CHARACTERIZATION OF GENES INVOLVED IN BIOSYNTHESIS OF SOME AMINO ACIDS IN *Rhizobium meliloti*

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Characterization of genes involved in biosynthesis of some amino acids in Rhizobium 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 February 1989 to July 1994 under the supervision of **Dr. G. S. Randhawa.**

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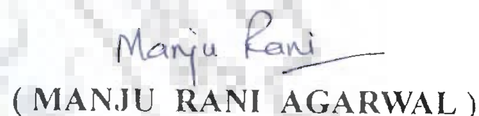

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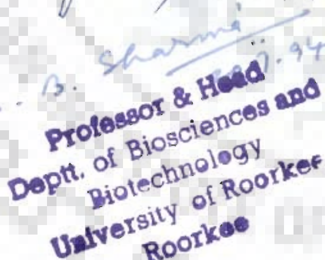

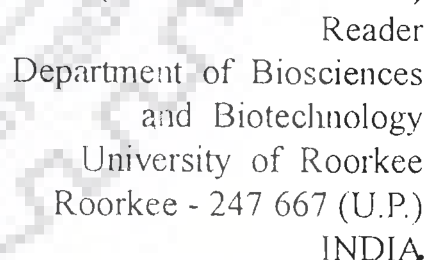

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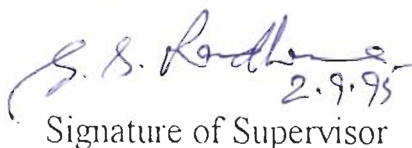

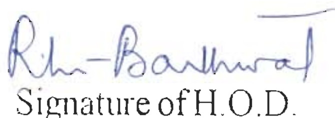

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ABSTRACT

The present study was undertaken to isolate and characterize amino acid auxotrophic mutants of *R. meliloti.* A total of thirty one amino acid auxotrophs of *R. meliloti* Rmd 201 were isolated. The current investigation deals with the nature of the metabolic blocks and the role of these mutants in the symbiotic process.

For obtaining auxotrophic mutants, *R. meliloti* ZB201 (Cys^-, Rif^r) was subjected to random Tn5 mutagenesis. Transposon Tn5 carrying suicide vector pGS9 was used for Tn5 mutagenesis. Conjugation (patch mating) was carried out between *R. meliloti* ZB201 and *E. coli* WA803 (pGS9) to obtain Tn5 derivatives. The mating time of sixteen hours was found sufficient for getting good frequency of Tn5 derivatives. A total of five thousands kanamycin resistant Tn5 exconjugants were isolated. All the Tn5 derivatives were found to be chloramphenicol sensitive, indicating positive suicide of pGS9 vector. Sixteen auxotrophic mutants were obtained when the growth of Tn5 derivatives was tested on minimal medium. These auxotrophs were found to be highly unstable and moreover their nutritional requirement could not be specified. Tn5 mutagenesis was done again with *R. meliloti* Rmd201 (Str^r derivative of *R. meliloti* AK631). Tn5 derivatives were obtained at a frequency of 0.4×10^{-4} transconjugants/recipient. Six thousand Km^r mutants were isolated and tested for nutritional auxotrophy. Seventeen auxotrophs were identified: $proline^-$ (three), $isoleucine^-$ (one), aspartic $acid^-$ (two), $methionine^-$ (one), $tyrosine^-$ (one), glutamic $acid^-$ (one), $leucine^-$ (one), $cysteine^-$ (one), $phenylalanine^-$ (two), $valine^-$ (one), $threonine^-$ (two) and $histidine^-$ (one).

Auxotrophic mutants of *R. meliloti* Rmd201 were also isolated by N-methyl-N′-nitro-N-nitrosoguanidine (NTG) mutagenesis followed by penicillin enrichment. A concentration of 100 µg/ml was used to yield six thousand NTG induced mutants. Thirty auxotrophs were isolated and tested for nutritional requirements. Sixteen mutants were found to possess multiple auxotrophies. Fourteen auxotrophs for amino acids were obtained as follows : five for threonine, two for proline, three for

lysine, one for asparagine and tyrosine and one for each valine, tryptophan and methionine.

All the amino acid auxotrophs of R. meliloti were subjected to reversion analysis. The revertants of seventeen Tn5 derived auxotrophs were found to be Km^s indicating precise excision of Tn5 at a frequency of 10^{-10}. NTG induced auxotrophs also reverted at the same frequency.

All the purified auxotrophic mutants were tested for their symbiotic properties by inoculating them on Medicago sativa seedlings grown aspetically on nitrogen free agar slants in test tubes. After eight weeks, data on individual plants were recorded for nodule number, nodule colour, nodule size, nodule shape, plant height, dry plant weight and total nitrogen per plant. Out of thirty one auxotrophic mutants, one auxotroph (proline$^-$) was found to be Nod$^-$, seven auxotrophs (threonine$^-$, valine$^-$, histidine$^-$, aspartic acid$^-$, proline$^-$, leucine$^-$ and phenylalnine$^-$) were found to have reduced nitrogen fixing ability and two auxotrophs (tryptophan$^-$ and lysine$^-$) were found to be more efficient in nitrogen fixation. When the revertants were similarly examined, they resembled the wild type strain Rmd201, indicating that changes in the symbiotic behaviour of the above strains could be attributed to their auxotrophic mutations. In all the above plant experiments, the bacteria were isolated from the nodules to confirm that nodules were colonized by the same strain with which the plant was inoculated.

The symbiotically altered auxotrophic mutants were tested for their symbiotic ability to fix nitrogen by inoculating them on nitrogen free agar supplemented with their auxotrophic requirement. The symbiotic properties of all the auxotrophs except four (requiring aspartic acid, lysine, tryptophan and threonine) were restored on supplementation with their nutritional requirement. The results with these four auxotrophs indicate either exogenous supply of amino acid is not accessible to the bacteroids in the nodules or the involvement of a particular intermediate of biosynthetic pathway in the symbiotic process.

The plasmid constructs pGR1 and pGR3 containing known symbiotic functions were transferred to different auxotrophs having altered symbiotic ability. Both pGR1 and pGR3 could not restore the symbiotic nitrogen fixation ability indicating that the symbiotic genes on nod-nif region present on megaplasmid were not mutated in these auxotrophic mutants.

Cross feeding and intermediate supplementation of amino acid auxotrophs were done to identify the metabolic blocks in the amino acid biosynthetic pathways. From the results, it appears that auxotrophs were having defects in the different steps of the biosynthetic pathways.

The clones complementing the different auxotrophic mutations were isolated from the gene bank of R. meliloti by doing triparental crosses. For five auxotrophs, recombinant clones could not be obtained. The reason for this could be that these clones were not represented in the gene bank.

The soluble proteins of whole cell extracts from the wild type and its auxotrophic mutants was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).No marked difference between protein patterns of auxotrophic mutants and the wild type was found.

These auxotrophs were tested for their ability to utilise various sugars, response to high osmolarity and high temperatures. The number of arabinose$^-$, xylose$^-$, lactose$^-$, galactose$^-$ and maltose$^-$ auxotrophs were three , two, one, one and one, respectively. Two auxotrophs were osmosensitive and one was temperature sensitive.

The symbiotically affected auxotrophs were also tested for their surface properties. Two auxotrophs were Exo$^-$, two were β(1-3) glucan$^+$, two were Cel$^-$ and one was found unable to produce lipopolysaccharides. These results show the pleiotropic nature of these auxotrophic mutations.

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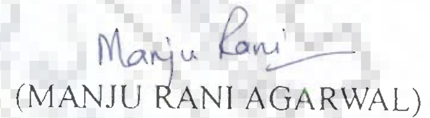

(MANJU RANI AGARWAL)

LIST OF ABBREVIATIONS USED

1.	Ade	=	Adenine
2.	Ara	=	Arabinose
3.	BHR	=	Broad host range
4.	C.D.	=	Critical difference
5.	Cel	=	Cellulose fibrils
6.	Chl	=	Chloramphenicol
7.	Cma	=	Chromosome mobilizing ability
8.	Cys	=	Cysteine
9.	DOC	=	Sodium deoxy cholate
10.	EMS	=	Error mean square
11.	EPS	=	Exopolysaccharide
12.	EXO	=	Exopolysaccharide
13.	Fix	=	Nitrogen fixation
14.	Gm	=	Gentamycin
15.	hsn	=	Host specific nodulation
16.	Kb	=	Kilo base
17.	Kd	=	Kilo dalton
18.	Km	=	Kanamycin
19.	Lac	=	Lactose
20.	LB	=	Luria Bertani
21.	Leu	=	Leucine
22.	LPS	=	Lipopolysaccharide
23.	Met	=	Methionine
24.	Mob	=	Mobilization
25.	MSY	=	Mannitol salt yeast extract

26. Mtl = Maltose
27. Neo = Neomycin
28. Nif = Structural gene for nitrogenase enzyme
29. Nod = Nodulation
30. NTG = N-methyl-N′-nitro-N-nitrosoguanidine
31. OriT = Origin of transfer
32. PAGE = Polyacrylamide gel electrophoresis
33. PNM = Plant nutrient medium
34. Pro = Proline
35. Rif = Rifampicin
36. RMM = Rhizobial minimal medium
37. SDS = Sodium dodecyl sulphate
38. Str = Streptomycin
39. Tet = Tetracycline
40. Thi = Thiamine
41. Tra = Transfer
42. TY = Tryptone yeast extract
43. Xyl = Xylose
44. YEMA = Yeast extract mannitol agar

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INTRODUCTION

Our planet's atmosphere comprises of three main gases, namely oxygen, carbon dioxide and nitrogen, out of which nitrogen alone shares about 80% by volume. Given this relative abundance of elementary nitrogen it is surprising to know that in agricultural areas, where sunlight and water supply are not limiting for crop growth, productivity is determined mainly by the availability of nitrogen in the soil. This is explained by the fact that the atmospheric nitrogen is chemically nonreactive and not accessible to plants as such. A crucial step in the incorporation of it into the soil involves its conversion into reactive or accessible forms like nitrate, nitrite and ammonia. Commercial production of nitrogen fertilizers consumes a large amount of non-renewable fossil resources. Moreover, a major fraction of the nitrogenous fertilizers used in agriculture is lost as nitrates that pollute the ground water reserves, rivers, lakes and other water bodies and pose a serious health hazard. In contrast, nitrogen fixing organisms can be produced at very low cost and provide nitrogen in the vicinity of crop root system where it is required. Therefore, expanded use of biological nitrogen fixation can lead to sustainable agriculture.

Biological nitrogen fixation by microorganisms is an important link in the nitrogen cycle of the planet. It involves the conversion of the atmospheric nitrogen to ammonia by a group of prokaryotes called diazotrophs. This natural process is cheap, pollution free, forms an abundant source of nitrogen and also an attractive alternative to chemical fertilizers.

The ability to fix dinitrogen is reported to be distributed among several prokaryotic

taxonomic groups, including Azotobacteriaceae, Enterobacteriaceae, Rhodospirillaceae, Bacillaceae, Rhizobiaceae, Actinomycetaceae and Cyanobacteria (32). Among these organisms, the physiological conditions under which fixation of nitrogen occurs vary but the enzymatic apparatus involved in nitrogen fixation appears to be largely similar (203). In relation to the direct application of biological nitrogen fixation to agriculture, the important question is how to make available to the plant the ammonia fixed by the micro-organisms, without it's much dissipation.

These bacteria on the basis of their degree of association with plants, can be classified into three main groups (i) free living (Klebsiella pneumoniae, Azotobacter vinelandii, Rhodospirillum rubrum, etc.) (ii) associative (Azospirillum spp.) and (iii) symbiotic species (Rhizobium, Frankia, etc.). The last group, the symbiotic species, is of special significance to agriculture, since it contributes a lot to the nitrogen economy of crop plants. According to one estimation, by Burns and Hardy (32), more than 25% of the terrestrial nitrogen fixation is accounted for by Rhizobium-legume symbiosis. Rhizobia are the agriculturally most important nitrogen fixing organisms. They form intimate symbiotic relationship with leguminous plants. They induce formation of nodules on the legume roots. In the nodules, the bacteria grow at the expense of photosynthates transported from shoot. The bacteroids, a transformed form of Rhizobium in legume root nodules, reduce nitrogen to ammonia. This ammonia is assimilated by the host plant which in turn compensates the bacteria by meeting their energy needs (145). Rhizobia in the root nodules are known to fix nitrogen at a higher rate than that of free living nitrogen fixing bacteria or azospirilla that form loose associations with plants (132). Since most of the grain and fodder legume crop plants are nodulated by rhizobia, the importance of legume-Rhizobium symbiosis is immense in agriculture.

Genetic investigations are in progress to dissect the complex series of

interactions involved in Rhizobium-legume symbiosis. Recently, with concomitant development of recombinant DNA technology, it has become possible to analyse and understand the molecular basis of this plant-microbe interaction. A two pronged approach has been adopted to understand the mechanism of symbiosis—analysis of the role of the host plant and analysis of the role of the bacteria. In plants, due to their biological complexity, the progress has not been rapid. Nevertheless, considerable information about their role has been obtained. No less than twenty proteins of host origin have been identified in the nodules and a number of genes encoding these proteins have been identified and characterized (255). This is not the complete picture, very little is known about the regulation of expression of these plant genes.

With bacteria, the progress has been rapid. It has been shown that rhizobia carry plasmids of large and small sizes and the genetic determinants for symbiotic functions are largely present on plasmids. A large number of genes involved in nitrogen fixation, nodule development and regulation have been identified (255). These genes are generally located on megaplasmids. Some of these plasmids have been shown to be self transferable (114). Several cell surface molecules have been implicated in early stages of infection process. The exopolysaccharide component of cell surface is one molecule, whose production is controlled by several genes that are also located on a megaplasmid which is different from the one having nod, nif and fix genes. Circular maps of the chromosome of R. meliloti, R. leguminosarum, R. phaseoli and R. trifolii have been given in some detail (19,133). Gene banks have been constructed for several strains of Rhizobium (61,83). The use of Rhizobium as a biofertilizer depends upon it's specificity to a crop, effectivity in nitrogen fixation, competitive ability against other rhizosphere microorganisms and ability for survival and growth under stress conditions. There is a need to understand the genetic basis of all the different steps of nodulation process, growth and adaptive responses. Much additional work is required for this purpose, with the use of such rhizobia where advance can be made rapidly.

Among the two groups of rhizobia, fast growers and slow growers, the former nodulate the important crops such as pea, clovers, beans and alfalfa. The fast growing rhizobia serve as better genetic system because relatively lesser time is required for their colony formation. Among the fast growers, certain strains of R. meliloti are known to grow relatively faster. This organism forms symbiotic association with three genera of Medicago group: Medicago, Melilotus and Trigonella. Therefore, R. meliloti provides a relatively more efficient genetic system for genetic dissection of their role in the complex process of symbiotic nitrogen fixation. Moreover, the host plants of R. meliloti, alfalfa and methi, are small in size. This facilitates assaying of bacterium plant interactions under laboratory conditions. These two advantages make R. meliloti a very useful genetic system.

Indeed, most of the recent work which has resulted in advancing the knowledge about genetics of Rhizobium has been in R. meliloti and R. leguminosarum. R. japonicum has also been worked upon to some extent. In all these organisms, the approach of site directed mutagenesis (204) has been utilized. But, this approach limits the analysis to functions specified by genes already cloned on the basis of the known involvement in symbiosis or genes linked to them. Therefore, this approach needs to be supported by the approach of random mutagenesis followed by isolation of mutants having desired phenotype by screening. Random mutagenesis can be achieved by using conventional mutagens or transposons.

Since Rhizobium does not fix nitrogen or express other symbiotic phenotypes in the free living state, chemical or physical mutagenesis must be followed by screening of several thousands of bacterial colonies through individual plant assays. The use of transposons can simplify this problem (i) because transposons such as Tn5 carrying resistance (Km^r and Neo^r) would tag the gene into which they get inserted to cause mutation. Thus, the cells having Tn5 inserted into their genomes can be identified and

screened. Moreover, several mutagenesis procedures have failed to induce a range of defined mutations including metabolic and symbiotic deficiencies. Transposon Tn5 mutagenesis has resulted in the isolation of a variety of auxotrophic and symbiotic mutants (105,199,206).

In the symbiotic relationship between Rhizobium and its host legume, a complex set of biochemical interactions take place during the development and maintenance of nitrogen fixing nodules on the plant roots (239,255). Some of these interactions probably involve surface macromolecules such as extracellular polysaccharides and lipopolysaccharides. In addition, small molecules are exchanged by the symbionts both during nodule development and in the mature nodule. The bacteria depend on their host for carbon and energy, but it is unknown what other metabolites they require or are available to them from the plant host. In addition, some functions of rhizobia indirectly influence symbiosis. These include metabolic pathways for the synthesis of amino acids (206,208,209), nitrogenous bases (154,173) and vitamins (218) and the reactions leading to production of cell surface components (147).

Thus analysing the symbiotic capabilities of auxotrophic mutants is one way of identifying some of the metabolic requirements for establishing a successful symbiotic relationship. Symbiotically defective auxotrophic mutants have been isolated from several fast growing species of Rhizobium, e.g. R. leguminosarum (178,181,217), R. meliloti (56,75,153,154,210) and R. trifolii (182,218). It was shown by Malek et al. (153,154) and Sadowsky et al. (206) that mutations in some of the biosynthetic histidine genes lead to symbiotic defect.

Several observations about the failure of amino acid auxotrophs to participate in symbiosis have been made, yet little is known about the specific component of the pathway responsible for this. Thus studies on the symbiotic properties of ineffective,

auxotrophic mutants of Rhizobium could enable differentiation of the stages leading to the establishment of the symbiotic association and the role of metabolites in this process. Though a few genes involved in the biosynthesis of amino acids have been identified and studied but knowledge is far from complete. For example the genes involved in the biosynthesis of isoleucine, threonine and valine have still not been studied in Rhizobium. Hence this work on the molecular genetics of the biosynthesis of amino acids and their role in symbiotic nitrogen fixation in Rhizobium was undertaken with following objectives:

1. To isolate a large number of auxotrophic mutants of R. meliloti.
2. To screen these auxotrophic mutants for their specific amino acid requirements.
3. To determine the nature of metabolic blocks in amino acid biosynthetic pathways.
4. To characterize these auxotrophic mutants with reference to symbiotic nitrogen fixation i.e. nodulation and nitrogen fixation.
5. To clone specific genes for amino acid biosynthesis by complementation.
6. To observe the pleiotropic effects of these auxotrophic mutations.

LITERATURE REVIEW

Gram negative soil bacteria of the genus Rhizobium interact with legumes to produce root nodules. Rhizobium meliloti infects and forms nodules on alfalfa (lucerne, Medicago sativa) and two related genera, Melilotus and Trigonella. Nodule development is initiated in R. meliloti - alfalfa symbiosis by bacterium-plant interactions which result in the deformation of root hairs, elaboration of a characteristic invasion structure (the infection thread), and stimulation of cell divisions in the host (25). These differentiated root structures furnish the necessary environment for rhizobia to reduce atmospheric nitrogen to ammonia. The fixed nitrogen is exported to plants, where it is assimilated. The host plant in turn reduces the atmospheric carbon dioxide to carbohydrates by photosynthesis and translocates these products to the nodules where the rhizobia use them to meet their energy requirements. Bacterial genes required for symbiotic function have been identified through mutations in many species and often have been shown to be located on large indigenous plasmids of various Rhizobium species (112). The symbiotic interaction of the rhizobia with the legumes is highly specific since a species of Rhizobium is able to induce nodules on one or a small group of legumes.

There are two major groups of root nodule inducing rhizobia:

i) Slow growing, alkali producing bacteria belonging to the genus Bradyrhizobium and

ii) Fast growing, acid producing bacteria that belong to the genus Rhizobium (115).

This division of root nodule inducing nitrogen fixing bacteria into two groups is based on their properties in both free living and symbiotic state. They differ in their numerical taxonomy (18), DNA base ratios (53), nucleic acid hybridization (87), composition of intracellular gum (68), carbohydrate utilization (231), bacteriophage sensitivity (167) and antibiotic susceptibility (237) characteristics. The bacteria

belonging to the genus Bradyrhizobium infect a vast number of legume species and also show some explanta nitrogenase activity, whereas the bacteria of the genus Rhizobium infect only a small number of closely related legumes (72). Normally the fast growing rhizobia enter the host plant via root hair while slow growing enter different hosts by different means (43).

2.1 DEVELOPMENT AND METABOLISM OF NODULES

Development of root nodules is a complex process in which the bacteria and the plant participate in a variety of activities(81), such as cell morphogenesis and various metabolic functions (169,256). Several distinct steps have been recognised in the development of root nodules (26). These are:

2.1.1 Pre-infection

In order to colonize the root, bacteria must first get there. Two possible mechanisms have been suggested to account for the presence of rhizobia near the host root. First the chemotaxis (33,152). Like many bacteria, rhizobia may move towards certain compounds of leguminous seed and root exudate. Recent studies have suggested that rhizobia may also respond to flavonoids. Plant flavones have been reported to act as signal molecules inducing the expression of bacterial nodulation (nod) genes (80,135). Subsequently, the bacteria are attached to the host root hairs. This is the crucial step in the host range determination and is achieved by recognition of specific exopolysaccharides of the rhizobial cell surface by the host lectins (59,104,150). The lipopolysaccharide structure is changed stably in response to various environmental conditions including the bacteroid environment (245). Once the bacteria are attached to the root surface, they induce the deformation of root hairs, called "Shepherd's Crook" curling (51). Root cortex cells are then stimulated to divide (169).

2.1.2 Infection

Bacteria attached to root hairs penetrate them through an infection thread which is derived from the plant cell wall (254). These infection threads then develop, ramify

and penetrate individual target cells of the nodules (34,169).

2.1.3 Nodule Formation

Bacteria in the target cells are enveloped in a plant derived plasma membrane, as they are released into the plant cytoplasm (192). The bacteria then grow in size, loose the capacity to divide, and are called bacteroids. The membrane covering the bacteroids is called the peribacteroid membrane (241).

2.1.4 Nodule Metabolism

A number of genes of plant and bacteria are expressed during nodule development and also in mature nodules (52,69). The bacteroids synthesise the key enzyme nitrogenase which is responsible for fixation of molecular nitrogen.

Bacteroids imbibe the carbon sources produced by the host plant. Sucrose and glucose are taken up very slowly by the bacteroids, while fructose, malate, succinate and fumarate are used up rapidly. Glutamate is taken up rapidly whereas several other aminoacids are absorbed slowly. Several anions, like nitrate (NO_3^-), chloride (Cl^-) and cations, like iron (Fe^{++}), and molybdenum (Mo^{+++}) are transported across the peribacteroid membrane to the bacteroids (50).

There is no evidence so far of a carrier mediated transport of ammonia from the bacteroids to the plant cells. Ammonia efflux during nitrogen fixation occurs by diffusion through a concentration gradient (50,99). Haeme is transported out of the bacteroids into the plant cytoplasm, where it combines with the plant produced globin to form leghaemoglobin. Leghaemoglobin facilitates diffusion of oxygen to the bacteroids for respiration without endangering the inactivation of oxygen labile nitrogenase complex.

