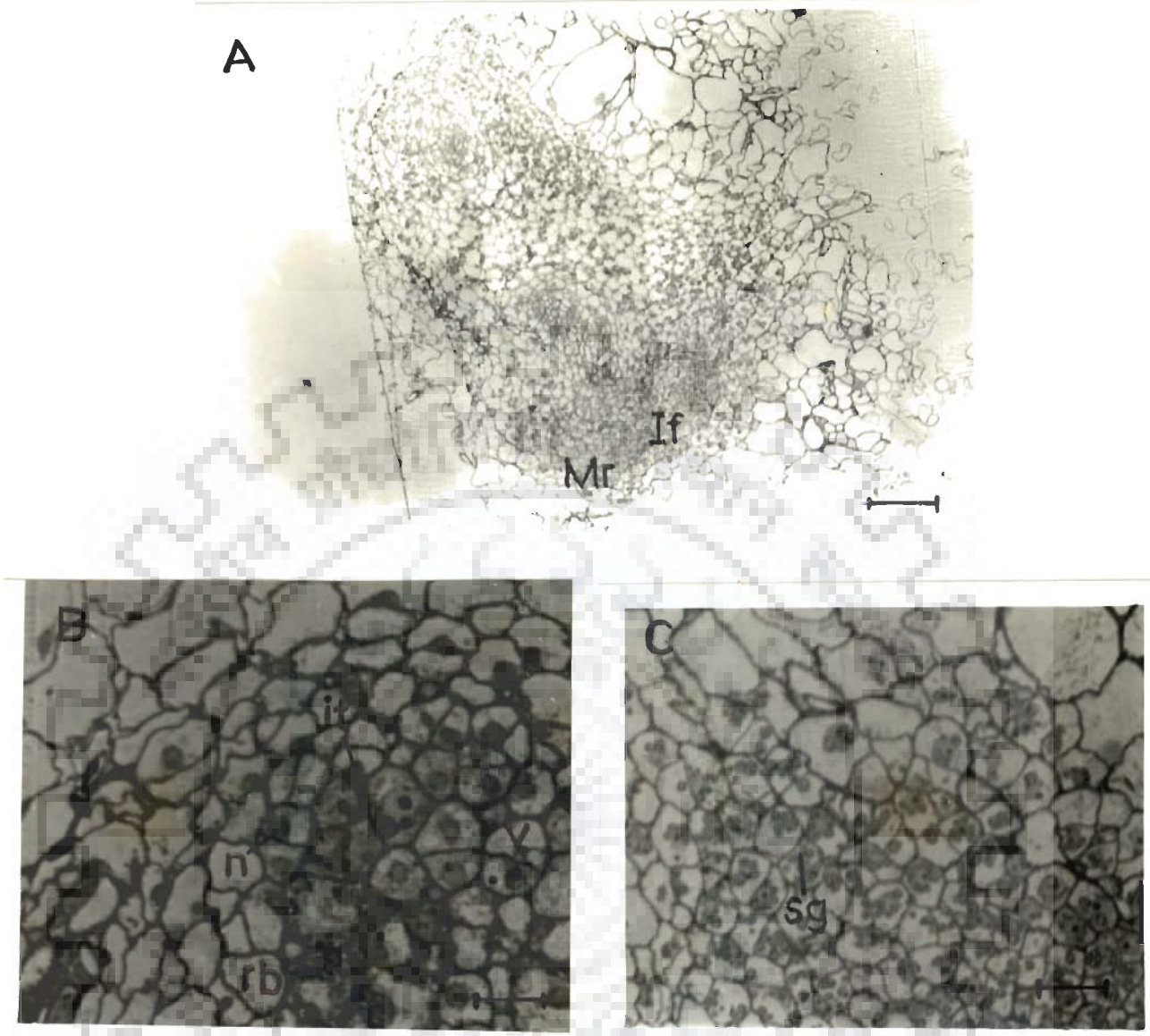


Plate 11: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain RH14, a *leuB* auxotroph of *Sinorhizobium meliloti* Rmd201, **A.** A nodule section showing a few advanced features over the parental strain Rmd201 induced nodules, Bar: 100µm (x 100), **B.** Nodule cells in infection zone (If) showing infection threads (it) in intercellular spaces of nodule cells and prominent nuclei, vacuoles are also visible in these cells; released rhizobial bacteria (rb) are seen clearly in some of the nodule cells, Bar: 25µm (x 400), and **C.** Nodule cells in the basal half of the nodule are packed with starch granules (sg), Bar: 25µm (x 400).