2.2 GENETICS OF RHIZOBIUM

The fast growing rhizobia have large plasmids which range in size from 200-300 Kb in R. leguminosarum to about 1200-1500 Kb in R. meliloti. These megaplasmids are known to carry a majority of the genes required for the development of symbiosis

(12). Understanding the function and organization of rhizobial genes involved in symbiosis has largely been achieved through a study of symbiotic mutants generated by one or more of the following methods: i) Chemical mutagenesis ii) Transposon mutagenesis iii) Heat treatment.

2.2.1 Mutagenesis

2.2.1.1 Chemical Mutagenesis

With the use of several mutagens like ethylmethane sulfonate, nitrosoguanidine etc. abberant phenotypes were recovered, some of which are symbiotically defective (41,172,229).

2.2.1.2 Transposon Mutagenesis

Transposons are discrete segments of DNA which are capable of transposition from site to site by insertion into different regions with in a replicon or between different replicons. Most of them carry a drug resistance gene which can be used to mark their location in the bacterial DNA. Transposons cause mutations in the genes in which they get inserted.

Because of the insertion, transcription is interrupted, the target gene product is truncated and downstream genes are not expressed in the operon (125). Thus, the recent advent of transposon mutagenesis as a general genetic tool has made available a mutagenesis system which can provide a 'tag' for particular gene(s) of interest. The use of transposons as mutagens has helped largely in overcoming the problem faced for genetic analysis of symbiotic process (19,82,160,194).

2.2.1.2.1 Transposable Elements

Barbara McClintock discovered movable elements in maize in the 1940's and poceeded to demonstrate a remarkable array of properties associated with them—such as controlled chromosomal breakage, effect on gene expression, localized mutagenicity etc. Elements with similar properties were discovered in bacteria around 1968. These have been called insertion elements and transposons. Several reviews described various aspects of these kinds of transposable elements in bacteria (125). The transposons

having selectable traits have given new dimensions to molecular genetic analysis of many prokaryotes. A list of some such bacterial transposons is presented in the Table 1.

Transposons are discrete sequence of DNA that are incapable of self replication and can insert into DNA replicons, such as chromosomes or plasmids in the absence of rec A gene function (125). Some transposons carry drug resistance genes and thus insertions can be mapped as drug resistance marker.

The transposable elements can be divided into three distinct classes: Class I (IS-like modules and composite elements formed from them); Class II (Tn 3 and a family of related elements); Class III (transposing bacteriophage Mu).

The properties of transposons which are particularly important for their usefulness in bacterial genetics are as follows:

a) Translocatable drug resistance elements can be found inserted at a large number of sites on the bacterial chromosome.

b) Interrupted genes suffer complete loss of function.

c) The phenotype of the insertion mutation is completely linked to drug resistance in genetic crosses.

d) Insertion mutants can be recovered at high frequency after low level 'mutagenesis' by exposure to a translocatable element.

e) Insertion mutations revert by precise excision with concomitant loss of drug resistance. In no case the fate of the excised DNA is known, it is rarely, if ever reinserted elsewhere in the genome (2). Excision by these elements occur by pathway(s) which is independent of recA function and of transposon encoded function. Several observations support the idea. The precise excision occurs as a normal by product of the interaction of host function with direct or inverted repeat sequences (124).

f) Most transposons exert strong polar effect on expression of genes in a operon which lies distal to the site of inserted element.

g) Drug resistance insertions can generate deletions nearby.

h) Drug resistance elements can provide a portable region of homology.

Table 1 : Translocatable drug resistance elements.

Element	Approx.size (base pairs)	Terminal repitition (base pairs)	Polarity		Translocation		Deletion generation
			Yes/No	One/both orientations	Specificity	Frequency	
Tn1 (Amp^r)	4,800	140, inverted	Yes	One	Uncertain	Variable	Yes
Tn2 (Amp^r)	4,800	140, inverted	Yes	One	Low	Variable	-
Tn3 (Amp^r)	4,600	140, inverted	-	-	Low	-	–
Tn4 (Amp^r ,Str^r)	20,500	140, inverted	-	-	Low	-	-
Tn5 (Km^r)	5,700	1,460, inverted	Yes	Both	Low	Medium	Yes
Tn6 (Km^r)	4,100	direct	-	-	-	-	-
Tn7 (Str^r ,Tmp^r)	-	-	-	-	High	Low	-
Tn9 (Chl^r)	2,500	800, direct homologous to IS1	Yes	Both	Uncertain	Low	Yes
Tn10(Tet^r)	9,300	1,400, inverted homologous to IS3	Yes	Both	Medium	Low	Yes

Abbreviations :
Amp = Ampicillin
Chl = Chloramphenicol
Km = Kanamycin
Str = Streptomycin
Tmp = Trimethoprim
r = Resistant

i) Insertions behave as point mutations in fine structure mapping.

Translocatable drug resistance elements are useful in isolation of mutants (even where the mutant phenotype is not easily scored), in the construction of strains and other genetic manipulations (even when selection is difficult or impossible), in localized mutagenesis, in chromosomal mapping, in complementation tests, in construction of gene and operon fusions and in the selection and maintenance of chromosomal duplications.

Transposon Tn5 is a well studied representative of a class of movable genetic elements (17).It is 5,700 base pair long and it has a central unique region of about 2,700 bp which is flanked by two nearby identical inverted repeats designated IS50L and IS50R (116)(Fig. 1 & 2). The inverted repeats and a part of the central region of Tn5 have been sequenced (201).

The bacterial transposon Tn5 encodes two proteins, the transposase and a related protein, the transposition inhibitor, whose relative abundance determines, in part, the frequency of Tn5 transposition (191).

The transposon Tn5 encodes kanamycin resistance in a wide range of organisms from bacteria to mammals (16). The effectiveness of the kanamycin selection and the wide host range of the Km^r element, coupled with the relatively high transposition frequency of Tn5, have made Tn5 a widely used tool in genetic studies of monitoring gram negative bacteria. Tn5 has recently been shown to encode streptomycin resistance (Str^r) in Rhizobium species and Agrobacterium species (188). The Str^r determinant lies in the central region of Tn5, distal to Km^r gene and encodes a streptomycin phosphotransferase which is not expressed in E. coli (188). The differential expression of Str^r turned out to be very useful in mating experiments of Tn5 between E. coli and R. meliloti 41, since both streptomycin and kanamycin can be used for selection thus eliminating the relative high number of spontaneous kanamycin resistant strains (199).

The selectable resistance phenotype, high frequency of transposition, and degree of randomness in insertion specificity have made Tn5 the most widely used transposon in genetic manipulations in different bacteria (19,160,224). Recently, a collection

of Tn5 derived minitransposons has been constructed that simplifies substantially the generation of insertion mutants and the introduction of foreign DNA fragments into the chromosome of a variety of gram negative bacteria (54).

2.2.1.2.2 Random Tn5 Mutagenesis

Genetic Vehicles For Selection Of Insertion Mutations

In order to introduce drug resistance elements into the bacterial chromosome, a vehicle is needed which carries the desired element. It is also necessary to have some means of selection against the maintenance of the vehicle in the recipient bacteria. The only way the bacteria can become stably drug resistant, is for the drug resistance element to leave the vehicle and become inserted into the bacterial genome.

Initially derivatives of phage l or P22 were used as vehicles for insertion element (125). Unfortunately, this technique was limited by the narrow host range of these vectors.

Subsequently, the genetic analysis of Rhizobium symbiotic genes was greatly facilitated by the development of plasmid vectors, such as pJB4JI, for the introduction of transposons into the genome of these genes (19). This plasmid vector is a cointegrate of a conjugative P1 incompatibility group plasmid and bacteriophage Mu, with the Mu genome conferring the suicidal property to the plasmid by mechanisms that are not well understood (38). The plasmid pJB4JI has been used successfully in the isolation of transposon Tn5 induced symbiotic mutants of R. meliloti (12,31,82,160), R. trifolii (220) and R. phaseoli (174). It was found that sometimes Mu sequences from plasmid pJB4JI also get transferred along with Tn5 and those Mu sequences created problems during the genetic analysis of Tn5 mutants (82,160). However, pJB4JI was also found to induce secondary genetic changes . Hence, search for new suicide vector was made.

A suicidal plasmid vector was constructed which is composed of the p15A replicon which functions in E. coli but not in Rhizobium species and a region encoding the N type of bacterial mating system which is very efficient in mating between E. coli and Rhizobium species (221). This vector has proved useful for other gram negative

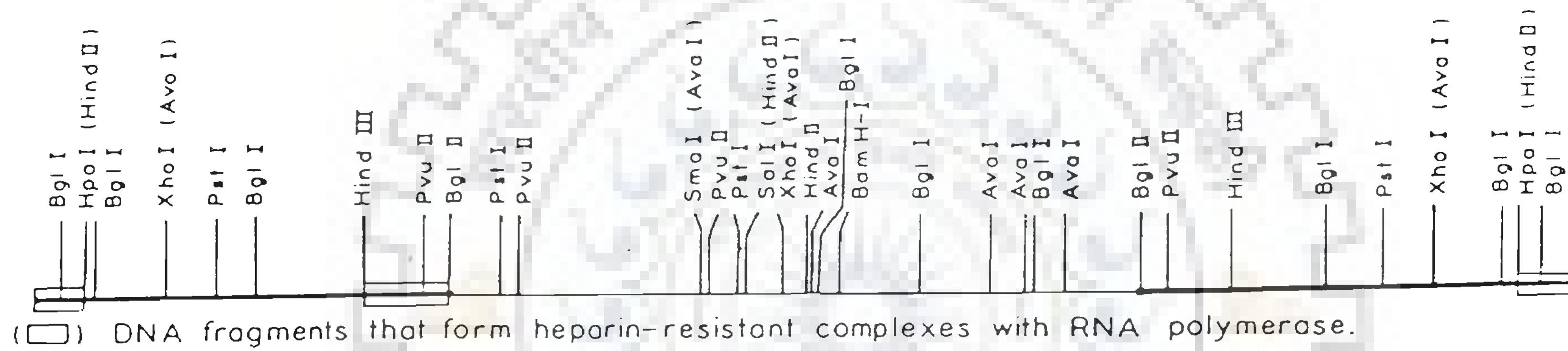
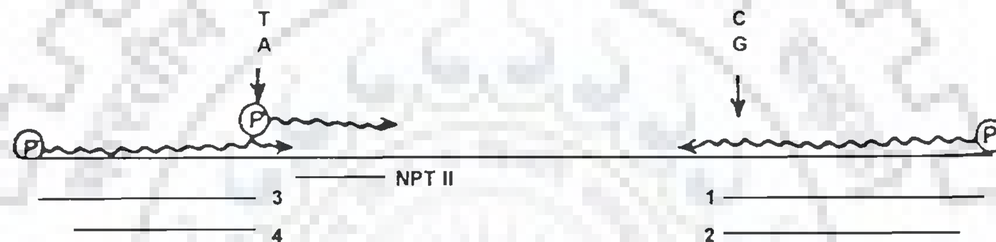


Fig. 1. Restriction map of Tn5



Inverted repeat, NPT II promoters and single base pair nonhomology are shown. One or both of the proteins (1 and 2) are required for transposition.

Fig. 2.The genetic organization of Tn 5

bacteria. Using this vector, several auxotrophic and symbiotic mutants have been isolated, in case of R. japonicum (199), R. fredii (122), and Cowpea rhizobia (158).

Simon et al. (227) reported the construction of suicide plasmid vectors for Tn5 mutagenesis. The ori T(mob) region of plasmid RP4 and transposon Tn5 were inserted into E. coli plasmid pBR322 or pACYC184 which can not replicate in Rhizobium. These suicide vector pSUP1011 were introduced into special E. coli strain in which tra genes of RP4 plasmid were integrated into chromosome. This special E. coli strain was used as donor to transfer the suicide vector to Rhizobium or any gram negative bacteria. The products of the chromosomally integrated tra genes helped in the mobilization of the suicide vector to Rhizobium by recognising oriT (mob) region on the vector. After entering Rhizobium this vector can not survive but Tn5 can survive by jumping to the Rhizobium genome. This vector has been used for Tn5 mutagenesis in Bradyrhizobium japonicum (233), R. fredii (122) and R. japonicum (5,13,105). A variant of Tn5, Tn PhoA can also be used to mutate a specific class of genes encoding secreted or membrane spanning proteins (147).

2.2.1.2.3 Site Directed Tn5 Mutagenesis

Rhizobium genes cloned in E. coli are amenable to site directed Tn 5 mutagenesis and their function is subsequently analysed in Rhizobium following homogenotization (88, 117,204). This approach gained prominence with the construction of the shuttle vector pRK290 by Ditta et al. (61). Now several derivatives of pRK290 like pLAFR1 with a lambda cos site and pRK404 with multiple linker site and small size are available (60,83).

2.2.1.3 Heat Treatment

Zurkowski and Lorkiewicg (262) gave a method for the isolation of non-nodulating mutants of R. trifolii. Non nodulating mutants of R. trifolii were found in cultures incubated at 35°C for 7 days. Zurkowski and Lorkiewicg (263) observed that in the above method, Nod$^-$ mutants were obtained by the loss of one plasmid. This heat treatment method with some modifications has proved to be very useful in obtaining

Nod⁻ mutants in other species of Rhizobium (12). In Rhizobium species that is able to nodulate legumes and nonlegume Parasponia species, the loss of whole symbiotic (sym) plasmid was observed by heat treatment, while in R. meliloti carrying regions of the sym plasmid carrying symbiotic genes were deleted (12). Later Nod⁻ or Fix⁻ mutants of R. japonicum were isolated by plasmid curing heat treatment(5).

2.2.2 Symbiotic Genes

In all fast growing Rhizobia, large plasmids upto 1500 Kb size can be seen (40,175). There are a number of genes involved in different steps of nitrogen fixation process from both partners of symbiotic association and the genes are activated only at a given stage of symbiotic development (256). It has also been established for the various fast growing rhizobia that some symbiotic genes are present on chromosome (82) while others, including the host range specificity genes are located on megaplasmids (101,114,185,263). In Rhizobium meliloti too nodulation as well as nitrogen fixation genes are plasmid born (12,197) and different plasmids are required for optimal symbiotic performance (28).

Genes involved in symbiosis have been broadly classified on the basis of their function, as genes for nodulation (nod genes), genes for nitrogen fixation (nif and fix general) and genes for cell surface components (exo genes, ndv genes etc.).

2.2.2.1 Nodulation Genes

Rhizobium induces the formation of nitrogen fixing nodules on the roots of leguminous plants(257). In R. meliloti a very large indigenous plasmid (megaplasmid) carries the genes conferring the ability to nodulate the host plant alfalfa (Medicago sativa). Nodulation (nod) genes are located on this plasmid in the vicinity of nitrogen fixation (nif) genes (12,31,197). Of a large number of genes involved in symbiosis, initiation of nodulation requires only a few genes. E. coli and Agrobacterium tumefaciens having short streches of Rhizobium genome are known to induce inffective nodules on alfalfa (100,146).

In R. meliloti the nodulation genes have been further identified on the basis of

their specificity, as common nod genes and host specific nodulation genes (hsn genes). They are clustered on an approximately 16 Kb long segment of a megaplasmid (134,242).

2.2.2.1.1 Common nod Genes

The common nod ABC genes (134) causing root hair curling and nodule induction are highly conserved among the rhizobia both structurally and functionally (65,142,198,249) and are involved in the synthesis of lipopolysaccharide signal molecule. nod B gene protein i.e. chitooligosaccharide deacetylase, involved in nodule signal synthesis has been identified (113). These genes are clustered on a 3 Kb segment which is about 30 Kb away from nif HDK cluster encoding nitrogenease polypeptides (131,146). Recently, two more common nod genes, i.e. nod M and nod N, have been identified. nod M encodes functions for efficiency of nod signal production and bacteroid maturation (9). nod D regulates the expression of nodulation genes in rhizobia(212). Expression of nod genes is activated by three allelic nod D genes in the presence of root exudates with flavonoid (109,183). The nod D gene thus defines the host range of Rhizobium (95).

2.2.2.1.2 Host Specific nod Genes

The second cluster of nodulation genes, viz., host specific nodulation (hsn genes) genes, is present about 6 Kb from the common nod region (64, 108, 146) towards the nif HDK cluster. Transfer of these genes between biovars may alter the range of host plant nodulation (238,235). This includes nod H and nod FEG and nod QP genes. R. meliloti nod H gene determines host range by producing a specific extracellular signal (74,193). Two copies of nod QP genes are involved in sulfate activation (215). Mutations in these genes lead to altered infection and nodulation of the homologous host, alfalfa. The mutations also elicit root hair curling of heterologous hosts like clover and vetch (108).

2.2.2.2 Nitrogen Fixation Genes

These are directly concerned with the enzymology of nitrogen fixation. These have been classified as nif and fix genes based on their structural and functional homology with the genes of Klebsiella pneumoniae. The nif genes have homology with the genes of Klebsiella while this is not true for fix genes (14). Both nif and fix gene mutants can nodulate but these nodules lack the capacity to fix nitrogen (Nod^+ Fix^-) (47).

2.2.2.2.1 nif Genes

nif HDK genes code for nitrogenase polypeptides. They exist in a single operon on a 6.3 Kb fragment of R. meliloti megaplasmid. They have also shown to be located together on megaplasmids in other species of fast growing rhizobia (31,197). The nif H, nif D, nif K genes form a single operon and are transcribed in the order of nif HDK (46,203,205,261). nif H encodes a 31 to 39 Kd protein which forms the component II of nitrogenase system. nif D encodes a 56-60 Kd polypeptides, the alpha subunit of component I of nitrogenase (47,205). nif K gene codes for a protein of 60 Kd polypeptide which forms the beta subunit of component I (63).

nif A is a regulatory gene for all known nif and fix genes (211). In all the fast growing rhizobia the nif A is located upstream of the nif HDK operon. In R. meliloti, it is located on a 1.8 Kb region, about 5.5 Kb upstream of the nif HDK cluster. It encodes a 55 Kd polypeptide which is a positive transcriptional activator for nitrogen fixation genes (30). R. meliloti nif A gene can be induced asymbiotically to levels exceeding those in the nodule bacteroids of microaerobic conditions (62).

It has been found in R. meliloti and other fast growing rhizobia that mutations in nif HDK operon lead to induction of nodules that are smaller in size and white in colour, lacking nitrogenase activity, nif HDK mutants have been sometimes called Nod^+ Fix^- (47).

2.2.2.2.2 fix Genes

The fix genes are those that are required for the development of ability in

bacteroids to fix nitrogen but which lack homology for nif genes of Klebsiella pneumoniae (46,186,205). The fast growing rhizobia have a cluster of fix ABCX genes located between nif HDK and nif A operons (205). The fix ABCX genes are conserved among Rhizobium and Bradyrhizobium. R. meliloti fix ABCX genes have a strong homology with R. leguminosarum and R. trifolii genes and a weak homology with Bradyrhizobium genes. The sequence of promoter region of these genes is almost identical in strains of R. meliloti (20). In R. meliloti this fix cluster is located 1.4 Kb downstream of nif HDK operon (186,205). This fix cluster comprises of four fix genes called fix A, fix B, fix C and fix X. These genes form a single operon (20,71) and are transcriptionally activated coordinately with all other known nif and fix genes via activation by nif A.

fix A: It encodes a polypeptide of 203 amino acids having 31.1 Kd molecular weight.

fix B: This gene codes for a polypeptide of 37.7 Kd molecular weight and consisting of 354 amino acids.

fix C: It codes for a 47.2 Kd protein having 436 amino acids.

fix X: This gene is located at a site distinct from the cluster of nif and fix genes formed by nif HDK, fix ABCX, nif A and nif B genes.

The fix F locus is at a distance of about 25 kb from the main nif-fix cluster in R. meliloti. This fix locus codes for 36 Kd protein which has a role in the functionality of nitrogenase. However, transcription and translation of nif HDK operon was independent of a functional fix F gene product, since both nif HDK mRNA and nitrogenase were found in effective nodules formed by fix F mutants.

2.2.2.3 Genes For Cell Surface Components

Presence of proper recognition molecules on the rhizobial surface is a must for the bacteria to complete infection. These surface components comprise of acidic exopolysaccharides, neutral β glucans, lipopolysaccharides (66) and to an extent the elements responsible for motility of the cells (3).

2.2.2.3.1 Exopolysaccharides

Acid calcaflour binding exopolysaccharides are needed for infection to occur (77,144). The exo genes determine exopolysaccharide synthesis. Wild type Rhizobium strains secrete an extracellular, water soluble, heteropolysaccharide known as exopolysaccharide or EPS. There is a direct corelation between EPS synthesis in bacteria and their ability to carry out symbiotic nitrogen fixation(148). It is proposed that EPS is involved in bacterial adhesion to host, leading to bacterial invasion (92,187). The Exo$^-$ mutants lack a wild type of exopolysaccharide and presumably as a consequence of deficiency have an altered response in nodulation. They also show altered behaviour towards phages and monoclonal antibodies (77,144). These Exo$^-$ mutants form Fix$^-$ nodules. In the Fix$^-$ nodules formed by them, no infection threads were observed. If any infection thread is formed they are in superficial intracellular spaces rather than within nodule cells (77).

Genes controlling the biosynthesis of exopolysaccharides have been mapped on a megaplasmid pRmeb which is different from the plasmid pRmea that carries nod, hsn and fix genes (10,79,230). Hybridization studies in R. meliloti revealed the exact location of all the six exo genes(79).

Locus	Location
exo A	pRmeb
exo B	pRmeb
exo C	chromosome
exo D	chromosome
exo E	pRmeb
exo F	pRmeb

Genetic mapping of exo genes on megaplasmid revealed the presence of exo A, exo B and exo F genes but position of exo E is not clearly known. The exo D gene encodes a novel function needed for alfalfa nodule invasion (190).

Finan et al. (79) have mentioned that pRmeb includes four loci involved in

exopolysaccharide synthesis as well as two loci involved in thiamine synthesis. Long et al. (147) have defined a thi and several exo genes located in a 50 Kb region of about 1500 Kb plasmid called pRmeb.

Long et al. (147) found these genes to be clustered on a 22 Kb region and comprise of twelve complementation groups. In addition, two genes were found which negatively regulate the synthesis of R. meliloti acidic, calcaflour binding exopolysaccharide (67). Recently few more exo genes (i.e. exo MONP) involved in exopolysaccharide biosynthesis have been identified (15). In addition to this calcaflour binding exopolysaccharide (EPS-I), R. meliloti also has a cryptic capacity to produce a second, non calcaflour binding exopolysaccharide (EPS-II) which is induced as a consequence of a chromosomal mutation (88). Later a gene pSi (polysaccharide inhibition) was identified on sym plasmid close to genes involved in nodulation and nitrogen fixation (23). This gene encodes a protein which might affect the processing, export or synthesis of EPS(143).

2.2.2.3.2 Beta-Glucans

A set of genes controlling beta (1-2) cyclic glucans production is required for proper infection to occur. Two of them ndvA and ndvB are located on the chromosome and have homology with agrobacterial chromosomal virulence loci (ChvA and ChvB) (70).

The ndvB locus of Rhizobium meliloti has been sequenced and found to be involved in the production of beta(1-2) glucan(110). Absence of beta (1-2) glucans has been correlated with the lack of flagellum motility of bacteria. Lack of motility can reduce symbiotic efficiency.

A major type of curdlans, beta(1-3) glucan can be specifically stained with aniline blue. These molecules, however are normally reported to be absent from rhizobial surfaces but may be present in related genus Agrobacterium (102).

Cellulose fibrils also have been shown to be present on rhizobial surfaces, by virtue of their capacity to bind the dye congo red (117,129).

2.2.2.3.3 Lipopolysaccharides (LPS)

LPS are the other variety of polysaccharides of the cell surface involved in the interaction of bacteria with the host plant (37, 140). Three genetic regions of R. leguminosarum necessary for LPS production have been identified and cloned. Recently, lps Z, a lipopolysaccharide gene involved in symbiosis of R. meliloti has been identified (29).

2.3 REGULATION OF SYMBIOSIS

2.3.1 Regulation of Nodulation Genes

Rhizobia, elicit on their leguminous hosts, in a specific manner, the formation of nodules in which they fix nitrogen. Rhizobial nod genes, which determine host specificity, infection and nodulation are involved in the exchange of low molecular weight signal molecules between the plant and the bacteria as follows: transcription of the nod operons is under the control of nodD regulatory proteins, which are specifically activated by plant flavonoid signals (109). The common and species specific structural nod genes are involved in turn in the synthesis of specific lipo-polysaccharides that signal back to the plant to elicit root hair deformations, cortical cell divisions and nodule meristem formation (55).

Though most genes are not expressed in cultured cells, nod D expresses constitutively. Other nod genes are induced only when the cells are exposed to plant exudates like flavonoids (107). The specificity of rhizobial response to the plant inducers has been corelated with the nod D alleles carried by Rhizobium (91,95,151). The nod D, acts as a positive regulator of nodulation gene expression (107). R. meliloti has three functional nod D genes that modulate the nodulation process in a host specific manner(49). The gene product of nod D3 has a role in mediating the ammonia regulation of early nod genes.

2.3.2 Regulation of Nitrogen Fixation Genes

Information about nif gene regulation in rhizobia is still incomplete. In free

living Klebsiella pneumoniae, regulation on nif gene transcription is through nif A gene product and ntr A encoded sigma factor. ntrC is known to activate nifA in the presence of ntr A (94). In Rhizobium too, activation of nif genes is through nifA but it is not activated by ntrC (244). nif A expression is also dependent upon low oxygen concentration (62) whose effect is mediated by the distantly linked fix LK and fix J genes (48). fix L can sense oxygen tension (57). This system is also responsible for the positive induction of fixK. Recently, R. meliloti nifA promoter has been analysed (1). In K. pneumoniae, nifL is also a regulatory gene which mediates the physiological effect of oxygen. No nif L homology has been found either in Rhizobium or Bradyrhizobium. It has been suggested that nif A in rhizobia may incorporate the functions of nif L as well (81).

2.4 USE OF MUTATIONS IN STUDY OF RHIZOBIUM

The application of mutant methodology to symbiotic nitrogen fixation by Rhizobium has got a sufficient utility in understanding the complex series of interactions involved in the symbiosis. A major difficulty in analysis is that the mutants of the genes of Rhizobium involved in symbiosis can not be selected directly in vitro. Some classes of mutants (such as those resistant to bacteriophages, resistant to antibiotics and antimetabolites, auxotrophs, polysaccharide deficient mutants and pleiotropic carbohydrate negative mutants) do affect directly or indirectly the symbiotic process (219). Most of the significant advances resulting from the study of Rhizobium mutants with altered symbiotic ability have been reviewed in this section.

2.4.1 Naturally Occurring Symbiotically Defective Strains

A number of naturally occurring Nod⁻ Rhizobium strains have been reported, but there has always been doubt about whether they are mutants of the relevant Rhizobium species since the only absolute taxonomic criterion for classification of a bacterium as Rhizobium is its ability to induce nodules on the roots of legumes (127).

Fix⁻ rhizobia can be isolated from root nodules arising on field grown legumes, but they have several disadvantages for genetic studies of symbiotically important genes.

One is the absence of the wild type parents for comparison.

2.4.2 Direct Isolation of Symbiotic Mutants

First attempt to induce mutations in Rhizobium by transforming DNA and other agents was made by Kleczkowska (126). Mutagenesis in R. japonicum strain using nitrosoguanidine (NTG) was carried out by Maier and Brill (1976). They found five mutant strains after screening about 2500 surviving clones. Two of the mutant strains failed to form nodules after two weeks. The other three mutants nodulated soybean but did not symbiotically fix nitrogen as revealed by acetylene reduction assays on the nodules induced.

Zurkowski and Lorkiewicz (262) described a method for isolation of non nodulating mutants of R. trifolii by incubation at elevated temperatures. Rolfe and Coworkers (194) demonstrated rapid screening of symbiotic mutants of R. trifolii using inoculated seedlings, incubated vertically on sealed petriplates for three weeks. Several mutants unable to nodulate or unable to fix nitrogen have been isolated: R. japonicum (5,105,172,236), R. meliloti (82,160), Bradyrhizobium japonicum (223), Cowpea rhizobia (158) and R. phaseoli (174).

2.4.3 Mutants Resistant to Antibiotics

Mutation to drug resistance occur due to changes in the outer layers of microbial cells. Schwinghamer (216) first noted a correlation between resistance to antibiotics known to inhibit cells wall synthesis and symbiotic defectiveness. Only detailed biochemical studies of cell walls from resistant mutants are those of Yu and Jordan (260). They studied viomycin resistant mutants of R. meliloti which were shown to accumulate phospholipids in their cell walls. These mutants were Fix^- and did not form bacteroids (97). Twentynine of thirty three mutant clones selected for viomycin were uniformly ineffective. Fifteen of sixteen clones selected for neomycin resistance were also ineffective.

Pankhurst (180) isolated mutants resistant to sixteen individual antibiotics in Lotus rhizobia and tested for symbiotic effectiveness. Resistance to nalidixic acid and

rifampicin (inhibitors of nucleic acid synthesis) and to D-cycloserine, novobiocin and penicillin (inhibitors of cell wall-cell membrane synthesis) was associated with the significant loss of effectiveness in 20-100% of the mutant while resistance to inhibitors of protein synthesis had no effect on symbiotic effectiveness.

In R. leguminosarum, a number of antibiotic resistant mutants were examined. Only a minority of rifampicin resistant mutants had ineffective symbiotic phenotype (178). In Cowpea Rhizobium mutants resistant to streptomycin and erythromycin were isolated (58) by NTG mutagenesis. The resistant mutants differed from parent in growth pattern, phage sensitivity, infectivity or efficiency. Some antibiotic resistant mutants of R. leguminosarum have also been isolated (229).Recently, Ayhan (8)has demonstrated the importance and use of antibiotic resistant mutants of Rhizobium in ecology.

2.4.4 Pleiotropic and Carbohydrate Negative Mutants

Kuykendall and Elkan (138) isolated R. japonicum derivatives on the basis of morphologically different colonies on YEM-HM salts medium. A clear difference in abilities to utilize D-mannitol, D-arbitol, D-glucose and D-fructose was observed alongwith variation in nitrogen fixing ability. Ronson and Primrose (196) isolated independent carbohydrate mutants of R. trifolii which were characterized as deficient in glucokinase (glk), fructose uptake (fup) and pyruvate carboxylase (pyc). All these mutants formed an effective symbiosis on red clover. C_4–dicarboxylate transport mutants of R. trifolii were found to form ineffective nodules on Trifolium repens (195) while succinate resistant mutants of R. leguminosarum failed to show nitrogenase activity within the pea root nodules. Gardiol and Coworkers (85) isolated a succinate dehydrogenase mutant of R. meliloti which failed to grow on succinate, glutamate, acetate, pyruvate or arabinose but grew on glucose, sucrose and fructose. The mutant showed delayed nodulation on lucerne and nodules were white and ineffective and revertants showed normal nodulation. Pleiotropic carbohydrate negative mutant strains of R. meliloti were isolated after NTG mutagenesis (42). All these mutants were

symbiotically effective. Carbohydrate utilization mutants of R. leguminosarum which were Nod+ Fix-, isolated by Arwas et al. (7). Symbiotically effective, fructose negative mutant of R. meliloti was isolated and studied (93). Carbohydrate minus mutants were isolated and isocitrate dehydrogenase gene was cloned by complementing R. meliloti library by McDermott and Kahn (157).

2.4.5 Phage resistant Mutants

Surface macromolecules and appendages serve as specific receptors for different phages. Mutant resistant to different phages are usually affected in distinct cell surface macromolecules and often have pleiotropic phenotypes (136). A phage resistant mutant of R. meliloti Rmd 201 was not able to utilize galactose as the sole source of carbon and was Fix- (121).

2.4.6 Polysaccharide Deficient Mutants

A mutant of R. leguminosarum was isolated by selecting for physically small cells. This mutant produced diminished amounts of extracellular polysaccharides (EPS) and failed to nodulate pea (207). It was further found that this mutant was unable to produce capsules (167).

Russa and Coworkers (202) studied LPS and EPS of nodulating and nonnodulating strains of R. trifolii. Some quantitative differences were found between the LPS in respect of the composition of neutral sugars. Involvement of gross EPS & LPS in nodulation have been ruled out (45,123), Although minor components of EPS may be involved. Mutants of R. leguminosarum biovar phaseoli defective in the synthesis of LPS induced aberrant nodules on beans which failed to fix nitrogen (253). R. trifolii defective in the biosynthesis of LPS produces abortive nodules on clover roots (90). By studying changes during bacteroid development, it has been seen that some lipopolysaccharides epitopes may disappear (84) where as new ones appear (228,252). In R. meliloti mutant strains that are affected in EPS synthesis have been shown to induce pseudonodules that are devoid of bacteria (77,144,165).

In summary, LPS is indeed involved in the symbiotic interaction with alfalfa

by (i) providing a structure able to recognize the plant root during bacterial adsorption (141),(ii) modulating strain competitiveness during infection (140),(iii) supporting a compatible association with the host plant independently or in conjuction with exopolysaccharide (189).

2.4.7 Some Specific Mutants Related to Symbiosis

Ames and Coworkers (4) isolated nonflagellated, nonmotile and nonchemotactic mutants in R. meliloti and nodulation ability was compared. A competitive advantage provided by bacterial motility and chemotaxis in formation of nodules has been established (3,33).

Mutants of R. melilioti altered in ammonium utilization which require trace amounts of glutamate to use ammonium as nitrogen source were found to be deficient in nitrogen fixation (176). Toro and Olivares (248) observed that small colonies appearing on heat treatment elicited pseudonodules on alfalfa roots.

2.4.8 Auxotrophic Mutants

Auxotrophy can be used in genetical research both as a marker and as a tool for probing the nature of induced defects in symbiosis. Particularly, studies on possible roles of specific bacteroid metabolites at various stages of symbiotic interaction are helped by the auxotrophic mutants and their prototrophic revertants. Some of the antimetabolites resistant mutants isolated by Schwinghamer (218) showed partial or multiple nutritional requirements. A D-histidine resistant mutant of R. trifolii was auxotrophic for riboflavin (Rib^-) and ineffective. The prototrophic revertants recovered were found to be effective.

The common auxotrophic mutants obtained in R. leguminosarum and R. trifolii (217) were deficient for adenine and thiamine. They were ineffective. Partial or full restoration of effectiveness in symbiosis often accompanied reversion to prototrophy in ineffective auxotrophs.

An association between nutritional requirement and effectiveness in N_2 fixation was demonstrated in case of R. meliloti mutants auxotrophic for adenine, cytosine, methionine, cysteine and glycine (210). All amino acid mutants were effective, glycine

dependent mutants being more effective than wild type. However, in another study, R. meliloti mutants requiring histidine, arginine and uracil and cysteine showed loss of effectiveness (153,154) and a mutant requiring isoleucine and valine was noninfective.

Fedorov and Zaretzkaya (75) isolated ethylmethane sulfonate induced mutants in R. meliloti. Those requiring methionine, cysteine, histidine and adenine with thiamine were not effective. Others requiring arginine, tryptophan and thiamine were capable of nitrogen fixation.

However, a large proportion of the other classes of auxotrophs, such as methionine, tryptophan and leucine auxotrophs, were described by Pain (178) as giving rise to effective nodules containing prototrophs. These nodules were clearly formed by prototrophic revertants. Thus many amino acid requiring mutants may be symbiotically defective as a secondary consequence of the auxotrophic marker, and prototrophic revertants have restored symbiotic competence.

When adenine was supplied in the plant growth medium, one of the thirty one avirulent adenine auxotrophs studied by Pain (178) formed nodules, albeit ineffective ones, on peas. Pankhurst and Schwinghamer (181) had previously studied a similar nonnodulating auxotroph of R. leguminosarum, which formed ineffective nodules on peas when adenine was supplied in the plant growth medium. Schwinghamer (218) studied an ineffective riboflavin requiring mutant of R. trifolii that was rendered symbiotically competent by the addition of riboflavin to the plant growth substrate. The development of nitrogen fixing bacteroids was shown to require either riboflavin or flavin mononucleotides (182).

From these studies, it is now clear that adenine is required for the establishment of nitrogen fixing symbiosis but, unlike riboflavin, exogenously supplied adenine probably does not enter the plant tissue in concentrations sufficient to restore the symbiotic fitness of adenine auxotrophs. Adenine auxotrophs of R. leguminosarum were found unable to nodulate pea plants (178,181,217). But adenine auxotrophs of R. meliloti were found to form nodules, but they are ineffective (75,210). This difference

may be species specific.

Leucine auxotrophs (56,153,154) and histidine auxotrophs (250) of R. meliloti were found to be ineffective. In both cases, supplementation of the plant growth medium with the required amino acid permitted release of the bacteria from the infection thread and normal proliferation, resulting in the development of a freely competent nitrogen fixing symbiosis (153,154,250).

Truchet et al. (251) used a leucine requiring mutant of R. meliloti with a leucine dependent expression of effectiveness, to study the details of the sequence of events in nodule formation. Reasoning that the restoration of symbiotic competence by exogenous leucine might be due to a nonspecific improvement in the nitrogen nutrition of the host, they tested the effect of adding urea to the plant growth medium. Intrestingly, the added urea brought about the development of nodules with the same external shape as the wild type nodules. Histological examination revealed a close similarity to those formed by the wild type strain. Under these conditions, Rhizobium remained in the infection threads. The differentiation of plant tissue to form nodules, or nodule organogenesis, thus appeared to be initiated by Rhizobium bacteria by a specific diffusible trigger.

Kohli and Vashishat (130) described a procedure for enrichment of auxotrophs in slow growing mungbean Rhizobium using a combination of ampicillin with lysozyme. They observed that mutants requiring adenine, arginine or histidine did not form nodules.

Four histidine auxotrophs were isolated by Sadowsky et al. (206) using random Tn5 mutagenesis. Two of the histidine mutants were found to be symbiotically defective and did not form nodules. When histidine was supplied to the plant growth medium, both nodulation deficient mutants formed effective nodules.

One Nod$^-$ auxotroph requiring adenine and thiamine was isolated by Mclaughlin et al. (158). This non-nodulating mutant induced root hair curling but did not show any nodule initiation or nodule development. A set of noninfective purine auxotrophs were

isolated by Noel et al. (173) and Newman et al. (170). Addition of AICAR (5-aminoimidazole 4-carboxamide riboside) to plant growth substrate, were capable of rescuing the infection by these mutants. Later Swamynathan and Singh (242) reported seven purine auxotrophs of *R. meliloti* which produced small, irregular nodules lacking any detectable nitrogenase activity on *Medicago sativa*. External supplementation of the plant growth substrate with purines or their biosynthetic intermediates failed to restore symbiosis. Kim et al. (122) isolated fourteen Tn5 insertion auxotrophs of *R. fredii*. All were able to nodulate but their symbiotic effectiveness was different.

Kerppola (119) isolated auxotrophic mutants requiring ornithine, pyrimidine, purine, asparagine, leucine, methionine and tyrosine. All the auxotrophs were found to form nodules but these nodules were unable to fix nitrogen. In all cases, supplementation of the nutritional defect restored symbiotic nitrogen fixation. Sathyanarayan (208), found a leucine auxotroph to be Fix^-, azide sensitive and unable to utilize glucose as the sole source of carbon. In the same study, a thiamine auxotrophs was found to be Fix^-, Exo^- and unable to utilize glucose. Te-Chein et al. (246) showed that symbiosis was not effected by proline auxotrophy.

These studies show that auxotrophy of *Rhizobium* that are symbiotically defective (as a consequence of their nutritional requirement), can be useful tools for studying the complex series of events in the formation of symbiotic nitrogen fixing nodules.

2.5 GENETIC ANALYSIS: APPROACHES USING COMPLEMENTATION STUDIES

The classical and recombinant DNA approaches of genetics have greatly facilitated the analysis and manipulation of bacterial genomes. These techniques have also been applied to rhizobia in understanding the complex symbiotic interaction process. The combination of transposon induced mutagenesis and molecular cloning (in vivo and in vitro) has permitted the isolation of genes involved in some of the many steps leading to symbiotic nitrogen fixation. In order to perform genetic complementation studies, two

techniques are valuable. First being the in vivo cloning of Rhizobium DNA (wild type or mutated segment) on a wide host range Rplasmid to construct Rprimes and the second is to generate the clone bank of Rhizobium DNA on a wide host range Rplasmid. These Rprimes as well as the clone banks can be used for complementation with the mutants to define different cistrons.

2.5.1 Plasmids Mediated Mobilization of Bacterial Chromosome

There are a number of bacterial genera for which systems of conjugative transfer of chromosome have been demonstrated and mapping of genes accomplished. The phenotypic ability of plasmids to transfer host chromosome has been called chromosome mobilization ability (cma).The best, and proabably the only, example of plasmid mediated mobilization (cma) for which mechanism of chromosome transfer has been established in some detail is that of 'F' in E. coli. It has been been established that 'F' can integrate stably into the E. coli chromosome at a variety of sites to create HFr strains each having a specific transfer origin and direction.

The Inc P-1 plasmids including RP4, R68, R68.45, pJB3JI and others have attracted particular attention in view of their wide host range which includes a number of organism of considerable scientific, economic and medical significance. One of the key questions relating to the role of the plasmids in chromosome mobilization is that the plasmid integration in the chromosome leading to the transfer of latter. Stable integration of plasmids into the bacterial chromosome is a rare phenomenon. On the basis of the fact that, in E. coli, F′ plasmids can affect mobilization of the chromosomes (149), an attempt to insert a piece of bacterial DNA into an Inc P-1 plasmid was made (111). Segments of R. leguminosarum chromosome had been inserted into RP4. It was found that, while such recombinant plasmids retained the transfer characteristics and wide host range of the parental plasmid, they were no more efficient in mobilizing chromosome than the parent plasmid RP4. A search was made for variants of various IncP-1 plasmids that would be effective in promoting chromosome transfer. The Rplasmids such as RP4, R68 and R68.45 differ from R68 by the duplication of the IS

element. IS 21 have a broad conjugal host range among fast growing rhizobia. RP4 and R68.45 have been used to determine linkage maps in Rhizobium (19,133,161). Among slow growing rhizobia, transfer of Rplasmids in some of the strains of R. japonicum (137,184) and Cowpea rhizobia have been shown (118). Later, the gene mobilizing Rplasmid pJB3JI (27), a kanamycin sensitive derivative of R68.45, was used to mobilize a Tn5 tagged 90 Kb region of the megaplasmid DNA carrying symbiotic genes of fast growing R. meliloti (11) and three Rprimes carrying symbiosis controlling DNA of the fast growing Rhizobium strain NG4234 have been characterized (168). Plasmid pJB3JI was also reported to be useful for Rprime formation of Bradyrhizobium japonicum strain and of R. trifolii (225).

2.5.2 Clone Banks of Rhizobium

Plasmids and cosmids have been used as vectors for cloning symbiotic genes of Rhizobium.

2.5.2.1 Plasmid Vectors

Amplifiable high copy number and a narrow host range plasmid vector pBR322 (21) has been used by some workers (220). The advantage of this vector is that the gene can be obtained in large amount because this vector can be amplified by adding chloramphenicol to the E. coli cells. One disadvantage of this vector is that only small fragments can be cloned. The other disadvantage is that this vector is not useful for complementation studies in Rhizobium, neither it can be transferred to Rhizobium by conjugation nor transformation. The cloned gene has to be recloned on some conjugative vector.

Later, Ditta and Coworkers (61) developed a broad host range cloning vehicle that can be mobilized at high frequency into gram negative bacteria. This cloning system consists of two plasmids derived from the P group plasmid pRK2, a 56Kb low copy number plasmid which confers resistance to tetracycline, neomycin, kanamycin and ampicillin and which is self transmissible and replicates within most gram negative bacteria. The first derivative of pRK2 is the 20 Kb plasmid pRK290, which confers

tetracycline resistance, is mobilizable but not self transmissible (Mob$^+$, Tra$^-$, Tet^r). The second derivative of pRK2, pRK2013 (76) contains the RK2 tra function and neomycin resistance genes ligated to a ColE1 replicon and can mobilize pRK290 in trans to a variety of gram negative recipients. This binary vehicle system is diagramed in Fig.5 . The vector pRK290 contains single recognition site for EcoRI and BglII. R. meliloti genomic DNA fragments were spliced in EcoRI site of pRK290 and the gene bank was used to transform E. coli. Triparental matings (204) were carried out to mobilize in trans the recombinant plasmid from E. coli to R. meliloti or other gramnegative recipietns using pRK2013 (Tra$^+$, Km^r) as the helper which can not replicate in Rhizobium. Several other broad host range vectors similar to pRK290 are: pRK291 with a BamHI and pRK292 with a HindIII cloning site instead of BglII, pRK293 with an extra Km^r gene, pRK252 which is smaller than pRK290 and pRK404 which is a derivative of pRK252 (60).

2.5.2.2 Costramids broad Host Range Cosmid Vectors

Further modification of broad host range vectors pRK290 has been done for efficient cloning of large size inserts by introducing into it a lambda 'cos' site to give a Mob$^+$, Tra$^-$ cosmid vector pLAFR1,is 21.6 Kb in size, has the unique EcoR1 site, selectable by Tet^r and is packageable in vitro into lambda heads for efficient transfer into E. coli by infection. Cosmid cloning vectors are specially useful for efficient cloning of large fragments of DNA of megaplasmids of Rhizobium difficult to purify and obtain in large quantities.

The clone banks have been used for carrying out genetic analysis using complementation studies to fish out and identify the particular gene of interest in R. japonicum (35), R. leguminosarum (247), and R. meliloti (6,119,244).