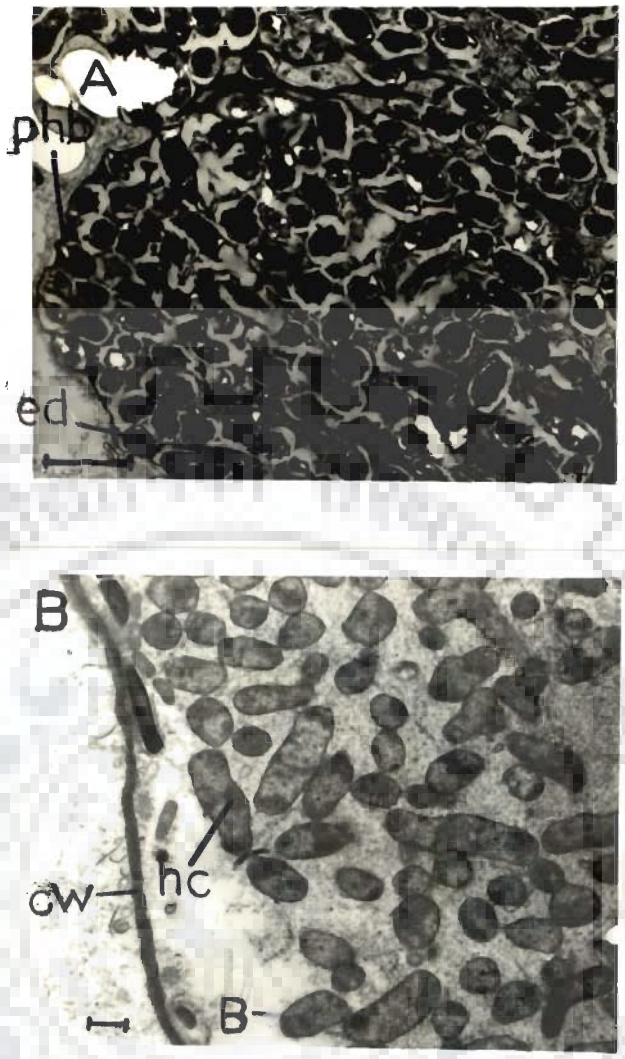


Plate 12: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain RH14, a *leuB* auxotroph of *Sinorhizobium meliloti* Rmd201, A. Higher magnification of infection thread (it) showing rhizobial bacteria (rb) with electron dense cytoplasm (ed); poly-β-hydroxybutyrate (phb) granules are seen in many of these rhizobial cells, Bar: 1μm (x 11900), and B. A part of nodule cell showing released bacteroids (B) from infection thread (it) with heterogeneous cytoplasm (hc), Bar: 1μm (x 5000).