Long et al. (146) developed a clone bank of R. meliloti in E. coli using pLAFR1 and conjugated en masse in R. meliloti Nod$^-$ mutants and selected for Nod$^+$ transconjuants and identified the inserts containing genes for nodulation.

2.6 MAPPING STUDIES IN DIFFERENT SPECIES OF RHIZOBIUM

Palomares et al. (179) used plasmid R68.45 to promote conjugal transfer of chromosomal markers in R. trifolii RS55. They analyzed two factor and three factor crosess among R. trifolii strains and constructed circular linkage map containing nutritional and resistance markers.

A molecular linkage map of two genetic regions (nif and nod) were constructed in R. trifolii strain by hybridization analysis. It was found that these two regions are located some 16 Kb apart on the 180 Kb symbiotic plasmid (213).

Linkage mapping in R. leguminosarum was done by using R68.45 for mapping and results indicated that Serl was unlinked with four other markers which were linked with a probable mapping order (met-14)-(trp-16)-(phe-1-)-(ura-14).

Double or multiple marked derivatives of R. meliloti 41 were mated with derivatives of R. meliloti carrying plasmid R68.45. Recombinants for single markers were selected and coinheritance of unselected markers was determined by replica plating. Linkage analysis of various marked pairs of R. meliloti were found out and order of markers was determined by calculating map distances between closely linked markers or by three factors crosses (133).

Development of a genetic recombination system in R. meliloti based on conjugation was reported. During the mating, the drug resistance factor RP4 can promote the conjugal transfer of chromosome markers to produce ultimately recombinant progeny. This system was used to construct a circular linkage map of R. meliloti. All the 25 sites on the map constitute a single linkage structure, and there is no evidence for additional linkage group (159,161).

A circular linkage map of R. meliloti chromosome obtained from R68.45 mediated crossess have been revised by co-transductional analysis, after general transduction by DF2 phage. Three short chromosomal regions were mapped by co-transduction. Comparison between conjugal and co-transductional data suggests that R68.45 mediated linkage measures are induced enough (39). Recombination

between the two R. meliloti strains ocurred at high frequency. When co-inheritance data for the three strains were transformed into additive map distances, the arrangements of markers showed striking similarilties.

Ruvkun and Ausubel (204) reported general method for site directed mutagenesis in prokaryotes. This method was also used to construct a physical genetic map of a subset of the R. meliloti nif genes.

Both genetic and physical map of the regions of pRmea carrying the nod, nif and fix loci have been published (14, 243). A circular linkage map of pRmeb has also been prepared (44).

In strain R. meliloli 41 Rprime plasmids carrying the essential nod genes and the nif structural genes were isolated (11) and the physical map of this region was constructed. (134).

Tn5-oriT has been used to promote conjugal transfer of the chromosome in R. meliloti (259) and to position succinate transport loci on the genetic map of this bacterium (78). In 1989, a chromosomal genetic map of Rhizobium sp. NGR234 was constructed by polar mobilization of chromosome using Tn5-Mob insertions (177).

Klfin et al. (128) devised a system for genetic mapping of the R. meliloti chromosome that utilizes Tn5-Mob, a derivative of Tn5 constructed by Simon (226) that carries the origin of transfer (oriT) of plasmid RP4. This system is applicable to any gram negative bacterium in which Tn5 can transpose and into which Inc P plasmids can conjugate.

The genetic and molecular maps of the chromosomes and symbiotic plasmids a and b that emerged for R. meliloti 1021, AK631 and 102F34 were found to be similar (44,89,117,190,234).

MATERIALS AND METHODS

The material used and the methods followed during the course of various experiments done are presented below:

3.1 STRAINS USED

Several strains were used for various purposes during the course of this investigation.

3.1.1 Bacterial Strains

The list of strains of Rhizobium meliloti and Escherichia coli K12 used in this study alongwith information on their characteristics is given in Table 2.

3.1.2 Legume Strains

Medicago sativa cv. T9 (Lucerne or alfalfa) was used in this study. The seeds were obtained from National Seeds Corporation, New Delhi.

3.2 COMPOSITION OF MEDIA USED

3.2.1 Media for Rhizobium meliloti

3.2.1.1 Complete Media

The following complete media were utilized for growing rhizobia.

3.2.1.1.1 Mannitol-Salts-Yeast Extract Medium (MSY)

This was used in liquid form as broth to grow cultures and in solid form as medium for plating.

Constituent	Stock Solution	Amount of Stock solution added per l	Final Concentration Obtained per l
Mannitol	20%	10ml	2.0g
$K_2HPO_4 . 3H_2O$	2%	10 ml	0.2g
KH_2PO_4	2%	10 ml	0.2g

Table 2 : Bacterial strains, R primes and plasmid constructs used.

Strain	Relevant Characters	Source/Reference
Rhizobium meliloti		
ZB201	Cys^- 46, Rif^r, Fur^r, derivative of AK631.	Forrai et al., 1983.
Rmd 201	Spontaneous Str^r derivative of AK631.	Khanuja and Kumar, 1988.
ZB178	Ade^- 15, Rif^r.	Kondorosi,A.
Escherichia coli WA803	Met^-, Thi^-.	Selvaraj and Iyer, 1983.
HB101	F^-, Ara^-, Xyl^-, Lac^-, Mtl^-, Met^-, Pro^-, Leu^-, Thi^-, SupE, $RecA^-$, Str^r.	Boyer and Roulland Dussoix, 1969.
Plasmids		
pGS9	Inc N, repP-15-A, Chl^r, Km^r	Selvaraj and Iyer, 1983
pRK290	Wide host range cloning vector. Tet^r, Ori(RK2).	Ditta et al., 1980.
R. meliloti genomic library	pRK290 with cloned fragments of genomic DNA of R. meliloti 102 F34.	Ditta et al., 1980.
pRK2013	Helper plasmid for mobilisation of pRK290. tra (RK2), ori (ColE1), Km^r.	Figurski and Helinski, 1979.
pJB3JI	Km^s derivative of pR68.45 capable of mobilising genomic segments of its host. Tet^r.	Brewin et al., 1980.
pGR1	pJB3JI prime carrying all known nod, hsn, nif and fix genes.	Banfalvi et al., 1981.
pGR3	pJB3JI prime carrying all known nod, hsn, and nif genes.	Banfalvi et al., 1981.
pPH1JI	Incompatible to pRK290 and pJB3JI. Gm^r.	Beringer et al., 1978.

Yeast Extract	10%	10 ml	1.0g
$MgSO_4 . 7H_2O$	1 M	0.8 ml	0.1g
$CaCl_2 . 2H_2O$	1M	0.4 ml	0.05 g
Water to make 1 l volume.			

To make solid medium, 15.0g agar was added per litre.

3.2.1.1.2 Tryptone-Yeast Extract Medium (TY)

Constituent	Amount used/l
Bactotryptone	5.0g
Yeast extract	3.0g
$CaCl_2 .2H_2O$	0.12g
Water to make 1 l volume	

15 g agar was added to make solid medium.

3.2.1.1.3 Yeast Extract Mannitol Agar Medium (YEMA)

The composition was as in MSY agar medium but mannitol was used 10.0g/l.

3.2.1.1.4 Yeast Extract Mannitol Swarm Plates

Swarm plates were used for motility tests. The composition of the media was the same as in YEMA except for the reduction of agar concentration to 0.3%.

3.2.1.2 Minimal Medium (RMM)

First stock solutions A and B were prepared. The solution A was 20% glucose or any other sugar at 20%. The solution B called 2XR salt solution (2XRSS) had the following composition:

Salt	Stock solution	Amount of stock solution added/l	Final concentration obtained per litre
$Na_2HPO_4 3H_2O$	4.5%	20ml	0.90g
$(NH_4)_2 SO_4$	20%	20ml	4.0g

$FeCl_3$	1%	0.4ml	4.0mg
$MgSO_4 .7H_2O$	1M	0.4ml	0.2g
$CaCl_2 .2H_2O$	1M	0.2ml	0.08g

Water to make 1 litre volume.

3.2.2 Media for Escherichia coli

3.2.2.1 Complete Medium: Luria Bertani Medium (164)

Constituent	Amount used /l
Bactotryptone	10g
Yeast extract	5g
Sodium chloride	10g
H_2O	1 l
Agar (for solid medium)	15 g

3.2.2.2 Minimal Medium : M9 Medium (156)

Minimal medium used was of the following composition per litre

(a) Na_2HPO_4 6.0g

(b) KH_2PO_4 3.0g

(c) NaCl 0.50g

(d) NH_4Cl 1.00g

The pH was adjusted to 7.4 with 1M NaOH. Whenever solid medium was required, 15g of bactoagar was added. After autoclaving add separately autoclaved

(e) 1M $CaCl_2$ 1.0 ml

(f) 1M $MgSO_4 .7H_2O$ 2.0 ml

(g) 20% glucose 10ml

3.2.3 Nitrogen Free Plant Growth Medium

For carrying out nodulation tests on alfalfa plants, the nitrogen free medium

developed by Engelke and Coworkers (73) was prepared as follows:

Twelve stock solutions (A to L) were prepared. The composition of these solution are given below:

Solution A: 2.0902 g K_2HPO_4, water 10 ml.

Solution B: 0.544g KH_2PO_4 , water 10 ml.

Solution C: 7.351 g $CaCl_2$, water 10 ml.

Solution D: 0.335g ferric citrate, water 10 ml.

Solution E: 6.162 g $MgSO_4$, water 10 ml.

Solution F: 4.3562g K_2SO_4 , water 10 ml.

Solution G: 0.0340g $MnSO_4$, water 20 ml.

Solution H: 0.026 g H_3BO_3 , water 20ml.

Solution I: 0.03 g $ZnSO_4$, water 20 ml.

Solution J: 0.002g $CuSO_4$, water 20 ml.

Solution K: 0.006g $CaSO_4$, water 20 ml.

Solution L: 0.006 g $NaMoO_4$, water 20 ml.

These stock solutions were autoclaved separately. To make 1l of plant growth medium, 10g of bactoagar was dissolved in 1l of water and autoclaved. One ml of each of the stock solutions was added to this autoclaved agar solution and pH was adjusted to 6.8. This resulting medium was then used to prepare plant growth medium slants.

3.3 Diluents

(i) **MSY Salt Solution (MSS)**: MSY medium minus sugar and yeast extract was used for diluting bacterial cultures.

(ii) **NaCl**: 0.9% NaCl solution was used for carrying out dilutions.

3.4 SUPPLEMENT TO MEDIA

3.4.1 Addition of Dyes

Aniline blue, calcafluor or congo red was added at a rate of 0.02% to the medium

before autoclaving.

Sodium deoxycholate (DOC) : DOC was added at a rate of 1mg/ml before autoclaving the medium.

3.4.2 Amino acids, Vitamins and Nitrogenous Bases

Stock solutions of amino acids, vitamins and bases were made in distilled water and autoclaved. Their required volumes were added to the autoclaved media.

For identifying the nature of auxotrophy, Holliday (103) pools with the following composition were used:

Pool 1 - Alanine, Glycine, Asparagine, Tyrosine, Biotin.

Pool 2 - Arginine, Histidine, Phenylalanine, Valine, Thiamine.

Pool 3 - Aspartic acid, Isoleucine, Proline, Adenine, Cobalamine.

Pool 4 - Cysteine, Leucine, Serine, Guanine, Uracil.

Pool 5 - Glutamic acid, Lysine, Threonine, Cytosine, Riboflavin.

Pool 6 - Glutamic acid, Methionine, Tryptophan, Thymine, Pantothenic acid.

Pool 7 - Alanine, Arginine, Aspartic acid, Cysteine, Glutamic acid.

Pool 8 - Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine.

Pool 9 - Asparagine, Phenylalanine, Proline, Serine, Threonine, Tryptophan.

Pool 10 - Tyrosine, Valine, Adenine, Guanine, Cytosine, Thymine.

Pool 11 - Biotin, Thiamine, Cobalamine, Uracil, Riboflavin, Pantothenic acid.

Each of the above chemicals was used at a concentration of 2mg/ml and 1ml of this pool solution was added to 100ml of RMM before pouring plates.

3.4.3 Sugars and Carbon Sources

Following sugars or carbon sources were employed in this study: Glucose, Lactose, Mannitol, Sucrose, Arabinose, Glycerol, Maltose, Mannose and Xylose.

3.4.4 Antibiotics

Stock solutions of antibiotics were filter sterilized and stored at 4^0 C and when required, desired volumes were added to the autoclaved medium that had been allowed to cool to about 50^0 C.

3.4.5 Salts

Required amouts of NaCl was added to the TY medium directly before autoclaving to test the NaCl tolerance of various strains.

3.5 Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out by the procedure described by Laemmli (139). The various solutions used were as following:

STOCK SOLUTIONS

Five stock solutions called (A to E) were prepared. The composition of these solutions are given below.

Solution A: Acrylamide-bisacrylamide (30:0.8)

It was prepared by dissolving 30g acrylamide and 0.8g bisacrylamide in water and made to 100ml with water.

Solution B: Stacking Gel Buffer Stock (0.5M Tris HCl, pH 6.8)

6.0g of Tris was dissolved in 40ml of water, titrated to pH 6.8 with 1M HCl, and brought to 100ml final volume with water.

Solution C: Resolving Gel Buffer Stock (3.0 M Tris HCl,pH 8.8)

36.3g of Tris and 48.0 ml of 1M HCl were mixed and brought to 100 ml final volume with water.

Solution D: 10% SDS

Solution E: 1.5% Ammonium persulphate

Electrophoresis Buffer: Tris-glycine, pH 8.3

It contained in final concentration, 0.025M Tris, 0.192M glycine and 0.1% SDS. It was prepared by adding 10ml of 10% SDS stock solution to 3g of Tris and 14.4g glycine and made to 1000ml with water.

SAMPLE BUFFER

The samples buffer contained in final concentration 0.0625 M Tris HCl buffer

(pH 6.8), 2% SDS, 10% glycerol and 5% β-mercaptoethanol.

Marker Dye: 0.001% Bromophenol blue

1 mg Bromophenol blue

1µl Glycerol

900 µl Water

Staining Solution

Coomassie brilliant blue R-250 0.05%

Methanol 40%

Acetic acid 10%

Mixed in the ratio of V/V/V and filtered before use.

Destaining Solution

Methanol 5%

Acetic acid 7%

Water

Working Solutions

Resolving Gel

The resolving gel of 10% acrylamide was prepared so that the final concentration of the buffer was 0.375M Tris HCl (pH 6.8) and 0.1% SDS. The gels were polymerized chemically by the addition of 0.025% by volume of tetramethylethylenediamine (TEMED) and ammonium persulphate. It was prepared as follows:

Solution A	10.0ml
Solution C	3.75 ml
Solution D	0.3 ml
Solution E	1.5 ml
TEMED	0.015 ml

water upto 30 ml.

Stacking Gel

The stacking gel of 3% acrylamide was prepared so that the final concentration was

0.125M Tris HCl (pH 6.8) and 0.1% SDS. The gel was polymerized chemically in the same way as for the separating gel. It was prepared as follows:

Solution A	2.5ml
Solution B	5.0 ml
Solution D	0.2 ml
Solution E	1.0 ml
TEMED	0.015 ml

water upto 20 ml.

3.6 METHODS

3.6.1 General Bacteriological Procedures

3.6.1.1 Culturing of Bacteria

Single colonies were obtained by plating or streaking cells of a strain on complete or minimal medium. When complete medium was used, incubation of four days was required at 30^0 C for the colonies to appear. When minimal medium was used, incubation of about one week was required. A fully grown R. meliloti colony usually contained about 10^7 cells.

Often spot tests were employed to study growth response of bacterial strains. For this purpose, cells from a colony were suspended in 0.05ml of 0.9% NaCl. About 10^5 to 10^6 cells were applied to the surface of agar with a loop to make a spot.

To start a liquid culture, a colony was transferred to 5 ml of broth in a tube using a loop. Then the tube was shaken in orbital shaker at 30^0C. At about 36 hours, the culture usually had about 5×10^8 cells/ml. Rhizobium meliloti and E. coli were incubated at 30^0C and 37^0C respectively.

3.6.1.2 Matings Between Bacteria

Patch matings were employed (133). TY broth cultures of donor and recipient bacteria were grown for 24 hours. They were mixed 1:1 and 0.05 ml of the mixture was spread on TY agar surface with the help of loop. Then the plate was incubated at 30^0C

for desired lengths of time. The patch of growth obtained was treated in two ways:

1. It was replica plated on selective medium.
2. The growth was scrapped using a sterile spatula and suspended in 1ml of MSS (sugar not added). The suspension was plated, directly and after dilution, on the selective medium. The plates were examined for exconjugants after five days of incubation at 30°C.

3.6.1.3 Serial Dilutions

Serial dilution were made in saline solution (0.9% sodium chloride) to obtain single bacterial colonies for many experiments. 0.1 ml of neat culture was added to the first of tube containing 0.9 ml of saline solution to obtain 10^{-1} dilution. After mixing it properly, 0.1ml culture from this tube was added to the next 0.9 ml saline solution tube to obtain 10^{-2} dilution. Similarly , further dilutions were made according to the requirements of the experiment.

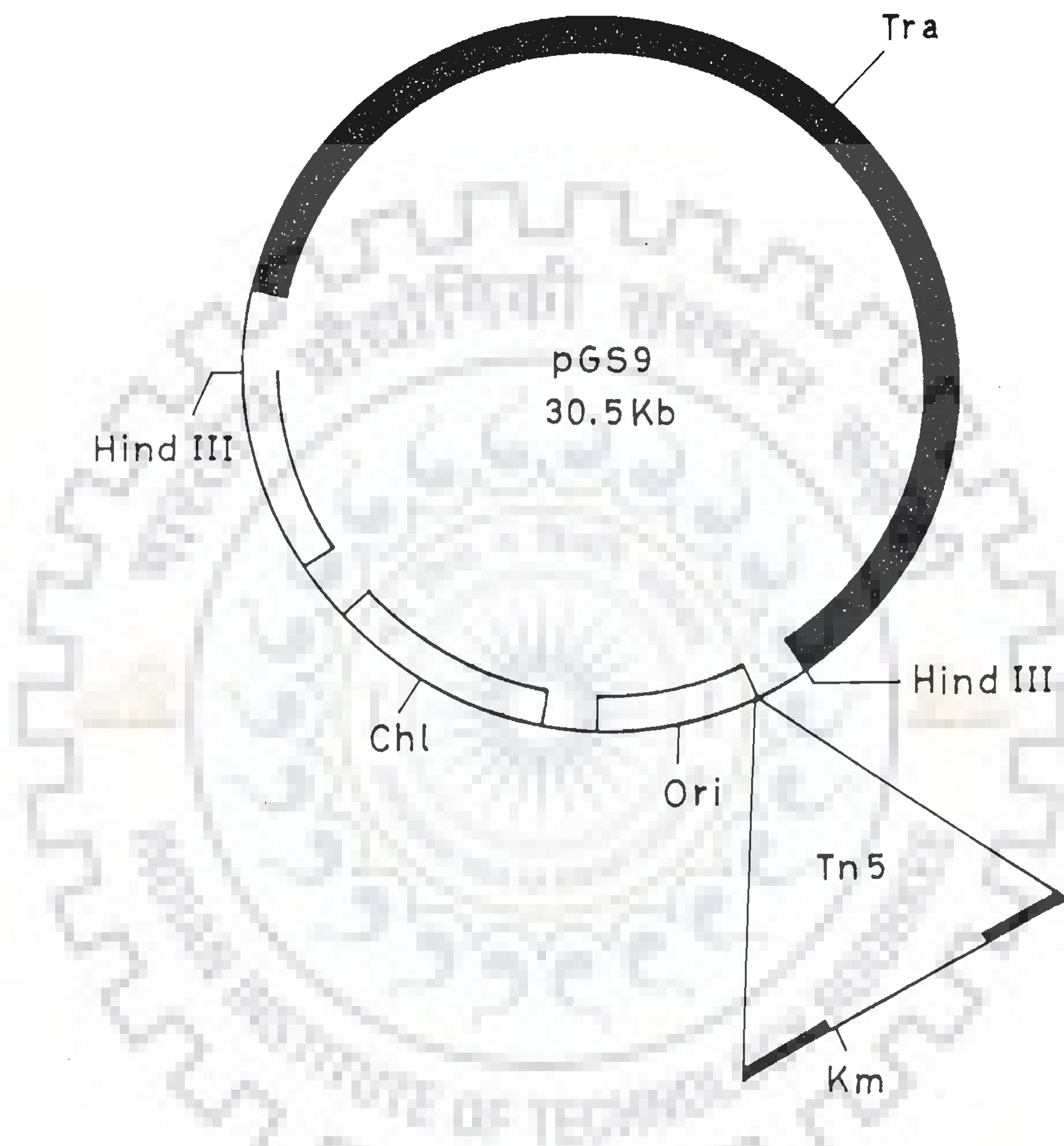
3.6.2 Induction and Isolation of Mutants

3.6.2.1 Transposon Tn5 Mutagenesis Procedures

Cultures of E. coli strain WA803 (pGS9), (Fig 3) and R. meliloti were grown to log phase in TY broth. The cells of the two strains were mixed 1:1 and the mixture was plated on TY for allowing mating as described above. Several patches were made using only 20μl of mixture per patch. The patched plates were incubated at 30°C for different time intervals to get a good frequency of Tn5 transconjugants (Fig 4).

After mating had been allowed, the growth from a patch was transferred to 1ml 0.9% saline solution in a 5ml tube. The suspension of mated cells was plated directly on one selective plate and after 10^{-1} dilution on another selective plates. In all matings, the donor and recipient culture were also plated on selective plates. The plates were incubated for five days.

There were 0 to 250 colonies on different plates when the suspension from a patch was plated directly. From a plate, derived from a patch, five or less number of colonies were purified twice on YEMA plates having Km and Str.



pGS9 has p15A replicon and N transfer system making it a conjugative plasmid suicidal in <u>Rhizobium</u>

Fig.3. Suicide vector pGS9 carrying Tn5.