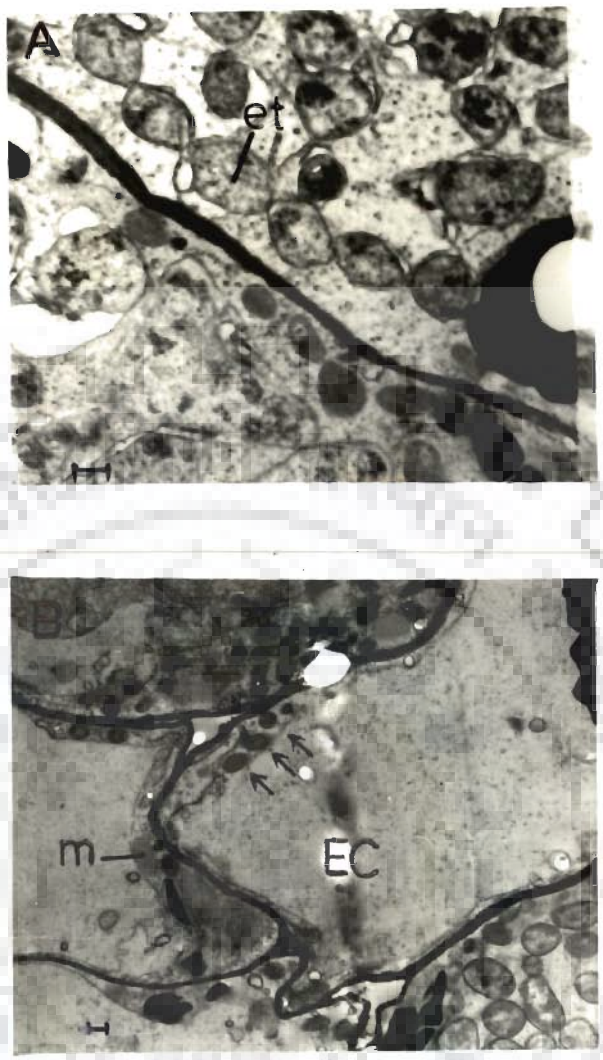


Plate 13: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain RH14, a *leuB* auxotroph of *Sinorhizobium meliloti* Rmd201, A. A part of nodule cell showing electron transparent cytoplasm (et) of rhizobial bacteria; broken peribacteroidal membrane (pbm) also indicating that these rhizobial bacteria are in lysed condition, Bar: 1μm (x 4700), and B. A part of nodule cells without bacteroids (EC) showing the cytoplasm restricted to the periphery and all cell organelles (indicated by arrows) are displaced near to cell wall, Bar: 1μm (x 2300).

4.13.4 *leuB* mutant RH14 supplied with 50µg/ ml leucine

4.13.4.1 Light microscopy

Distinct peripheral and central tissues were observed in the section of a nodule induced by the *leuB* mutant RH14 in plant nutrient medium supplemented with 50µg/ ml leucine. The central tissue of this nodule, like that of the parental strain Rmd201 induced nodule, comprised five distinct zones. However, the nitrogen fixation zone was poorly developed (Plate 14A). Very few nodule cells were filled with rhizobia in the nitrogen fixation zone (Plate 14B). Moreover, in this zone the arrangement of bacteroids around a large central vacuole was present in only some of the rhizobia filled nodule cells.

4.13.4.2 Electron microscopy

Rhizobial bacteria in the infection threads were electron dense and spherical (Plate 15A). Freshly released rhizobia in the nodule cells were also electron dense. Some of these rhizobial cells were elongated. The bacteroids in the nitrogen fixation zone were elongated and had heterogeneous cytoplasm (Plate 15B). Lysed bacteroids were seen in some nodule cells (Plate 15C).