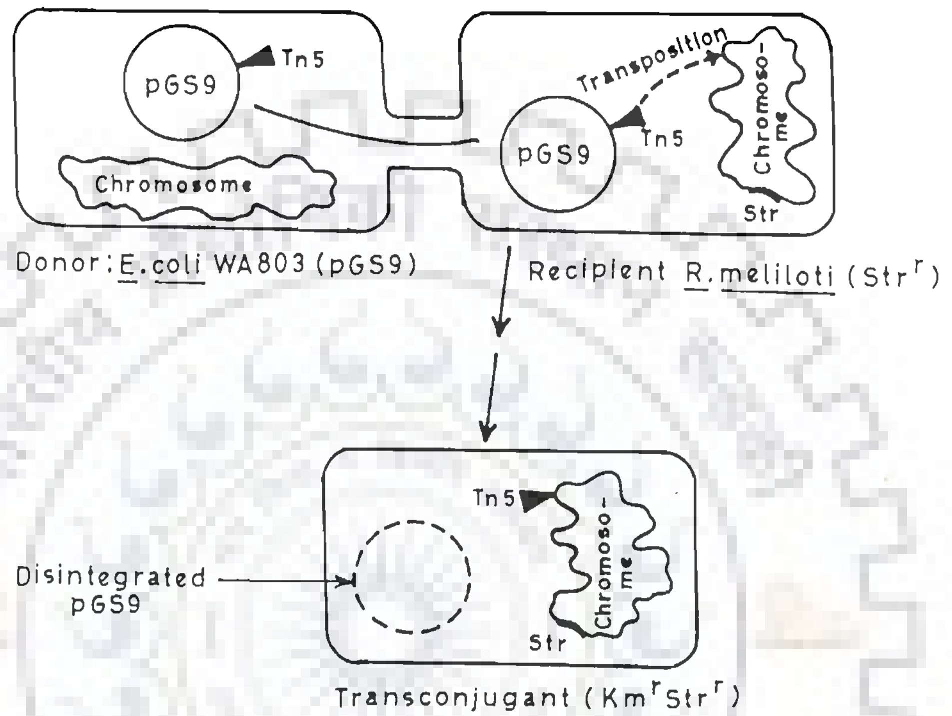


Fig.4. Diagramatic representation of Transposon Tn5 mutagenesis.

This way a collection of around 5000 mutants of R. meliloti from about 1200 patch matings, was built up. The mutants were maintained on YEMA plates having Km and Str.

All Tn5 induced Km^r mutants were then checked for the loss of the suicide plasmid. This was done by looking for the absence of vector encoded chloramphenicol resistance in these transconjugants.

3.6.2.2 N-methyl-N′-nitro-N- nitrosoguanidine (NTG) Mutagenesis (229)

Day 1:

5ml of MSY broth was inoculated with R. meliloti and the culture tube was incubated at 30°C in an orbital shaker.

Day 3:

(After 36 hours) A sample of the culture was diluted and plated on MSY plate to determine the titre. To the rest of culture, 0.01 ml of NTG solution (10mg/ml in acetone, freshly prepared) was added. The culture was divided into two equal parts and both the parts were kept at 30°C in an orbital shaker. At intervals, samples were taken to determine the percentage of survival of bacterial cells. When percentage of survival cells was around 10%, bacterial cells were washed two-three times in RMM salts (sugar not added). After washing the cells were transferred to MSY broth and this was incubated at 30° C in an orbital shaker.

Day 4:

Culture was centrifuged and the cells were washed twice with 5ml RMM salts. The cells were resuspended in 5 ml RMM salts and incubated overnight at 30° C in an orbital shaker.

Day 5:

Then penicillin (200µg/ml) and mannitol (2mg/ml) were added and incubated the culture overnight at 30°C.

Day 6:

The culture was plated on TY agar to find out its titre and plates were incubated

at 30°C.

Day 9:

Titre was estimated and sample was plated on TY plates such that thirty colonies appeared per plate and incubated at 30°C. These colonies were streaked on TY plates for purification. These purified colonies were finally used for screening of auxotrophs.

3.6.3 Screening for Auxotrophs

3.6.3.1 Replica Plating Method

The mutagenized culture was plated on TY plates to get about 100 colonies per plate. These plates were incubated at 30°C for two days. These plates were used as master plates for replica plating onto RMM plates. Both the sets of plates were incubated at 30°C for four days. The plates were then compared to identify the auxotrophs. Colonies growing on TY but not on RMM were picked up as auxotrophs and streak purified on complete medium three times.

3.6.3.2 Minimal Supplementation Method

Limited enrichment of minimal medium was achieved by adding 20mg/l of Difco, Bacto nutrient broth to RMM. The mutagenized culture was plated on enriched minimal medium plates so as to obtain about fifty colonies per plate. These plates were incubated at 30°C for five days and then the relatively small colonies were marked. These small colonies were purified and examined for auxotrophs.

3.6.4 Characterization of Auxotrophs

3.6.4.1 Nature of Auxotrophy

The mutants were suspended in a drop of 0.9% saline solution and spotted on different Holliday pools (103,229). The plates were incubated for three days at 30°C. Based on the growth on the pools, the kind of auxotrophy was determined. Those mutants that are not growing on any pool were considered to be multiple auxotrophs or auxotrophs whose requirements are not met by the nutrients included in the pools.

3.6.4.2 Reversion Analysis

Reversion frequencies of auxotrophic mutations were determined with cells

grown until the late logarithmic phase in TY medium at 30°C. The cells were washed twice with RMM medium and resuspended in the same medium to a concentration of about 10^9 cells per ml. Then the frequency of spontaneous revertants was checked by plating a known number of mutant cells on RMM plates and counting the number of prototrophic colonies appearing after five days incubation at 30° C.

In case of Tn5 induced auxotrophic mutants, these protrophs were replica plated onto RMM agar plates supplemented with kanamycin to determine whether Tn5 was retained or not.

In case of NTG induced mutants also, the frequency of induced revertants was similarly determined. The only additional step was the addition of 5μl nitrosoguanidine (10mg/ml) at the centre of the plates.

3.6.4.3 Genetic Linkage of Tn5 to Auxotrophic Mutations

To verify that the insertion of Tn5 was indeed the cause of the auxotrophic mutations, the linkage of the Tn5 conferred kanamycin resistance phenotype to the auxotrophic phenotype was determined (22,160). This was done by conjugating the Tn5 containing mutant strains with appropriate recipient strains, selecting for Km^r exconjugants, and then testing these for auxotrophy, according to the donor's mutant phenotype. In these experiments, genomic mobilization was mediated by R68 derived pJB3JI kanamycin sensitive plasmid (27), capable of promoting chromosome transfer in R. meliloti.

3.6.4.4 Symbiotic Properties

3.6.4.4.1 Nodulation Assays of the Auxotrophs

These assays were carried out in 20 cm x 2.5cm glass tubes. Twenty five-thirty ml of plant growth medium was added to the tubes. The tubes were autoclaved after stoppering with cotton plugs and slants were made. Whenever nutritional supplementation was required, a given amount of sterile nutrients was added to the tubes.

Alfalfa seeds (Medicago sativa cv. T9) were procured from the National Seeds

Corporation, New Delhi. The seeds were soaked in sterile water for 30 minutes. Then, they were surface sterilized with 0.1% $HgCl_2$ for one mintue followed by treatment with absolute alcohol for one minute. The seeds were then quickly washed several times with sterile water.Surface sterilized seeds were spread on 1.0% agar plate for germination and kept inverted in a dark place. Two seedlings about two days old were then transferred to each tube.

For inoculation, two days old culture of R. meliloti, strains grown in MSY broth was used. The culture was washed with sterile water and was suspended in 3 ml of sterile water. 0.5 ml of the suspension was dispensed into each tube having two days old seedlings.

The plants were kept under 2000 lux light with the roots protected from direct exposure to light with the help of black paper. A 16 hour light, 8 hour darkness cycle was maintained and temperature maintained was 25°C. For each R. meliloti strain being tested, four tubes containing two plants each were used. After eight weeks, data on individual plants were recorded for nodule number, nodule colour, nodule size, nodule shape, plant height and dry plant weight. Nitrogen content of plants was estimated by Kjeldahl's method.

3.6.4.4.2 Nitrogen Estimation by Kjeldahl's Method (163)

One gram of finely ground dried plant sample was taken in digestion flask in which 10 g of reaction mixture (10 parts of potassium sulphate and one part of copper sulphate) and 30 ml of concentrated sulphuric acid were added. The sample was digested for 2-3 hours till the solution became clear. A volume of 250 ml was made from the digested material. The digested material was distilled in Kjeldahl distillation set. Five ml of the digested solution was taken in the distillation set and 10 ml of 40% NaOH solution was added to it. The steam was passed through it and the distillate was collected in 4% boric acid solution which contained mixed indicator (Methyl red - Bromocresol green). Ammonia thus collected was titrated against N/100 sulphuric acid. Using the following formula nitrogen percentage was determined.

$$\% \text{ Nitrogen} = \text{Vol. of N/100 } H_2SO_4 \text{ used} \times \frac{.00014 \times \text{Total Vol. made after digestion}}{\text{Wt. of sample} \times \text{Vol. of digested soln. taken for distillation}} \times 100$$

3.6.4.5 Recovery of Bacteria from the Nodules

Roots bearing nodules were surface sterilized by dipping in absolute alcohol for 45 seconds and were quickly washed several times in sterile water. The nodules were then crushed in a drop of 0.9% NaCl solution using a sterile forceps. The milky suspension of cells thus obtained was streaked on MSY plate and incubated at 30^{0}C for two days.

3.6.4.6 Surface Properties

3.6.4.6.1 Reaction of Dyes

The bacterial strains to be tested were either spotted or streaked on YEMA plates having aniline blue or congo red or calcafluor and incubated at 30^{0}C for three days. Calcafluor staining was examined as fluorescence under long wavelength UV light while other stainings were observed in normal light.

3.6.4.6.2 Test for Production of Lipopolysaccharides (LPS)

The bacterial strains to be tested were spotted on TY plates having sodium deoxycholate at a rate of 1 mg/ml. The plates were then incubated at 30^{0}C for two days and those which showed growth on this medium were considered to be LPS producing.

3.6.4.6.3 Test of Motility

The bacterial strains to be tested were spotted on swarm plates and incubated at 30^{0}C for two days. The motility of the bacterial strains was determined by the spread of the colonies.

3.6.4.7 Sugar Utilization Properties

The bacterial strains to be tested were spotted along with the wild type strain on minimal medium (containing their nutritional requirements) supplemented one at a time, with various sugars. The plates were incubated at 30^{0}C and examined after 72 to 96 hours.

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3.6.4.8 Effect of Salts

The auxotrophic strains were streaked with the wild type on TY plate containing various concentrations of salts. Plates were incubated at 30°C and examined after 72 to 96 hours.

3.6.4.9 Growth Properties

Auxotrophic strains along with the wild type strain Rmd 201 were grown in 10ml of MSY medium. These exponentially growing cultures were transferred to fresh medium. Medium was then mixed properly and these subcultures were then incubated at 30°C in an orbital shaker. After every hour, aliquots were taken in three replicas and their optical density was determined at 600nm using MSY medium as blank. A semilog plot of the optical desnity versus time was plotted for each bacterial strain and the generation time was determined (164).

3.6.4.10 Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis

The soluble protein pattern of the wild type strain and auxotrophic mutants was studied by SDS-PAGE.

Cell-Free Extracts Preparation

Cells grown to exponential phase were collected by centrifugation for 20 minutes at 8000Xg, washed twice and resuspended in the sample buffer. Cells were disrupted by five 60s sonic treatments with 60s rest intervals at 0 °C. Cell-free extracts were obtained by 30 minute centrifugation at 27000Xg.

Gel Electrophoresis

The electrophoresis buffer was filled to the lower tank and the slab gels were fitted in their places in the apparatus. Cell-free extracts in sample buffer were heated by immersing them in boiling water for 2 minutes. Protein samples were mixed with tracking dye and loaded in the wells. The upper tank was filled with electrophoresis buffer. Electrophoresis was carried out with a current of 30mA per gel until the bromophenol marker reached the bottom of the gel. The gels were stained overnight. The gels were diffusion destained by repeated washing in 7% acetic acid and 5% methanol.

3.6.5 Handling the Plasmids

3.6.5.1 Transfer of Plasmids

Overnight cultures of the donor strain, the recipient strain and when needed, the helper strain were grown in TY broth. For making the conjugal cross, 0.1ml of each of these was taken and mixed on TY plates. Individual spots of each culture were also included. These plates were incubated at 30°C for 24-36 hours. The content of the TY plates were then replica plated to selective medium for exconjugant selection. The selective medium plates were incubated at 30°C for four days. The colonies appearing were streak purified on selective medium thrice.

3.6.5.2 Isolation of Clones From Genomic Library

The gene bank of R. meliloti 102F34 on the vector pRK290 developed by Ditta et al. (61) was used. It was made available as a confluent growth developed from a large number colonies of HB101 carrying different clones. The plasmid pRK290 has origin of transfer but does not carry tra genes. Therefore, it can be mobilized only if helper tra^{+} plasmid pRK2013 is simultaneously present (Fig. 5).

Overnight grown TY broth cultures of each of the different auxotrophs was mixed with the genomic library and pRK2013 in equal quantities in an eppendorf tube. The mating mixture was then plated on TY and incubated at 30°C for 24-36 hours. A suitable control was included by spotting separately, each of the cultures used in the cross. The growth on TY plates was then replica plated to the plates having RMM with tetracycline. These plates were incubated at 30°C for six days. The colonies that appeared on RMM + tetracycline plates were purified by streaking thrice on RMM + tetracycline plates.

3.6.6 Cross Feeding Assays

The auxotrophs were suspended in 0.9% sodium chloride and streaked on minimally supplemented RMM plates. Three auxotrophic strains (requiring same aminoacid) were streaked on each plate in such a manner that each streak formed a side of an equilateral triangle. The plates were then incubated for 15 days at 30°C. It was

observed that the strain was being cross fed had enhanced growth at a point nearer to the streak of the strain which was cross feeding it.

3.6.7 Stastistical Analysis

The data related to plant characteristics of wild type strain and its auxotrophic mutants were statistically analysed. The means of eight plants of a type were used.

Calculation of critical difference (C.D.):

C.D.= $t_{0.05} \times \overline{sd}$

where

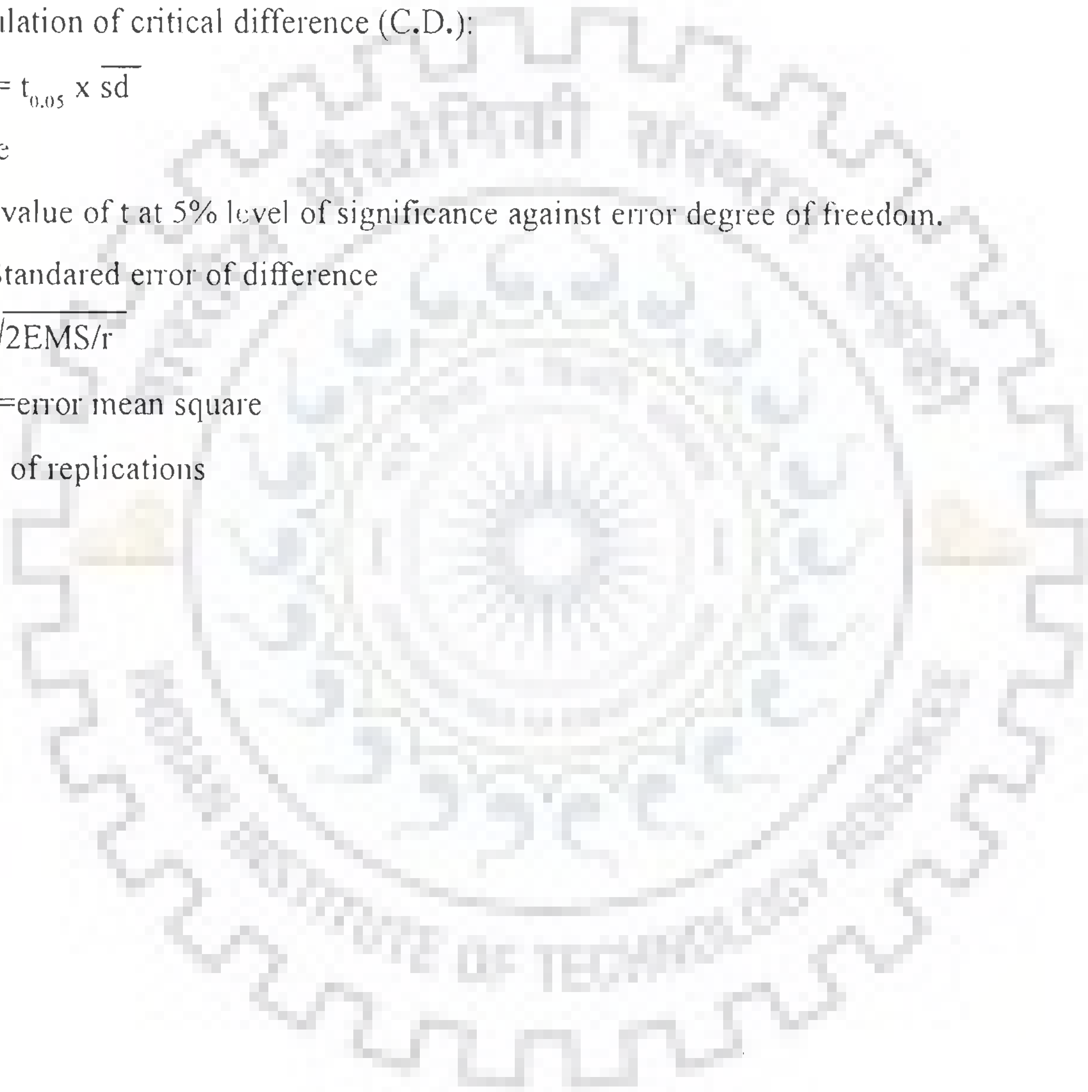
$t_{0.05}$=value of t at 5% level of significance against error degree of freedom.

$\overline{sd}$= Standared error of difference

$\overline{sd} = \sqrt{2EMS/r}$

EMS=error mean square

r=no. of replications



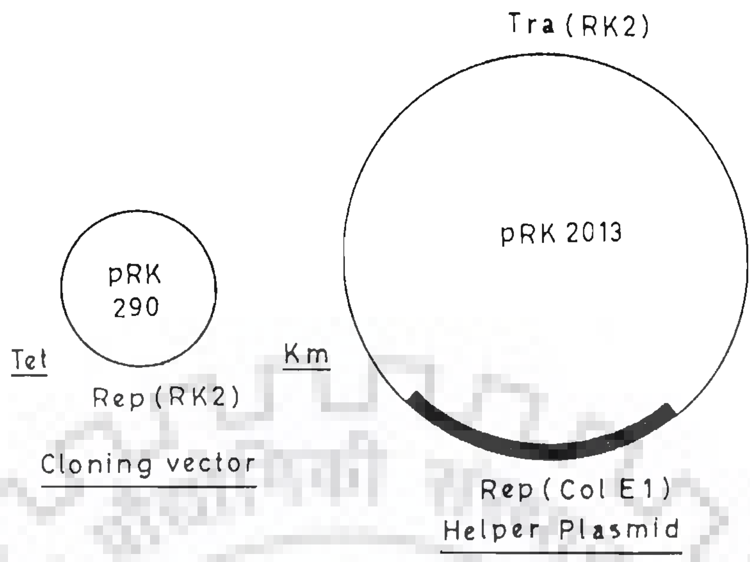


Fig.5. Binary vehicle system. (i) physical map of pRK 290, (ii) physical map of pRK 2013.

RESULTS

Gram negative bacterium Rhizobium meliloti effectively nodulates Medicago, Melilotus and Trigonella group of plants. R. meliloti was used for isolating several auxotrophs following treatments with transposon Tn5 and N-methyl-N′–Nitro-N-nitrosoguanidine (NTG). The results of isolation and characterization of auxotrophs are presented here.

4.1 ISOLATION OF RHIZOBIUM MELILOTI MUTANTS

4.1.1 Transposon Tn5 Induced Auxotrophic Mutants

The use of antibiotic resistant transposons as a mutagenic agent has been shown to be extremely powerful tool in the genetic analysis of Rhizobium. Therefore, to isolate auxotrophic mutants of Rhizobium, Tn5 mutagenesis was done.

Tn5 carried on plasmid pGS9 in the E. coli strain WA803 was used to generate mutants. The parental strains were patch mated for different time intervals and frequency of Tn5 exconjugants per recipient was calculated for each interval. The mating time of 16 hours generated good frequency of transconjugants (Table 3). After 16 hours, a very little increase occurred in Tn5 transfer frequency with time. Therefore, all the subsequent matings to get Tn5 mutants of R. meliloti ZB201 and R. meliloti Rmd201 were performed for 16 hours.

4.1.1.1 Tn5 Insertion Mutants of R. meliloti ZB201

E. coli WA803 carrying plasmid pGS9 (Figure 3) was conjugated with R. meliloti ZB201 (Rif^r). Km^r Str^r R. meliloti exconjugants were selected. The frequency of kanamycin resistant clones per recipient was found to be $4.2x10^{-4}$ which was greater than the frequency of spontaneous resistant mutants ($4.2x10^{-7}$). Tn5 encodes resistance to kanamycin and streptomycin in various species of Rhizobium (188, 222).

Table 3 : Frequency of Tn5 exconjugants of Rhizobium meliloti with different mating times.

Mating time (hours)	Frequency (exconjugants/ recipient)
8 hour	2×10^{-9}
12 hour	2×10^{-6}
16 hour	0.32×10^{-4}
20 hour	4.1×10^{-4}
24 hour	2×10^{-4}

Thus, by selecting both the markers (199), elimination of relatively high number of spontaneous kanamycin resistant strains can be done.

All the Tn5 exconjugants were found to be chloramphenicol sensitive. Since chloramphenicol resistant is coded by a non transposable gene present on the vector pGS9, this showed that pGS9 was not present as a free plasmid and no co-integrate formation had taken place.

The ZB201:Tn5 exconjugants were purified twice. A total of 5000 exconjugants were individually screened for auxotrophic mutants and thus sixteen auxotrophic mutants were isolated. These auxotrophic mutants were replica plated on minimal medium supplemented with cysteine and different Holliday Pools. But, by this method, their auxotrophic requirement could not be specified. Therefore, These auxotrophs were again replica plated on minimal medium supplemented with cysteine and amino acids and nucleotides individually to find out their specific auxotrophies. The results are shown in Table 4. It was found that these auxotrophs required either of several amino acids apart from cysteine to grow on RMM plates. Thus, second auxotrophy could not be specified. Moreover, The auxotrophs were highly unstable (reversion frequency was very high). So Tn5 mutagenesis was done again but this time a prototrophic strain of R. meliloti was used.

4.1.1.2 Tn5 Insertion Mutants of R. meliloti Rmd201

Rhizobium meliloti strain Rmd201 is a streptomycin resistant derivative of strain AK631. Rmd201 is a prototroph and effectively nodulates alflafa plants.

The frequency of kanamycin resistant exconjugants per recipient was found 0.4×10^{-4} . It was quite higher than that of spontaneous kanamycin resistant mutants (1.8×10^{-6}). This showed that transposon Tn5 was successfully introduced into R. meliloti Rmd201. All the Tn5 exconjugants were chloramphenicol sensitive indicating positive suicide of pGS9 vector.

From a plate, derived from a patch, five or less number of exconjugants were selected and exconjugants were purified twice.

Table 4 : Auxotrophic mutants of *Rhizobium meliloti* strain ZB201 obtained through Tn5 mutagenesis.