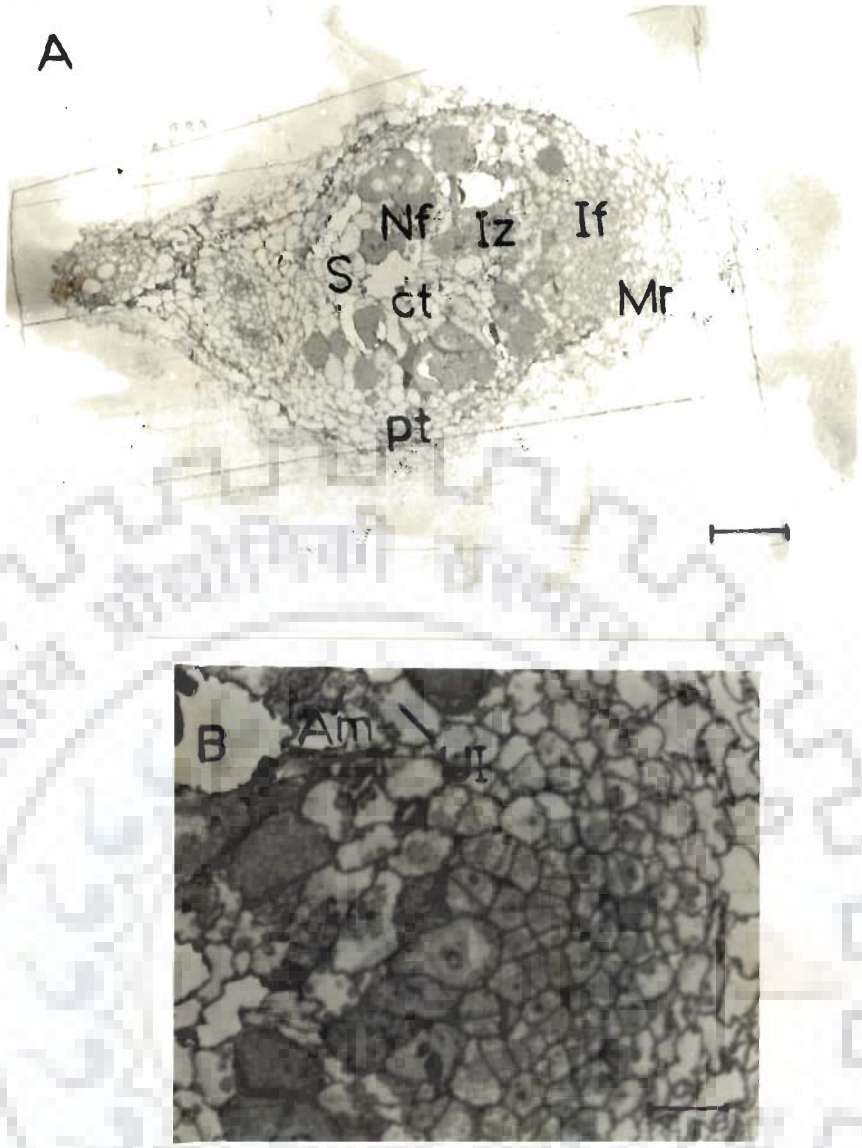
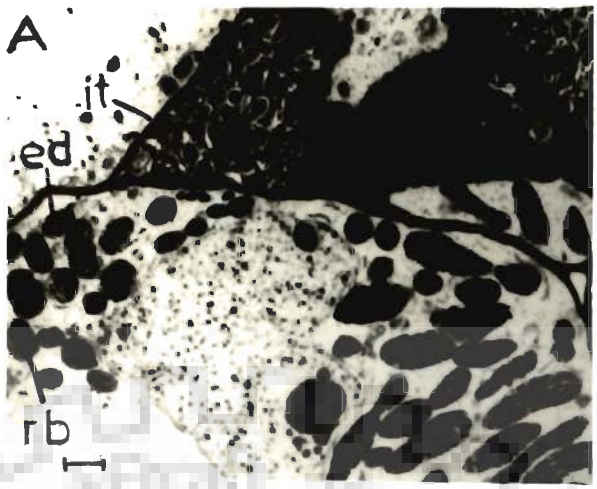
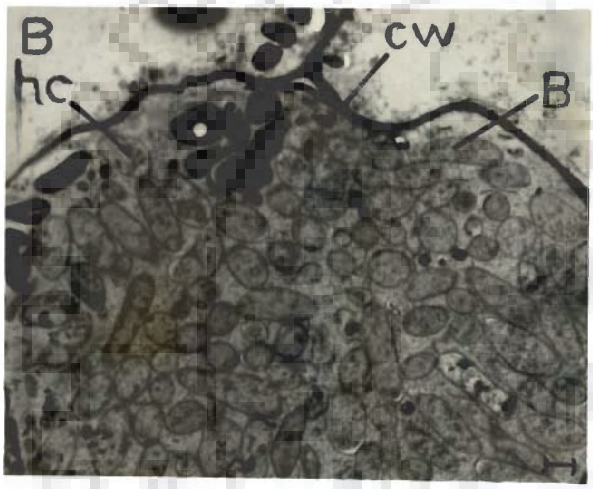


Plate 14: Light microscopic examinations of longitudinal-semithin sections of a nodule induced by the strain RH14, a *leuB* auxotroph of *Sinorhizobium meliloti* Rmd201 supplemented with 50µg/ml leucine, A. A nodule section showing distinct peripheral tissue (pt) and central tissue (ct) with different zones, *viz.*, meristematic zone (Mr), infection zone (If), interzone (Iz), nitrogen fixation zone (Nf) and senescence zone (S), Bar: 100µm (x 100), and B. A part of nodule section showing uninfected cells (UI) with amyloplasts (Am) and poorly developed nitrogen fixation zone, Bar: 25µm (x 400).





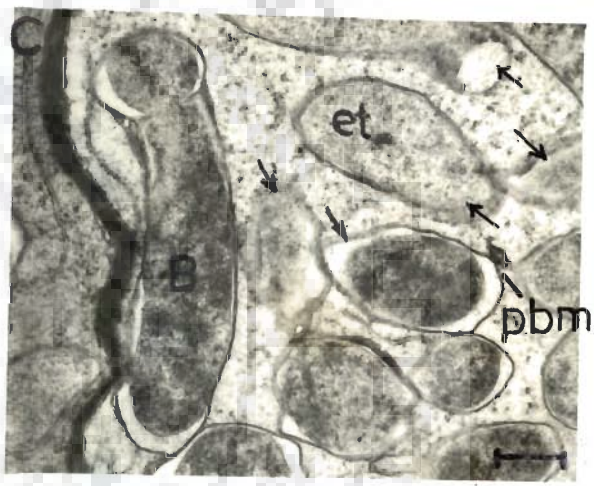


Plate 15: Transmission electron microscopic examinations of longitudinal-ultrathin sections of a nodule induced by the strain RH14, a *leuB* auxotroph of *Sinorhizobium meliloti* Rmd201 supplemented with 50µg/ml leucine, **A.** A part of nodule cells of infection zone showing infection threads (it) in intercellular spaces filled with electron dense rhizobial bacteria (rb); released rhizobial bacteria were also electron dense, Bar: 1µm (x 5200), **B.** A part of nodule cell in nitrogen fixation zone filled with elongated bacteroids (B) having heterogeneous cytoplasm (hc), Bar: 1µm (x 3500), and **C.** A part of nodule cell showing broken peribacteroidal membrane (pbm) and electron transparent cytoplasm (et) of some bacteroids (B), Bar: 1µm (x 8400).

Chapter 5

DISCUSSION

Random transposon Tn5 mutagenesis of *S. meliloti* Rmd201 strain yielded 5,450 kanamycin resistant Tn5 derivatives, out of which 30 were found to be auxotrophs. The frequency of occurrence of auxotrophs among Tn5-induced derivatives was 0.5%. Similar frequencies of occurrence of auxotrophs among Tn5 derivatives have been reported in rhizobia by the other workers (Meade *et al.*, 1982; Kim *et al.*, 1988; Prasad *et al.*, 2000; Vineetha *et al.*, 2001). The isolation of auxotrophs having diverse nutritional requirements indicated randomness of Tn5 insertions as reported in the previous studies.

Six genes, *viz.*, *ilvA*, *ilvB*, *ilvG*, *ilvC*, *ilvD* and *ilvE*, control five and four biochemical steps in the biosynthesis of isoleucine and valine, respectively. In the present work mutations have been obtained in *ilvB* / *ilvG*, *ilvC* and *ilvD* genes resulting in biochemical blocks at three steps in the biosynthesis of each of these amino acids. Six genes, *viz.*, *leuA*, *leuC*, *leuD*, *leuB*, *ilvE* and *tyrB* control four biochemical steps in the biosynthesis of leucine. In this study mutations have been obtained in *leuC* / *leuD* and *leuB* genes resulting in biochemical blocks at two steps in leucine biosynthesis. Hence in the present work symbiotic roles of about half the number of genes/enzymes/intermediates involved in the biosynthesis of isoleucine, valine and leucine have been studied.

The isoleucine + valine, and leucine auxotrophs were similar to the parental strain Rmd201 w.r.t. cell surface molecules and utilization of carbon sources. These results demonstrated that the symbiotic defects of symbiotically defective isoleucine + valine and leucine auxotrophs were not caused by a change in any of the above characteristics.

When each auxotroph containing chromosome mobilizing plasmid pJB3JI was mated with *S. meliloti* ZB555 recipient strain, 100% co-transfer of Tn5-encoded

kanamycin resistance and auxotrophy occurred. This result indicated that a single Tn5 insertion is responsible for auxotrophy in each auxotroph. *S. meliloti* strain Rmd201 used in this study is a derivative of strain AK631 which in turn is a compact colony mutant of Rm41 strain. In *S. meliloti* Rm41 strain, some leu mutations have been located on the chromosomal map (Kondorosi *et al.*, 1977) but none of the *ilv* genes has been mapped so far. In this work *ilvB* / *ilvG*, *ilvC* and *ilvD* genes have been found to be linked to *ade-15* marker.

All *ilvD* mutants induced nitrogen fixing nodules on alfalfa plants and nitrogen fixing efficiencies of these mutants were similar to the nitrogen fixing efficiency of the parental strain Rmd201. Aguilar *et al.* (1991) have also reported normal symbiotic activity of the *ilvD* mutant of *S. meliloti* 1021 isolated by them. These results indicated that the expression of *ilvD* gene does not have a direct role in the symbiosis of *S. meliloti* with alfalfa plant, and the alfalfa plant is able to provide both isoleucine and valine amino acids to rhizobia during nodule formation and function.