Auxotrophic Mutants	Auxotrophy for Amino acid/Vitamin/Nucleotide base
MA 1	Cysteine and Glycine/Histidine/Uracil
MA 2	Cysteine and Thiamine/Serine/Glycine
MA 3	Cysteine and Serine/Histidine/Aspartic acid
MA 4	Cysteine and Glycine/Serine/Leucine
MA 5	Cysteine and Aspartic acid/Phenylalanine/Leucine
MA 6	Cysteine and Histidine/Glycine/Leucine/Phenylalanine
MA 7	Cysteine and Aspartic acid/Leucine/Histidine/Glycine
MA 8	Cysteine and Thiamine/Glycine/Leucine
MA 9	Cysteine and Histidine/Leucine/Threonine
MA 10	Cysteine and Serine/Proline/Leucine
MA 11	Cysteine and Thiamine/Serine/Glycine/Histidine
MA 12	Cysteine and Histidine/Uracil/Glycine
MA 13	Cysteine and Glycine/Valine/Proline/Phenylalanine
MA 14	Cysteine and Histidine/Glutamine/Leucine
MA 15	Cysteine and Serine/Glycine/Thiamine
MA 16	Cysteine and Histidine/Glycine/Leucine/Serine

This way a collection of 5580 transposon exconjugant from several independent patch matings was built up. These transposon derivatives were replica plated to get auxotrophic mutants. Thus, 17 auxotrophic mutants were isolated at a frequency of 0.3%. These auxotrophic mutants were replica plated on plates made of RMM (minimal medium) plus nutritional pools 1 to 11 to find out their specific auxotrophies. The following classes of auxotrophs have been identified (Table 5): proline$^-$ (three), isoleucine$^-$(one), aspartic acid$^-$(two), methionine$^-$(one), tyrosine$^-$(one), glutamic acid$^-$ (one), cysteine$^-$(one), phenylalanine$^-$(two), valine$^-$(one), threonine$^-$(two), leucine$^-$(one) and histidine$^-$(one).

4.1.2 Nitrosoguanidine Mutagenesis of R. meliloti Rmd201

N-methyl-N′-nitro-N-nitrosoguanidine is a potent mutagen widely used in the field of microbial genetics (162) because it induces a high frequency of mutations. Thus, nitrosoguanidine induced mutants of Rmd201 were also isolated.

Strain Rmd201 was treated with nitrosoguanidine at a concentration of 100 μg/ml. At intervals, samples were removed to determine the number of viable cells. Table 6 and Figure 6 show the survival of bacterial cells at different time intervals. It is clear from the table and figure that after a treatment of three hours with nitrosoguanidine, only 11% cells were viable. Thus, the optimal time of nitrosoguanidine treatment was found three hours to get good frequency of mutants. Enrichment of auxotrophs was done by penicillin treatment.

The mutagenised colonies were purified by streaking on TY thrice. Only ten or less than ten mutants were taken from one mutagenic treatment. This way, by doing several rounds of NTG mutagenesis, 6000 mutants were isolated.

These mutants were screened for auxotrophs by two methods, (a) well grown colonies were replica plated from complete medium (MSY) to minimal medium (RMM) to identify the colonies which failed to grow on RMM, and (b) colonies that were relatively small on minimally supplemented RMM were suspected to be auxotrophs.

Using these two methods, 30 auxotrophic mutants of R. meliloti strain Rmd201

Table 5 : Auxotrophic mutants of *Rhizobium meliloti* strain Rmd201 obtained through Tn5 mutagenesis.

Auxotrophic Mutants	Amino acid Auxotrophy
MA 30	Proline
MA 31	Isoleucine
MA 32	Aspartic acid
MA 33	Methionine
MA 34	Tyrosine
MA 35	Aspartic acid
MA 36	Proline
MA 37	Glutamic acid
MA 38	Leucine
MA 39	Cysteine
MA 40	Phenylalanine
MA 41	Valine
MA 42	Threonine
MA 43	Proline
MA 44	Phenylalanine
MA 45	Histidine
MA 46	Threonine

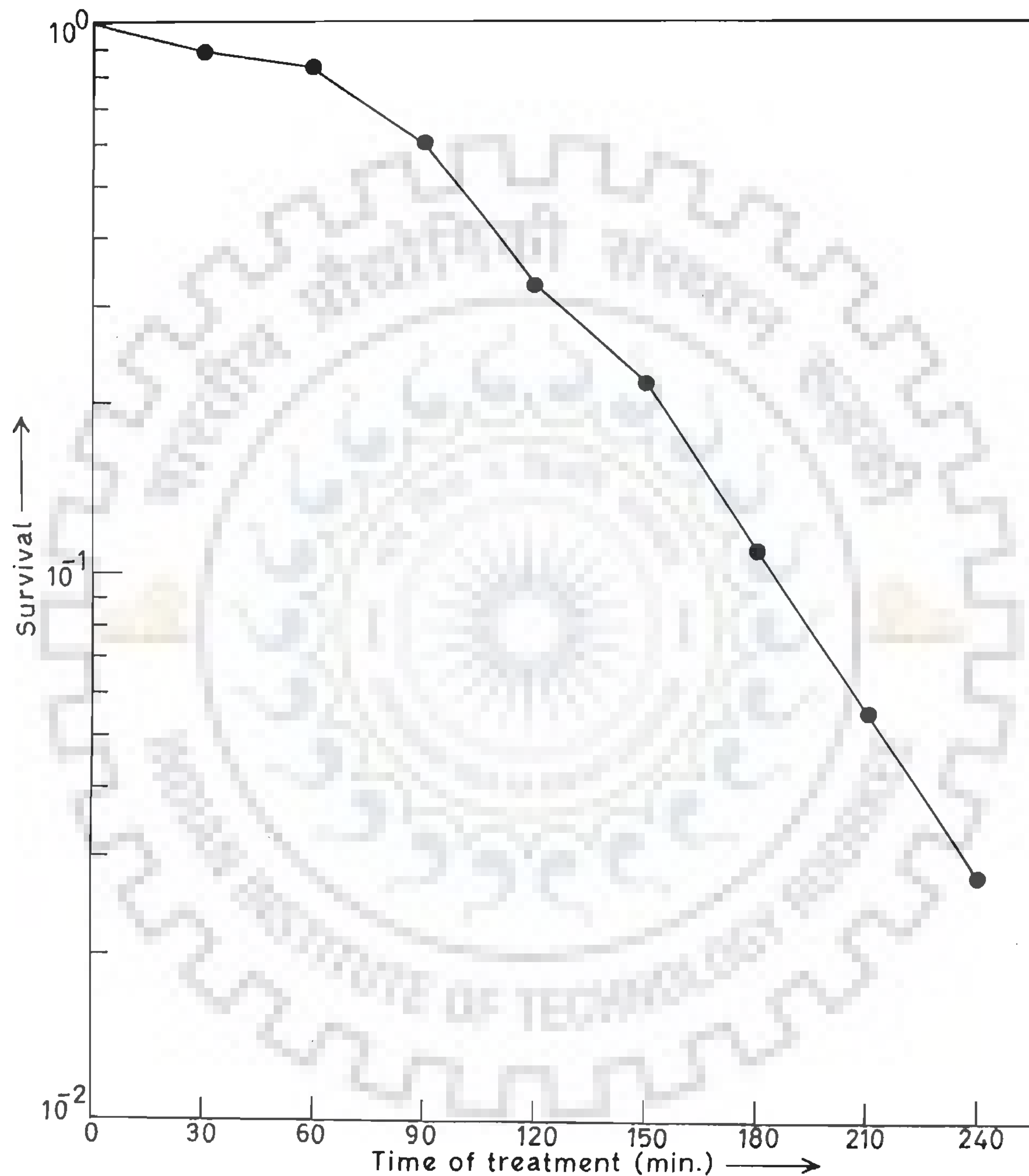


Fig. 6 Survival of Rhizobium meliloti after exposure to N-methyl-N′-nitro-N-nitrosoguanidine at a concentration of 100 µg/ml for various time intervals.

Table 6 : Effect of exposure time of N-methyl - N′-nitro-N -nitrosoguanidine (NTG) on survival of *Rhizobium meliloti* Rmd201.

Exposure time (minutes)	Survival (percentage)
30	89
60	84
90	61
120	33
150	22
180	11
210	5.6
240	2.8

were isolated. Thus the frequency of getting NTG induced auxotrophic mutants was found to be 0.5%. Out of 30 mutants, 16 were multiple auxotrophs as their growth could not be restored on RMM by adding any of the Holliday Pools. However, the nutritional requirement of the fourteen mutants could be specified (Table 7). Fourteen auxotrophs for amino acids were obtained as follows : five for threonine, two for proline, three for lysine, one for asparagine and tyrosine and one for each valine, tryptophan and methionine.

4.2 CHARACTERISTICS OF AMINO ACID AUXOTROPHS

4.2.1 Reversion Analysis

To determine the nature of the mutational lesion(s) responsible for the auxotrophy, study of their revertants was imperative.

4.2.1.1 Reversion Analysis of Tn5 Induced Auxotrophs

All the auxotrophic mutants were tested for reversion to prototrophy by plating them on RMM medium. It was found that all the mutants are revertable at almost similar rates (Table 8). However, prototrophic revertants could not be found when cells were cultured in the presence of kanamycin (Km) and streptomycin (Str). All the prototrophic revertants examined were sensitive to Km and Str and most likely arose from the excision and loss of Tn5 when grown under nonselective conditions (221).

4.2.1.2 Reversion Analysis of NTG Induced Auxotrophs

For reversion analysis, each of the fourteen auxotrophs was plated on RMM with and without NTG to determine the spontaneous and induced rate of reversion. It was found that all the mutants were revertible at almost similar rate and NTG enhanced the rate of reversion in all cases (Table 9).

4.2.2 Genetic Linkage of Tn5 to Auxotrophic Mutations

Verification of the Tn5 associated nature of the mutation was obtained using coinheritance studies during gene transfer experiments. The results of these experiments are listed in Table 10. Using this system, 100% cotransfer of the Tn5 encoded antibiotic

Table 7 : Auxotrophic mutants of Rhizobium meliloti strain Rmd201 obtained through NTG mutagenesis.

Auxotrophic Mutants	Amino acid Auxotrophy
MA 51	Threonine
MA 52	Threonine
MA 53	Asparagine and Tyrosine
MA 54	Proline
MA 55	Lysine
MA 56	Threonine
MA 57	Valine
MA 58	Lysine
MA 59	Threonine
MA 60	Lysine
MA 61	Tryptophan
MA 62	Threonine
MA 63	Proline
MA 64	Methionine

Table 8 : Reversion frequency of auxotrophic mutants of R. meliloti Rmd201 obtained through Tn5 mutagenesis.

Auxotrophic Mutants	Spontaneous reversion frequency
MA 30	7.2×10^{-10}
MA 31	6.4×10^{-10}
MA 32	8.0×10^{-10}
MA 33	8.2×10^{-10}
MA 34	3.4×10^{-10}
MA 35	4.5×10^{-10}
MA 36	6.0×10^{-10}
MA 37	6.8×10^{-10}
MA 38	5.8×10^{-10}
MA 39	5.8×10^{-10}
MA 40	6.4×10^{-10}
MA 41	6.9×10^{-10}
MA 42	5.9×10^{-10}
MA 43	7.0×10^{-10}
MA 44	7.3×10^{-10}
MA 45	7.8×10^{-10}
MA 46	6.3×10^{-10}

Table 9 : Spontaneous and induced reversion frequencies of auxotrophic mutants of R. meliloti Rmd201 obtained through NTG mutagenesis.

Auxotrophic Mutants	Reversion Frequency	
	Spontaneous	Induced with NTG
MA 51	7.8×10^{-10}	3.2×10^{-9}
MA 52	7.2×10^{-10}	2.9×10^{-9}
MA 53	5.4×10^{-10}	3.4×10^{-9}
MA 54	5.8×10^{-10}	3.4×10^{-9}
MA 55	6.2×10^{-10}	2.5×10^{-9}
MA 56	8.2×10^{-10}	2.6×10^{-9}
MA 57	6.4×10^{-10}	3.4×10^{-9}
MA 58	5.0×10^{-10}	3.3×10^{-9}
MA 59	7.2×10^{-10}	2.6×10^{-9}
MA 60	7.3×10^{-10}	3.5×10^{-9}
MA 61	6.3×10^{-10}	2.4×10^{-9}
MA 62	8.3×10^{-10}	4.0×10^{-9}
MA 63	6.8×10^{-10}	2.8×10^{-9}
MA 64	6.2×10^{-10}	3.2×10^{-9}

Table 10: Genetic linkage of Tn5 with auxotrophic phenotypes of different transposon induced Rhizobium meliloti Rmd201 auxotrophs.

Donor	Relevant donor pheotype	Recipient	Selected Phenotype	% of selected colonies which were Km^r	% of Km^r exconjugants with donor phenotype
MA 30 (pJB3JI)	Proline$^-$	ZB178	Rif^r,Tet^r	98	100
MA 31 (pJB3JI)	Isoleucine$^-$	ZB178	Rif^r,Tet^r	99	100
MA 32 (pJB3JI)	Aspartic acid$^-$	ZB178	Rif^r,Tet^r	74	100
MA 33 (pJB3JI)	Methionine$^-$	ZB178	Rif^r,Tet^r	80	100
MA 34 (pJB3JI)	Tyrosine$^-$	ZB178	Rif^r,Tet^r	95	100
MA 35 (pJB3JI)	Aspartic acid$^-$	ZB178	Rif^r,Tet^r	60	100
MA 36 (pJB3JI)	Proline$^-$	ZB178	Rif^r,Tet^r	80	100
MA 37 (pJB3JI)	Glutamic acid$^-$	ZB178	Rif^r,Tet^r	70	100
MA 38 (pJB3JI)	Leucine$^-$	ZB178	Rif^r,Tet^r	98	100
MA 39 (pJB3JI)	Cysteine$^-$	ZB178	Rif^r,Tet^r	92	100
MA 40 (pJB3JI)	Phenylalanine$^-$	ZB178	Rif^r,Tet^r	70	100
MA 41 (pJB3JI)	Valine$^-$	ZB178	Rif^r,Tet^r	75	100
MA 42 (pJB3JI)	Threonine$^-$	ZB178	Rif^r,Tet^r	79	100
MA 43 (pJB3JI)	Proline$^-$	ZB178	Rif^r,Tet^r	82	100
MA 44 (pJB3JI)	Phenylalanine$^-$	ZB178	Rif^r,Tet^r	89	100
MA 45 (pJB3JI)	Histidine$^-$	ZB178	Rif^r,Tet^r	69	100
MA 46 (pJB3JI)	Threonine$^-$	ZB178	Rif^r,Tet^r	80	100

Abbreviations :

Km^r = Kanamycin resistant
Tet^r = Tetracycline resistant
Rif^r = Rifampicin resistant

resistances and the auxotrophic phenotype was observed in conjugation experiments. This indicates that the auxotrophic phenotype in R. meliloti Rmd201 was Tn5 induced.

4.2.3 Symbiotic Properties

4.2.3.1 Nodule Characteristics

Initiation of nodulation of Medicago sativa seemed to be normal with most of the mutant strains. However, later, clear differences emerged in the morphology of the nodules elicited by these mutants from those of the nodules formed by the wild type. The nodules induced by Rmd201 were cylindrical, 2-3 mm long, and pink in colour. The nodules characteristics of the auxotrophic mutants are listed in Table 11 and Table 12. One of the Tn5 induced mutants, viz., MA 43 (proline auxotroph) could not form any nodule on alfalfa plant. Nine auxotrophs, viz., MA 32, MA 36, MA 38, MA 40, MA 45, MA 55, MA 57, MA 61 and MA 62 formed nodules which were irregular in shape and small in size. The nodules formed by seven auxotrophs, viz., MA 32, MA 36, MA 38, MA 40, MA 45, MA 57 and MA 62 were white in colour. Plants inoculated with MA 61 had more number of nodules. When the revertants were, however, similarly examined, they behaved like the wild type strain Rmd201 (Table 15).

4.2.3.2 Plant Characteristics

The plant height, dry plant weight and nitrogen content per plant were recorded after eight weeks of inoculation.

Plant height : The data from Table 13 and Table 14 show that minimum and maximum plant heights were recorded in auxotrophic mutants MA 57 (5.0 cm) and MA 55 (14.0 cm). The plants inoculated with strain Rmd201 were 8.0 cm tall. The plant height with strains MA 32, MA 36, MA 38, MA 40, MA 45, MA 57 and MA 62 were 5.2, 6.3, 5.5, 6.3, 6.3, 5.0 and 5.0 cm, respectively. The plant height in case of all these seven mutants was significantly less than the plants inoculated with parental strain. The plants inoculated with two mutant strains i.e. MA 55 and MA 61 were significantly taller than the plants with parent strain. The plant height in case of the mutant strains except these nine did not differ significantly with the plant inoculated with parental strain (Fig.7).

Table 11: **Characteristics of nodules induced by the wild type Rhizobium meliloti Rmd201 and its Tn5 induced auxotrophic mutants on Medicago sativa plants.**

Strain	Time of visualization of first nodule in terms of days after inoculation	Average No.*/plant	Shape	Size (mm)	Colour	Location on the root
Rmd201	12-14	7	Cylindrical	2-3	Pink	P.R.,L.R.
MA 30	12-14	7	Cylindrical	2-3	Pink	L.R.
MA 31	10-12	8	Cylindrical	3	Pink	L.R.
MA 32	14-18	5	Irregular	1-2	Pinkish white	L.R.
MA 33	10-12	6	Cylindrical	2-3	Pink	L.R.
MA 34	12-14	5	Cylindrical	2-3	Pink	L.R.
MA 35	12-14	6	Cylindrical	1-2	Pink	L.R.
MA 36	10-12	8	Irregular	0.5 -1	White	L.R.
MA 37	12-14	6	Cylindrical	1-2	Pink	P.R.
MA 38	15-18	8	Irregular	very small	White	P.R.
MA 39	12-14	10	Cylindrical	1-2	Pink	P.R.,L.R.
MA 40	14-18	4	Irregular	1-2	Pinkish white	P.R.,L.R.
MA 41	10-12	8	Cylindrical	1-2	Pink	P.R.
MA 42	12-14	6	Cylindrical	2-3	Pink	P.R.,L.R.
MA 43	–	–	–	–	–	–
MA 44	10-12	8	Cylindrical	2	Pink	P.R.,L.R.
MA 45	12-14	5	Irregular	1-2	Pinkish white	L.R.
MA 46	12-14	6	Cylindrical	2-3	Pink	L.R.

* nodule number is average of eight plants.

Abbrevations:

P.R. = Primary root, L.R. = Lateral root

Table 12: Characteristics of nodules induced by the wild type Rhizobium meliloti Rmd201 and its NTG induced auxotrophic mutants on Medicago sativa plants.

Strain	Time of visualization of first nodule in terms of days after inoculation	Average No.* / plant	Shape	Size (mm)	Colour	Location on the root
Rmd201	12-14	7	Cylindrical	2-3	Pink	P.R.,L.R.
MA 51	10-12	8	Cylindrical	2-3	Pink	P.R.
MA 52	10-12	7	Cylindrical	2-3	Pink	P.R.
MA 53	12-14	7	Cylindrical	1-2	Pink	L.R.
MA 54	10-12	8	Cylindrical	2-3	Pink	P.R.
MA 55	8-10	9	Irregular	2-3	Pink	P.R.,L.R.
MA 56	12-14	7	Cylindrical	2-0	Pink	P.R.
MA 57	14-16	6	Irregular	1-2	Pinkish white	L.R.
MA 58	10-12	9	Cylindrical	0.5- 1	Pink	P.R.,L.R.
MA 59	12-14	7	Cylindrical	2-3	Pink	P.R.
MA 60	12-14	6	Cylindrical	2-3	Pink	P.R.,L.R.
MA 61	8-10	13	Irregular	1-2	Pink	P.R.,L.R.
MA 62	15-17	7	Irregular	1-2	White	P.R.
MA 63	10-12	9	Cylindrical	2-3	Pink	P.R.
MA 64	12-14	7	Cylindrical	1-2	Pink	L.R.

* nodule number is average of eight plants.

Abbrevations:

P.R. = Primary root
L.R. = Lateral root

Table 13 : Characteristics of Medicago sativa plants inoculated with the wild type Rhizobium meliloti Rmd201 and its Tn5 induced auxotrophic mutants.

Strain	Plant height* (cm)	Dry plant weight* (mg)	Percent nitrogen* content per plant (Microkjeldhal)	Total nitrogen* content per plant (mg)
Control (uninoculated)	4.0	8.0	1.69	0.1352
Rmd201	8.0	13.4	2.23	0.2988
MA 30	8.4	11.5	2.19	0.2516
MA 31	8.0	11.4	2.29	0.2610
MA 32	5.2	8.8	1.8	0.1584
MA 33	8.3	11.8	2.3	0.2714
MA 34	8.9	12.2	2.18	0.2660
MA 35	7.5	11.8	2.21	0.2608
MA 36	6.3	9.0	1.9	0.1710
MA 37	7.5	13.1	2.19	0.2869
MA 38	5.5	8.2	1.82	0.1492
MA 39	8.3	12.8	2.29	0.2931
MA 40	6.3	9.0	1.78	0.1602
MA 41	8.2	11.9	2.2	0.2618
MA 42	8.4	11.9	1.98	0.2356
MA 43	6.0	8.0	1.7	0.1360
MA 44	8.4	13.0	2.25	0.2925
MA 45	6.3	9.5	1.72	0.1634
MA 46	9.0	12.5	2.2	0.2750
C.D. at 5% level	1.6317	2.19	0.2649	0.0699

* Average of eight plants.

Table 14 : Characteristics of *Medicago sativa* plants inoculated with the wild type *Rhizobium meliloti* Rmd201 and its NTG induced auxotrophic mutants.

Strain	Plant height* (cm)	Dry plant weight* (mg)	Percent nitrogen* content per plant (Microkjeldhal)	Total nitrogen* content per plant (mg)
Control (uninoculated)	4.0	8.0	1.69	0.1352
Rmd201	8.0	13.4	2.23	0.2988
MA 51	7.8	11.9	2.19	0.2606
MA 52	8.4	12.9	2.1	0.2709
MA 53	8.2	13.2	2.16	0.2851
MA 54	7.9	13.0	2.2	0.2860
MA 55	14.0	16.2	2.79	0.4520
MA 56	7.5	12.6	2.29	0.2885
MA 57	5.0	8.8	1.75	0.1540
MA 58	7.8	13.4	2.2	0.2948
MA 59	8.8	11.5	2.2	0.2530
MA 60	8.2	13.8	2.18	0.3008
MA 61	13.1	16.2	2.96	0.4795
MA 62	5.2	9.0	1.7	0.1530
MA 63	7.6	12.8	2.1	0.2688
MA 64	8.1	11.2	2.0	0.2240
C.D. at 5% level	2.8977	2.6549	0.38	0.1072

* Average of eight plants.

Table 15 : Symbiotic properties of the wild type Rhizobium meliloti Rmd201 and amino acid auxotrophic revertants.

Strain	Time of visualization of first nodule in term of days after inoculation	Nodule Characteristics				Plant height* (cm)	Dry plant weight* (mg)	Percent nitrogen* content per plant	Total nitrogen* per plant (mg)
		Average No*/ plant	Shape	size (mm)	Colour				
Rmd201	12-14	7	Cylindrical	2-3	Pink	8.0	13.4	2.23	0.2988
MA 32r	12-15	6	Cylindrical	2-3	Pink	7.5	13.0	2.26	0.2938
MA 36r	12-14	8	Cylindrical	1-2	Pink	8.1	13.1	2.27	0.2973
MA 38r	12-15	7	Cylindrical	1-2	Pink	7.9	13.0	2.19	0.2847
MA 40r	14-16	6	Cylindrical	2-3	Pink	7.8	13.8	2.19	0.3022
MA 43r	12-14	8	Cylindrical	2-3	Pink	8.0	13.0	2.2	0.2860
MA 45r	12-14	7	Cylindrical	1-2	Pink	7.5	13.2	2.2	0.2904
MA 55r	10-12	7	Cylindrical	2-3	Pink	9.0	13.8	2.26	0.3118
MA 57r	12-14	8	Cylindrical	1-2	Pink	7.9	13.0	2.19	0.2847
MA 61r	10-12	8	Cylindrical	2-3	Pink	9.2	13.8	2.27	0.3132
MA 62r	12-15	7	Cylindrical	2-3	Pink	7.8	13.1	2.19	0.2869

Abbreviations :

r = Revertants

* = Average of eight plants

Fig.7 Alfalfa plants (8weeks old) inoculated with wild type *Rhizobium meliloti* Rmd 201 and auxotrophic mutants.

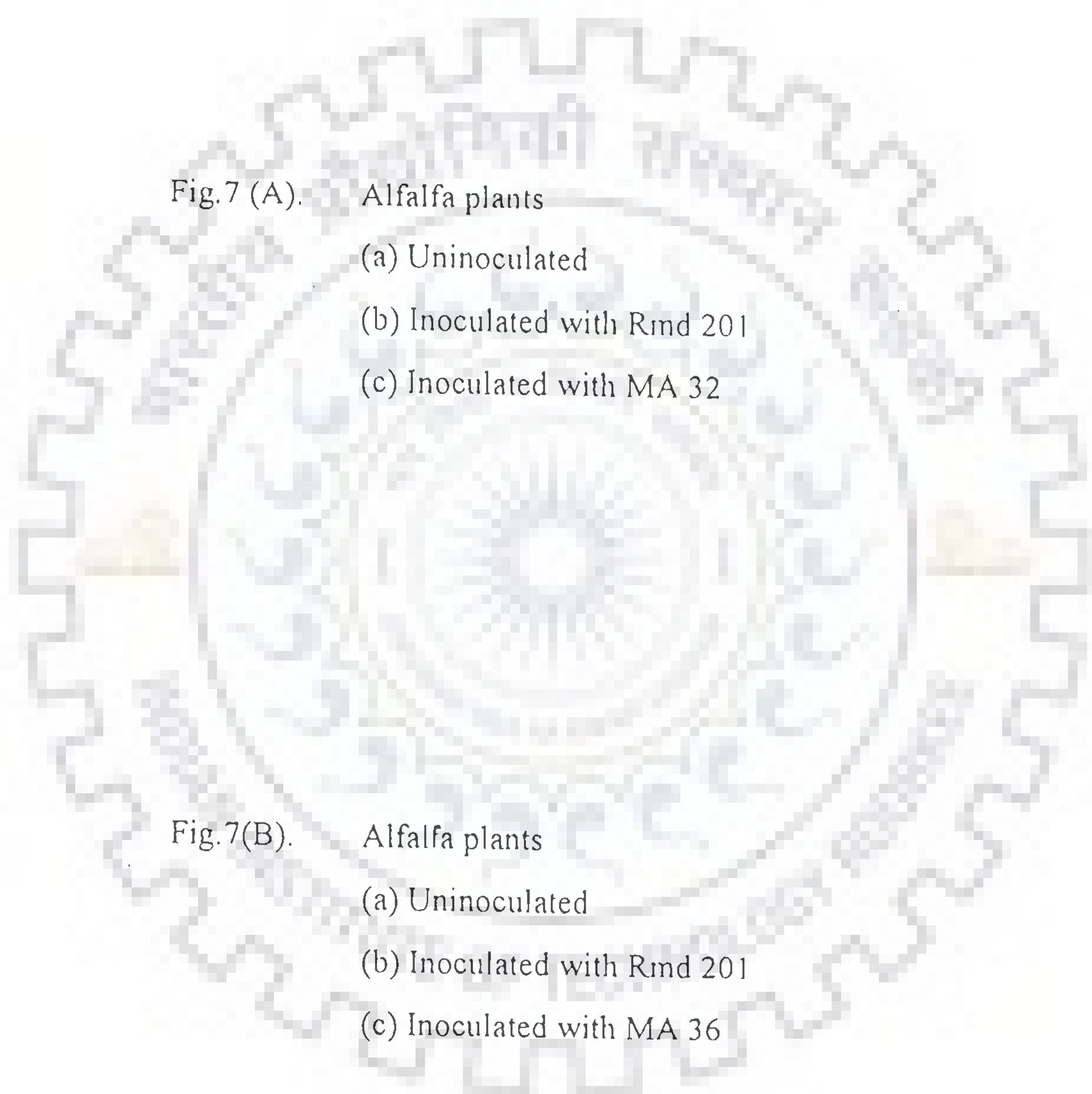


Fig.7 (A). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 32

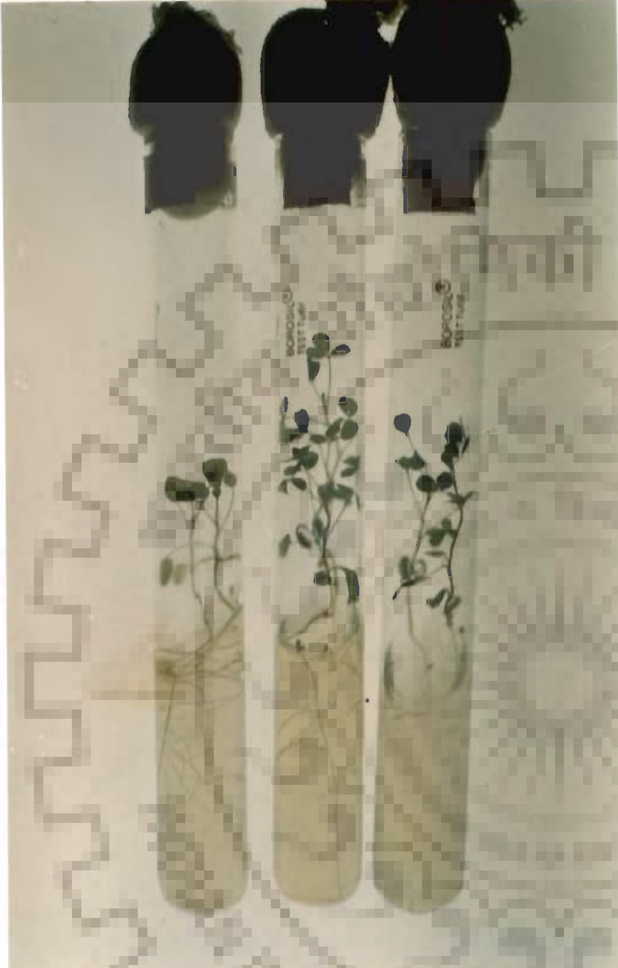
Fig.7(B). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 36

Fig.7(A)



a b c

Fig.7(B)

a b c

Fig.7(C). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 38

Fig.7(D). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 40

Fig.7(C)

a b c

Fig.7(D)

a b c

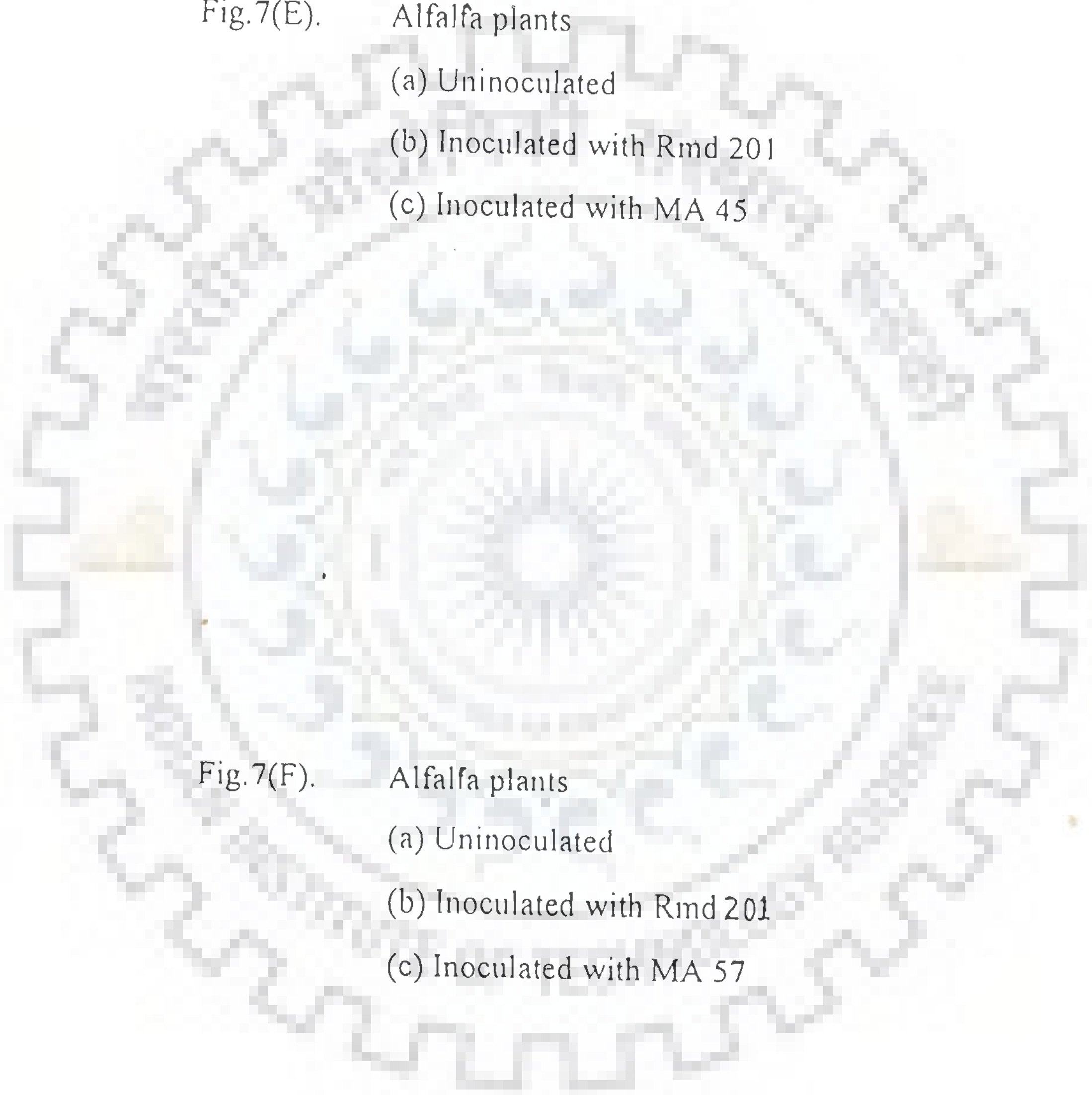


Fig.7(E). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 45

Fig.7(F). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 57

Fig.7(E)

a b c

Fig.7(F)

a b c

Fig.7(G). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 62

Fig.7(G)

a b c

Fig.7(H). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 55

Fig.7(I). Alfalfa plants

(a) Uninoculated

(b) Inoculated with Rmd 201

(c) Inoculated with MA 61

Fig.7(H)

a b c

Fig.7(I)

a b c

Dry plant weight : The data given in Table 13 and 14 indicate that the dry plant weight ranged from 8.2 mg (MA 38) to 16.2 mg (MA 55 and MA 61). In case of parental strain Rmd201, the dry plant weight per plant was 13.4 mg. The plants inoculated with MA 32, MA 36, MA 38, MA 40, MA 45, MA 57 and MA 62 were having significantly less dry plant weight in comparison to the parent strain. Two strains MA 55 (16.2 mg) and MA 61 (16.2 mg) were superior to the parent strain for dry matter production. The differences for dry matter production among all mutants strains except these nine as well as from the parent were nonsignificant.

Nitrogen content per plant : The table 13 & 14 show that the plants inoculated with MA 38 had minimum nitrogen content per plant (0.1492 mg). The maximum nitrogen content recorded was 0.4795 mg in case of MA 61. The plants with parent strain had 0.2988 mg nitrogen content per plant. The nitrogen content in case of MA 32, MA 36, MA 38, MA 40, MA 45, MA 57 and MA 62 strains was significantly less than the parent strain. However, the plants inoculated with MA 55 and MA 61 had significantly more nitrogen content than the parent strain.

Percent nitrogen content of the plant : The nitrogen percent in plants varied from 1.7 in MA 62 to 2.96 in MA 61. The plants with parent strain had 2.23% nitrogen. The plants with strains MA 32, MA 36, MA 38, MA 40, MA 45, MA 57 and MA 62 had significantly less percent nitrogen than the plants with Rmd201 strain. The plants with MA 55 and MA 61 were significantly superior to the plants with parent strain for percent nitrogen.

Thus data with plants indicates that mutants strains, viz., MA 31, MA 36, MA 38, MA 40, MA 45, MA 57 and MA 62 have reduced nitrogen fixing ability than the parent strain Rmd201. The two strains,i.e. MA 55 and MA 61 appear more efficient with respect to their symbiotic ability. When the revertants of these auxotrophic mutants were similarly examined, they behaved like the parent strain Rmd201 (Table 15).

4.2.3.3 Colonization of Nodules

From the nodules, the bacteria were isolated and grown on different media to

determine their identity. Bacteria with nutritional requirements similar to those used for inoculation of the plants were obtained from the nodules. This confirmed that the nodules were colonized by the same strain with which the plant was inoculated.

4.2.3.4 Exogenous Feeding of Amino acids to Plants

Feeding of nutritional requirement of the symbiotically affected auxotrophic mutants, to plant nutrient medium was carried out. As seen from the data of Table 16, there was no change in the symbiotic properties of four of the mutants, viz., MA 32, MA 55, MA 61 and MA 62. The symbiotic defect was restored in all other six auxotrophs, viz., MA 36, MA 38, MA 40, MA 43, MA 45 and MA 47 by the addition of their nutritional requirement to the plant nutrient medium.

4.2.4 Surface Properties of Symbiotically Affected Amino acid Auxotrophs

Surface components such as lipopolysaccharides, β-glucans and extracellular polysaccharides have an important role in the success or failure of symbiosis (41, 86, 144). Experiments were carried out to determine if pleiotropy of these amino acid mutations influenced some of the surface characteristics of R. meliloti cells. This was examined by use of several different chemical dyes.

4.2.4.1 Calcaflour Staining

This is one of the routine methods followed to determine the loss or reduced production of acidic exopolysaccharides from the cell surface. The rhizobia that flouresce under long wavelength UV light when grown on medium containing calcaflour are considered capable of producing acidic exopolysaccharides. All the auxotrophs except MA 36 and MA 40, flouresced as brightly as the parent strain Rmd201 (Table 17). This implied that the production of acidic exopolysaccharide of strains MA 36 and MA 40 is aberrant, while, in the other strains, the exopolysaccharides synthesis has not been affected by the amino acid auxotrophies.

4.2.4.2 Reaction With Aniline Blue

This dye has been reported to specifically bind to curdlan type of polysaccharides, of which beta (1-3) glucan is a major component (166). Since the parent strain Rmd201

Table 16 : Effect of nutritional supplementation of plant growth medium on symbiotic efficiency of amino acid auxotrophs.

Strain	Nutritional status in the plant growth medium	Nodule Characteristics				Plant height* (cm)	Dry plant weight* (mg)	Percent nitrogen* content per plant	Total nitrogen* per plant (mg)
		Average No.* /plant	Shape	size (mm)	Colour				
Control	Minimal	-	-	-	-	4.0	8.0	1.69	0.1352
Rmd201	Minimal	7	Cylindrical	2-3	Pink	8.0	13.4	2.23	0.2988
MA 32	Aspartic acid	5	Irregular	1-2	Pinkish white	5.1	8.9	1.84	0.1638
MA 36	Proline	7	Cylindrical	2-3	Pink	7.5	12.2	2.19	0.2672
MA 38	Leucine	8	Cylindrical	1-2	Pink	8.2	13.0	2.3	0.2990
MA 40	Phenylalanine	6	Cylindrical	2-3	Pink	7.8	13.5	2.2	0.2970
MA 43	Proline	6	Cylindrical	1-2	Pink	8.3	12.8	2.25	0.2880
MA 45	Histidine	7	Cylindrical	2-3	Pink	7.9	13.2	2.1	0.2770
MA 55	Lysine	9	Irregular	2-3	Pink	14.0	15.7	2.45	0.4318
MA 57	Valine	7	Cylindrical	2-3	Pink	8.1	12.9	2.29	0.2954
MA 61	Tryptophan	7	Cylindrical	2-3	Pink	13.0	16.2	2.94	0.4762
MA 62	Threonine	7	Irregular	1-2	White	5.0	9.1	1.70	0.1547

* = Average of eight plants.

Table 17: Exopolysaccharide production characteristics of the wild type *Rhizobium meliloti* Rmd201, its symbiotically affected auxotrophic strains.

Rhizobium strain	Exo phenotype
Rmd201 (wild type)	Exo^{+}
MA 32	Exo^{+}
MA 36	Exo^{-}
MA 38	Exo^{+}
MA 40	Exo^{-}
MA 43	Exo^{+}
MA 45	Exo^{+}
MA 55	Exo^{+}
MA 57	Exo^{+}
MA 61	Exo^{+}
MA 62	Exo^{+}

Abbreviations :

Exo^{+} = Exopolysaccharide production

Exo^{-} = No exopolysaccharide production

failed to be stained by aniline blue, there is an absence of beta curdlans on its surface. It was, however, seen that all the auxotrophic mutants except MA 38 and MA 45, could not be stained with aniline blue (Table 18). This indicates that these two auxotrophs i.e. MA 38 and MA 45 have the new capability to produce the beta curdlans. The revertants, however, had lost the staining ability, confirming that the production of beta curdlans by these two auxotrophs is a result of the pleiotropic effect of the mutations.

4.2.4.3 Reaction With Congo Red

The results of the staining of various R. meliloti strains with congo red are presented in Table 18. The parental strain Rmd201 took up the dye and produced red colonies. With the amino acid auxotrophs, it was seen that all the mutants except for MA 38 and MA 45 had the capacity to take up the red colour. The revertants of MA 38 and MA 45 were able to take the stain and formed red colonies.

Long term storage of strains on medium with congo red allows one to differentiate acid producing strains from the non acid producing strains. The medium around the colonies and they themselves will turn deep purple if the strain is acid producing (129). There was no change in the colour of the colonies and the medium around them, when the parental strain and auxotrophic strains were stored on the medium with congo red.

4.2.4.4 Sodium Deoxy Cholate (DOC) Sensitivity of The Mutants

Failure to produce lipopolysaccharides (LPS) by Rhizobium results in inability to form effective nodules (171). Further, LPS confer on Rhizobium resistance to DOC. Therefore, the sensitivity of the auxotrophs and the parent strain to DOC was examined. The parent strain Rmd201 and all the auxotrophs except MA 43 tested were equally tolerant to DOC (Table 19). This indicates that LPS proudction has not been impaired by auxotrophy except in MA 43. Revertants of MA 43 were equally resistant to DOC showing the pleiotropic effect of the proline mutation.

4.2.4.5 Motility of The Mutants

Lack of flagella can be a reason for failure of chemotactic response of rhizobia and finally for ineffective nodules. Absence of beta (1-2) glucan has been linked with the

Table 18: Staining characteristics of the wild type *R. meliloti* Rmd201, its symbiotically affected auxotrophic strains with aniline blue and congo red dye.

Rhizobium strain	Aniline blue	Congo red
Rmd201	-	+
MA 32	-	+
MA 36	-	+
MA 38	+	-
MA 40	-	+
MA 43	-	+
MA 45	+	-
MA 55	-	+
MA 57	-	+
MA 61	-	+
MA 62	-	+

Abbreviations :

+ = Stained

- = Not stained

Table 19: Sodium deoxy cholate (DOC) sensitivity and motility characteristics of the **Rhizobium meliloti** Rmd201 and its symbiotically affected auxotrophic strains.

Rhizobium Strain	DOC Sensitive/Resistant	Motility
Rmd201	Resistant	Motile
MA 32	Resistant	Motile
MA 36	Resistant	Motile
MA 38	Resistant	Motile
MA 40	Resistant	Motile
MA 43	Sensitive	Motile
MA 45	Resistant	Motile
MA 55	Resistant	Motile
MA 57	Resistant	Motile
MA 61	Resistant	Motile
MA 62	Resistant	Motile

lack of motility (86). Therefore, the motility of the auxotrophs was compared with that of the wild type (Table 19). The colony size after two days of growth on swarm plates was examined and all the mutants were found to be motile. This also implies that the beta (1-2) glucan production is unhindered by the auxotrophy.

4.2.5 Sugar Utilization

All the auxotrophs were tested for their ability to utilize various sugars. For this purpose, mutants and the wild type were spot tested for growth on Rhizobium minimal medium containing different carbon sources, one at a time, such as : glucose, arabinose, lactose, galactose, maltose, xylose, mannose, mannitol, sucrose and glycerol (Table 20 & 21). Arabinose could not be utilized by MA 34, MA 53 and MA 58 strains. MA 41 and MA 57 could not grow on xylose. Lactose, galactose and maltose could not be utilized as a sole carbon source by MA 63, MA 37 and MA 31 strains, respectively. The rest of the mutants were able to utilize the various carbon sources like the wild type. The revertants were able to utilize different sugars as the wild type strain.

4.2.6 Response to High Osmolarity

The mutants were tested for their ability to grow on TY medium supplemented with 0.4M, 0.5M, 0.8M and 1.0M NaCl (Table 22). The wild type was found to grow on TY medium containing upto 0.5M NaCl but not on medium containing 0.8M NaCl. MA 31 and MA 38 gave an osmosensitive phenotype since they did not grow on TY + 0.5M NaCl. The revertants of these two strains behaved like the parent strain.

4.2.7 Response to High Temperatures

The wild type was observed to grow on TY medium at 28^0 C and 37^0 C. However, it did not grow at/or above 42^0 C. Among the auxotrophic mutants MA 34 was unable to form colonies at 37^0 C, but grew well at 28^0 C. None of the mutants was resistant to high temperature (42^0 C).

4.2.8 Growth Properties

The generation time of the different auxotrophs isolated from R. meliloti Rmd201 in complete medium is presented in Table 23. The results show that the generation time

Table 20: Sugar utilization properties of the wild type Rhizobium meliloti Rmd201 and its Tn5 induced amino acid auxotrophs.