All *ilvC* mutants were Nod^- and the Nod^+ phenotype was not restored by the supplementation of the plant nutrient medium with isoleucine and valine, or α-keto-β-methylvalerate and valine. These results showed that the two intermediate products, *viz.*, α,β-dihydroxy-β-methylvalerate and/or α,β-dihydroxyisovalerate, which are produced by the *ilvC* encoded enzyme acetohydroxy acid isomeroreductase, may have a role in nodule formation. Another possibility is that the enzyme acetohydroxy acid isomeroreductase is able to recognize a substrate other than those in isoleucine and valine pathways, and the product of its conversion is required for nodule formation.

Plant flavonoids induce the expression of rhizobial *nod* genes and the *nod* gene products synthesize Nod factors which result in the deformation and curling of root hairs, and division of cortical cells in the roots of the legume plant (Schlaman *et al.*, 1989; Journet *et al.*, 1994; Díaz *et al.*, 2000). Hence it is obvious that in the *ilvC* mutants either Nod factor is not formed or it is not functional. Aguilar and Grasso (1991) have reported that in the *ilvC* mutant isolated by them, *nodABC* genes were not activated by the inducer luteolin and hence Nod factor was not formed. The *ilvC* mutants isolated during this work appear to synthesize, in response to plant signal, a defective Nod factor since these mutants were found to induce root hair curling. As no root hair curling was noticed in the single *ilvB / ilvG* mutant, the expression of *ilvB* and *ilvG* genes also seems essential for the synthesis of a functional Nod factor.

All leucine auxotrophs of *S. meliloti* isolated by earlier workers were Nod^+ Fix^- (Truchet *et al.*, 1980; Kerppola and Kahn, 1988b; Nichik *et al.*, 1995). The leucine auxotrophs of *S. meliloti* isolated during this work were also Nod^+ Fix^-. These findings suggest that either the alfalfa host does not provide leucine to rhizobial bacteria in nodule or a specific enzyme(s) and/or intermediate(s) of leucine biosynthetic pathway of *S. meliloti* has a direct role in nitrogen fixation. All leucine auxotrophs induced ineffective nodules in the plant nutrient medium supplemented with leucine. Either leucine from the plant medium is not reaching rhizobia inside the nodules or a specific enzyme(s) and/or intermediate(s) of leucine biosynthetic pathway of *S. meliloti* is directly involved in nitrogen fixation. The uptake problem for leucine seems unlikely since Truchet *et al.* (1980) have reported the restoration of nitrogen fixation in the nodules induced by leucine auxotrophs by supplementing leucine in the plant nutrient medium. The mutated

leucine biosynthetic gene was not identified by these workers. The mutations in these leucine auxotrophs were likely to be in the genes involved in the last or penultimate step of leucine biosynthesis. These genes were apparently not having a direct role in symbiosis. During this work nitrogen fixation was not observed in the nodules induced by leucine auxotrophs in the α-ketoisocaproate supplemented plant medium. The restoration of nitrogen fixation is not expected since *leuC* / *leuD* and *leuB* genes appear to have a direct role in symbiosis.

Among the plants inoculated with auxotrophs, a few plants had nodules containing prototrophic revertants. Revertant rhizobial cells formed as a result of spontaneous excision of transposon Tn5 obviously had a selective advantage over auxotrophic cells in nodule formation because the frequencies of nodule occupancy by revertants were much higher than the frequency of spontaneous excision of Tn5.

Histological studies revealed that the nodules induced by the leucine auxotrophs did not develop fully like those induced by the parental strain. The nodules induced by *leuB* mutants were structurally more advanced than the *leuC* / *leuD* mutant induced nodules. In the former case rhizobial bacteria were released from the infection thread into plant cells whereas in the latter case the rhizobia did not come out of the infection threads. The reason for this result may be that *leuC* and *leuD* encoded enzyme isopropylmalate dehydratase converts an unknown substrate into a product which is required for the release of rhizobial bacteria from the infection threads into plant cells. Another reason for the above result may be that the leucine biosynthetic pathway intermediate β-isopropylmalate, which is formed by the action of isoproplylmalate dehydratase, has a role in rhizobial release into plant cells. Other workers (Truchet *et al.*,

1980; Nichik *et al.*, 1995) have reported that in the nodules induced by leucine auxotrophs release of rhizobia from the infection threads did not occur. However the positions of biochemical block in these auxotrophs were not known.

Histological structure of the nodule induced by *leuB* mutant RH14 was improved to some extent by the supplementation of the plant nutrient medium with leucine. The host plant does not appear to provide sufficient amount of leucine to rhizobia in nodules. But this result also does not rule out the direct symbiotic role of *leuB* gene.

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