Designation of Rhizobium strain	Rhizobium minimal medium (RMM) contianing sole carbon sources as									
	Glucose	Arabinose	Lactose	Galactose	Maltose	Xylose	Mannitol	Mannose	Sucrose	Glycerol
Rmd201	+	+	+	+	+	+	+	+	+	+
MA 30	+	+	+	+	+	+	+	+	+	+
MA 31	+	+	+	+	-	+	+	+	+	+
MA 32	+	+	+	+	+	+	+	+	+	+
MA 33	+	+	+	+	+	+	+	+	+	+
MA 34	+	-	+	+	+	+	+	+	+	+
MA 35	+	+	+	+	+	+	+	+	+	+
MA 36	+	+	+	+	+	+	+	+	+	+
MA 37	+	+	+	-	+	+	+	+	+	+
MA 38	+	+	+	+	+	+	+	+	+	+
MA 39	+	+	+	+	+	+	+	+	+	+
MA 40	+	+	+	+	+	+	+	+	+	+
MA 41	+	+	+	+	+	-	+	+	+	+
MA 42	+	+	+	+	+	+	+	+	+	+
MA 43	+	+	+	+	+	+	+	+	+	+
MA 44	+	+	+	+	+	+	+	+	+	+
MA 45	+	+	+	+	+	+	+	+	+	+
MA 46	+	+	+	+	+	+	+	+	+	+

Abbreviations :

+ = Growth

- = No growth

Table 21: Sugar utilization properties of the wild type Rhizobium meliloti Rmd201 and its nitrosoguanidine induced auxotrophic mutants.

Designation of Rhizobium strain	Rhizobium minimal medium (RMM) contianing sole carbon sources as									
	Glucose	Arabinose	Lactose	Galactose	Maltose	Xylose	Mannitol	Mannose	Sucrose	Glycerol
Rmd201	+	+	+	+	+	+	+	+	+	+
MA 51	+	+	+	+	+	+	+	+	+	+
MA 52	+	+	+	+	+	+	+	+	+	+
MA 53	+	-	+	+	+	+	+	+	+	+
MA 54	+	+	+	+	+	+	+	+	+	+
MA 55	+	+	+	+	+	+	+	+	+	+
MA 56	+	+	+	+	+	+	+	+	+	+
MA 57	+	+	+	+	+	-	+	+	+	+
MA 58	+	-	+	+	+	+	+	+	+	+
MA 59	+	+	+	+	+	+	+	+	+	+
MA 60	+	+	+	+	+	+	+	+	+	+
MA 61	+	+	+	+	+	+	+	+	+	+
MA 62	+	+	+	+	+	+	+	+	+	+
MA 63	+	+	-	+	+	+	+	+	+	+
MA 64	+	+	+	+	+	+	+	+	+	+

Abbreviations :

+ = Growth

- = No growth

Table 22: Growth properties of the wild type R. melilotiRmd201 and its auxotrophic mutants at different temperatures on/and TY media of different osmolarities.

Strain(s) able to grow on TY +0.5M NaCl but not on TY +0.8M NaCl	Strain(s) unable to grow on TY +0.5M NaCl but grow on TY +0.4M NaCl	Strain(s) able to grow at 28°C and 37°C but unable to grow at 42°C	Strain(s) unable to grow at 37°C and 42°C but able to grow at 28°C
Rmd201 MA 30 MA 32 to MA 37 MA 39 to MA 46 MA 51 to MA 64	MA 31 MA 38	Rmd201 MA 30 to MA 33 MA 35 to MA 46 MA 51 to MA 64	MA 34

Table 23: Growth of parent Rmd201 and its auxotrophic mutants on MSY medium.

Strain	Generation time* (minutes)
Rmd201	160
MA 30	150
MA 31	155
MA 32	152
MA 33	155
MA 34	160
MA 35	155
MA 36	155
MA 37	160
MA 38	150
MA 39	155
MA 40	160
MA 41	165
MA 42	155
MA 43	155
MA 44	160
MA 45	165
MA 46	165
MA 51	150
MA 52	155
MA 53	150
MA 54	160
MA 55	160
MA 56	165
MA 57	165
MA 58	170
MA 59	170
MA 60	160
MA 61	155
MA 62	155
MA 63	150
MA 64	160
C.D. at 5% level	19.7869

* Mean of three replicates

of auxotrophs did not differ significantly from that of the parental strain i.e. Rmd201.

4.2.9 SDS-PAGE Electrophoresis

The soluble proteins of whole cell extracts from the parent strain Rmd201 and the auxotrophic mutants were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to see if there was any visible change in their protein pattern. The figure 8 shows that there is no marked difference in protein pattern of auxotrophs and the parent strain Rmd201.

4.3 GENETIC ANALYSIS OF AUXOTROPHIC MUTANTS

4.3.1 Complementation Analysis

4.3.1.1 Screening of Clones From The Genomic Library

The R. meliloti strain AK631 which was the parent of Rmd201 and the R. meliloti strain 102F34 have been utilized extensively in several laboratories in the molecular genetic studies of the symbiotic properties of Rhizobium. The genetic make ups of these strains appear similar in respect of the symbiotic genes as well as the chromosomal maps. Therefore, it was decided to use the gene bank of 102F34 strain to complement the auxotrophic mutants from Rmd201.

The pRK290 gene bank derived from the BglII digest of the strain 102F34 and maintained in E. coli strain HB101 was mass conjugated to the rhizobial auxotrophic mutants individually in triparental matings. HB101 (pRK2013) was used as a source of helper plasmid. The auxotrophs which received the appropriate wild type clone were selected on RMM having tetracycline. For the five auxotrophic mutants, viz., MA 39, MA 42, MA 51, MA 52 and MA 64, no exconjugants with complementing clones were obtained.

Attempts were made to transfer these wild type clones back to E. coli by mating the rhizobial cells having these complementing clones with HB101 as the recepient. The strain pRK2013 was used as the helper. This however, did not succeed.

Confirmatory proof that these clones have the complementation ability came

from plasmid curing experiments. The recombinant plasmid pRK290 : aa^+ in each of the complemented auxotrophic strains was cured by introducing pPH1J1, a plasmid incompatible to pRK290 (19). The strains from which the plasmid was cured, behaved like the parent auxotrophs, implying that the recombinant plasmid pRK290:aa^+ were responsible for the wild type phenotype of the auxotrophs, carrying them.

4.3.1.2 Transfer of Plasmid Constructs to Purine Auxotrophs

The plasmid construct pGR1 carries the common nod genes, hsn genes, nif genes and fix genes of the sym plasmid (pSym a), while pGR3 carries all these genes except for the fix genes (11). These two plasmid constructs were transferred to different auxotrophic mutants having altered symbiotic ability in comparison to parental strain Rmd201, to determine whether this symbiotic phenotype was due to a mutation in any of the known fix genes. Both pGR1 and pGR3 were unable to restore the symbiotic ability (Table 24) indicating that the symbiotic genes on nod-nif region present on the megaplasmid were not mutated in these auxotrophic mutants.

4.3.2 Biochemical Nature of The Auxotrophic Mutations

A knowledge of the specific step of the pathway in which the auxotroph is defective can be useful in linking the intermediate(s) not produced, with the pleiotropic phenotype of the mutant. The auxotrophic mutations affect a large number of characters including symbiosis. Determination of the precise block in the biosynthetic pathway therefore can be of great help in explaining pleiotropy. Two approaches were employed to determine the nature of the biochemical defect of mutations.

4.3.2.1 Feeding of Metabolic Intermediates

After an amino acid that restored growth to a particular mutant had been identified, available intermediates in the biosynthetic pathway for that amino acid were tested to further define the nature of the auxotrophy. Results with different available intermediates are presented in the Table 25.

The auxotrophic mutant MA 31 (isoleucine auxotroph) was able to grow when RMM was supplemented with α–ketobutyric acid. But it could not grow when threonine

Fig.8. SDS-polyacrylamide gel elecrophoresis of whole cell extract from Rhizobium meliloti Rmd 201 and its amino acid auxotrophic mutants.

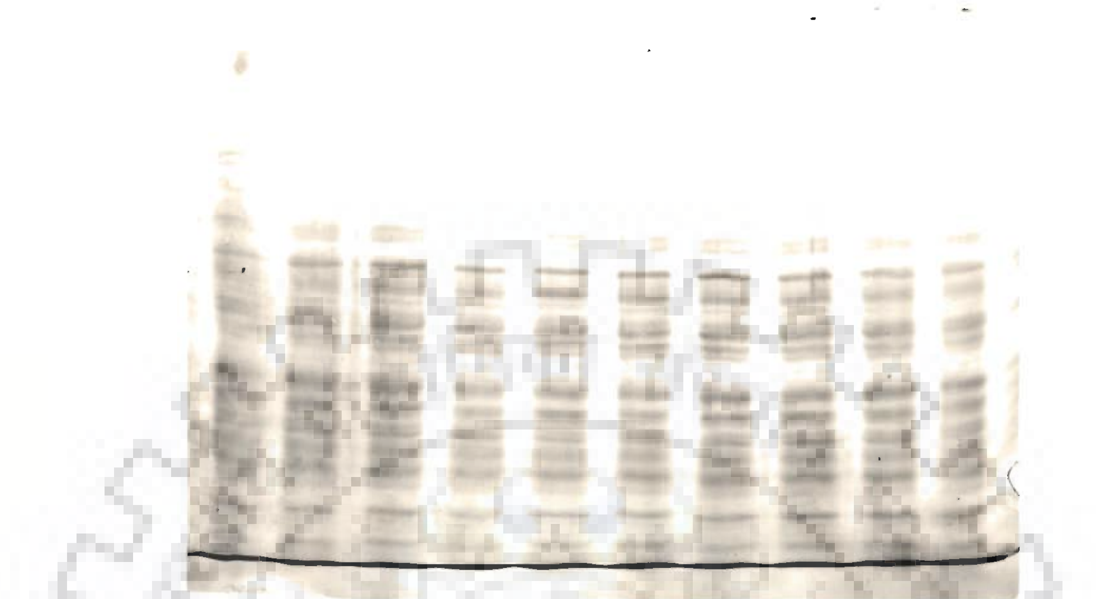
Fig.8(A). Lane

(a) Rmd 201
(b) MA 30
(c) MA 31
(d) MA 32
(e) MA 33
(f) MA 34
(g) MA 35
(h) MA 36
(i) MA 37
(j) MA 38

Fig.8(B). Lane

(a) Rmd 201
(b) MA 39
(c) MA 40
(d) MA 41
(e) MA 42
(f) MA 43
(g) MA 44
(h) MA 45
(i) MA 46
(j) MA 51

Fig.8(A)



Lane a b c d e f g h i j

Fig.8(B)

Lane a b c d e f g h i j

Fig.8(C). Lane

(a) Rmd 201

(b) MA 52

(c) MA 53

(d) MA 54

(e) MA 55

(f) MA 56

(g) MA 57

(h) MA 58

(i) MA 59

(j) MA 60

Fig.8(D). Lane

(a) Rmd 201

(b) MA 61

(c) MA 62

(d) MA 63

(e) MA 64

Fig.8(C)

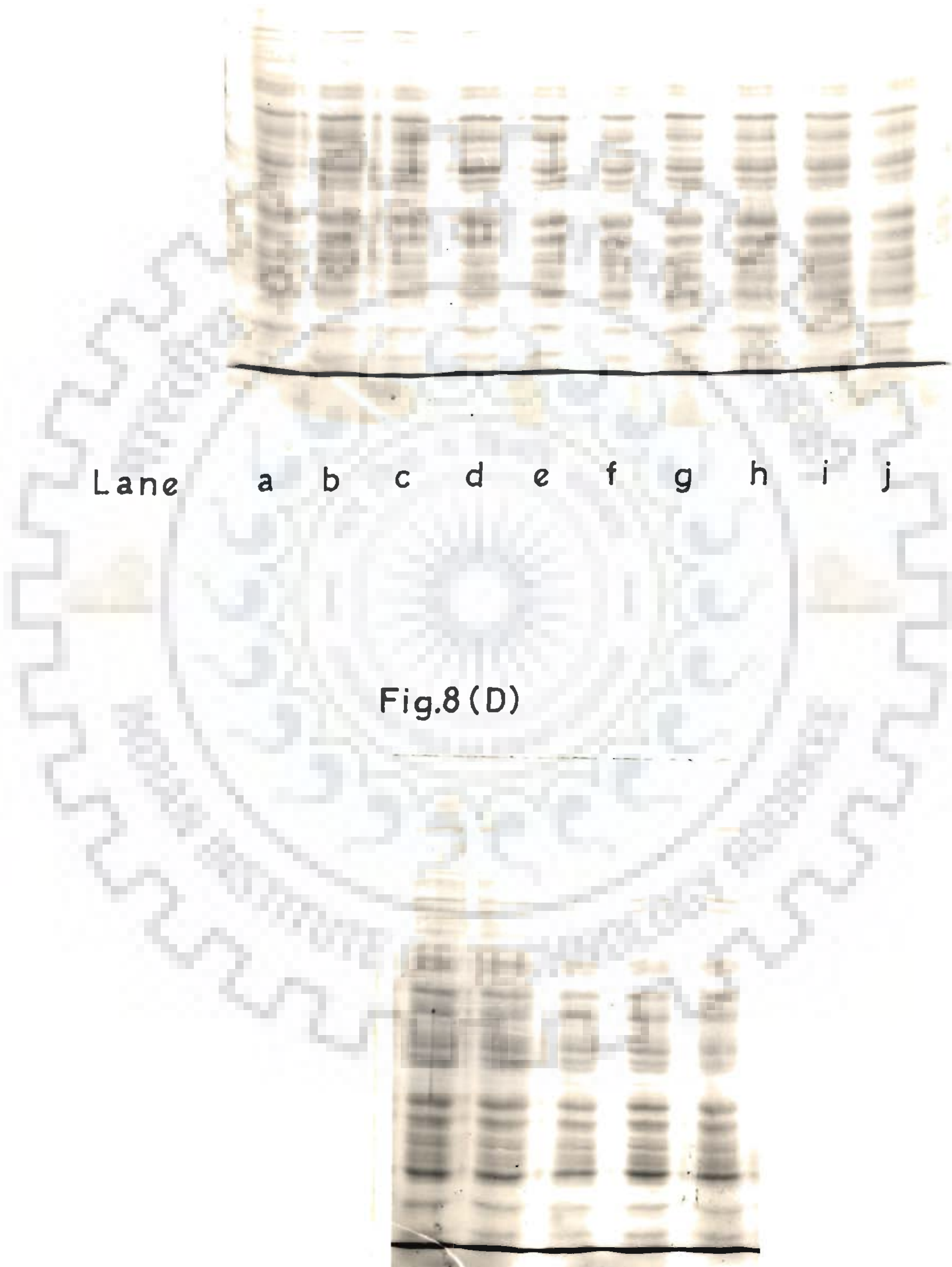


Fig.8(D)

Lane a b c d e

Table 24 : Symbiotic properties of amino acid auxotrophs with R prime pGR1 and pGR3.

Strain	Nodule Characteristics				Plant * height (cm)	Dry plant* weight (mg)	Percent nitrogen* content per plant	Total* nitrogen per plant (mg)
	Average No* / plant	Shape	size (mm)	Colour				
Rmd201	7	Cylindrical	2-3	Pink	8.0	13.4	2.23	0.2988
MA 32 : pGR1	5	Irregular	1-2	Pinkish white	5.0	8.7	1.7	0.1479
MA 32 : pGR3	6	Irregular	1-2	Pinkish white	5.3	8.9	1.82	0.1620
MA 36 : pGR1	8	Irregular	1-2	White	6.5	9.1	1.8	0.1638
MA 36 : pGR3	7	Irregular	1-2	White	6.2	9.2	1.89	0.1738
MA 38 : pGR1	8	Irregular	1-2	White	5.4	8.2	1.79	0.1468
MA 38 : pGR3	6	Irregular	1-2	Pinkish white	5.5	8.4	1.84	0.1546
MA 40 : pGR1	7	Irregular	1-2	Pinkish white	6.0	8.8	1.79	0.1575
MA 40 : pGR3	8	Irregular	1-2	Pinkish white	5.9	9.2	1.7	0.1564
MA 43 : pGR1	-	-	-	-	6.2	8.2	1.7	0.1394
MA 43 : pGR3	-	-	-	-	6.6	8.1	1.69	0.1369
MA 45 : pGR1	7	Irregular	1-2	Pinkish white	6.7	9.4	1.74	0.1636
MA 45 : pGR3	5	Irregular	1-2	Pinkish white	6.4	9.4	1.89	0.1777
MA 55 : pGR1	9	Irregular	2-3	Pink	14.0	15.0	2.79	0.4185
MA 55 : pGR3	9	Irregular	2-3	Pink	13.9	15.7	2.8	0.4396
MA 57 : pGR1	7	Irregular	1-2	Pinkish white	6.5	8.4	1.75	0.1470
MA 57 : pGR3	6	Irregular	1-2	Pinkish white	6.7	8.7	1.74	0.1514
MA 61 : pGR1	12	Irregular	1-2	Pink	13.0	16.0	2.94	0.4704
MA 61 : pGR3	13	Irregular	1 -2	Pink	13.2	16.0	2.95	0.4720
MA 62 : pGR1	7	Irregular	1-2	Pinkish white	5.4	8.9	1.9	0.1691
MA 62 : pGR3	6	Irregular	1-2	Pinkish white	5.2	9.0	1.89	0.1701

* = Average of eight plants.

Table 25: Growth factor requirements of auxotrophic mutants of *Rhizobium meliloti* Rmd201.

Strain	Auxotrophic requirement	Intermediate supplied	
		Growth	No Growth
MA 31	Isoleucine	α– ketobutyric acid	Threonine
MA 34	Tyrosine	Tyrosine	Prephenic acid
MA 39	Cysteine	Cysteine	Cystathionine
MA 38	Leucine	α–ketoisocaproic acid	α–ketoisovaleric acid
MA 45	Histidine	Histidinol, Histidine	Histidinol Phosphate
MA 61	Tryptophan	Tryptophan	Anthranillic acid
MA 33 & MA 64	Methionine	Methionine	Cystathionine
MA 40	Phenylalanine	Phenylpyruvate	Prephenic acid
MA 44	Phenylalanine	Phenylalanine	Phenylpyruvate
MA 41 & MA 57	Valine	Valine	α–ketoisovaleric acid
MA 58	Lysine	2,3- dihydrodipicolinate	Aspartic semialdehyde
MA 55	Lysine	Diaminopimelate	2,3- dihydrodipicolinate
MA 60	Lysine	Lysine	Diaminopimelate
MA 30 & MA 43 & MA 54	Proline	Glutamyl semialdehyde	Glutamate
MA 36 & MA 63	Proline	Proline	Glutamyl semialdehyde
MA 51 & MA 42	Threonine	Aspartyl phosphate	Aspartic acid
MA 56	Threonine	Aspartic semialdehyde	Aspartyl phosphate
MA 46 & MA 52	Threonine	Homoserine	Aspartic semialdehyde
MA 59 & MA 62	Threonine	Threonine	Homoserine

was added to RMM. This is likely to result from a defect in threonine deaminase (Fig.9).

Tyrosine requiring auxotrophic mutant could grow on tyrosine but not on prephenic acid. Thus, this mutant appears to be defective in either of two steps required for conversion of prephenic acid to tyrosine (Fig. 10).

Tryptophan mutant MA 61 could not grow on anthranillic acid indicating probable defect in conversion of anthranillic acid to tryptophan (Fig. 10).

Mutant MA 40 (i.e. phenylalanine auxotroph) could not utilize prephenic acid but could utilize phenylpyruvate. This shows that this is likely to be defective in prephenate dehydratase. The growth of MA 44 could not be restored on supplementation with phenylpyruvte indicating probable defect in phenylpyruvate aminotransferase (Fig.10).

α–ketoisocaproic acid restored the growth of leucine auxotroph MA 38 but α–ketoisovaleric acid did not restore the growth, this mutant is likely to be defective in either of three steps required for conversion of α–ketoisovalerate to α–ketoisocaproic acid (Fig. 11).

Valine auxotrophs MA 41 and MA 57 could not grow on α–ketoisovalerate showing the probable defect in valine aminotransferase (Fig.11).

The mutant MA 45 (histidine auxotroph) grew on minimal medium supplemented with histidine or histidinol but failed to grow when supplied with histidinol phosphate. This indicates that the step mediated by the enzyme histidinol phosphatase may be affected (Fig.12).

The proline mutants could be the result of block(s) in any of the four steps required for conversion of glutamate to proline. Glutamyl semialdehyde restored the growth of only MA 30, MA 43 and MA 54 on RMM. Thus these three mutants may be defective in one of the two steps required for the conversion of glutamate to glutamyl semialdehyde.The growth of MA 36 and MA 63 on only proline supplemented RMM shows that they are likely to be defective in two steps required for the conversion of glutamyl semialdehyde to proline (Fig. 13).

The methionine auxotrophs MA 33 and MA 64 could not grow on cystathionine, showing that the two steps involved in conversion of cystathionine to methionine is likely to be defective (Fig. 9).

Cysteine auxotroph MA 39 also did not grow on cystathionine indicating the probable defect in the step required for conversion of cystathionine to cysteine (Fig.9).

In case of lysine auxotrophs, 2,6 diaminopimelate restored the growth of MA 55 and MA 58 on RMM but the growth of MA 60 was not restored. This shows that MA 60 may be defective in one of the two steps required for the conversion of diamino-pimelate to lysine. Dihydrodipicolinate restored the growth of MA 58 only. This means MA 55 is having defect in either of three steps required for conversion of dihydrodi-picolinate to diaminopimelate. MA 58 could not grow on aspartic semialdehyde and is likely to be defective in dihydropicolinate synthase (Fig.9).

Threonine auxotrophs could be the result of block(s) in any of the five steps involved in conversion of aspartic acid to threonine.Homoserine could restore the growth of all auxotrophs except for MA 59 and MA 62. Thus the defect in MA 59 and MA 62 may be in one of the two steps required for conversion of homoserine to threonine. Aspartic semialdehyde could restore the growth of only MA 42, MA 51, and MA 56 but not of MA 46 and MA 52. The result indicates that MA 46 and MA 52 are having defect likely in homoserine dehydrogenase. MA 52, MA 51 and MA 56 are likely to have a defect in two steps required for conversion of aspartic acid to aspartic semialdehyde (Fig. 9).

4.3.2.2 Cross Feeding Studies

Mutant cross feeding studies have been important for identifying the specific steps of the pathway affected by the mutation. The cross feeding studies with proline auxotrophs indicated that the five proline mutants can be divided into two groups (Table 26). The first group consists of MA 30, MA 43 and MA 54 which appear to be defective in some early steps of the proline biosynthesis as these are crossfed by the other two mutants. The second group consists of MA 36 and MA 63 which cross feed the earlier three belonging to group one and hence, must be having block somewhere in the later steps of the pathway.

Table 26: Cross feeding assays of the proline auxotrophs.

Recipient / Donor	MA 30	MA 36	MA 43	MA 54	MA 63
MA 30	-	-	-	-	-
MA 36	+	-	+	+	-
MA 43	-	-	-	-	-
MA 54	-	-	-	-	-
MA 63	+	-	+	+	-

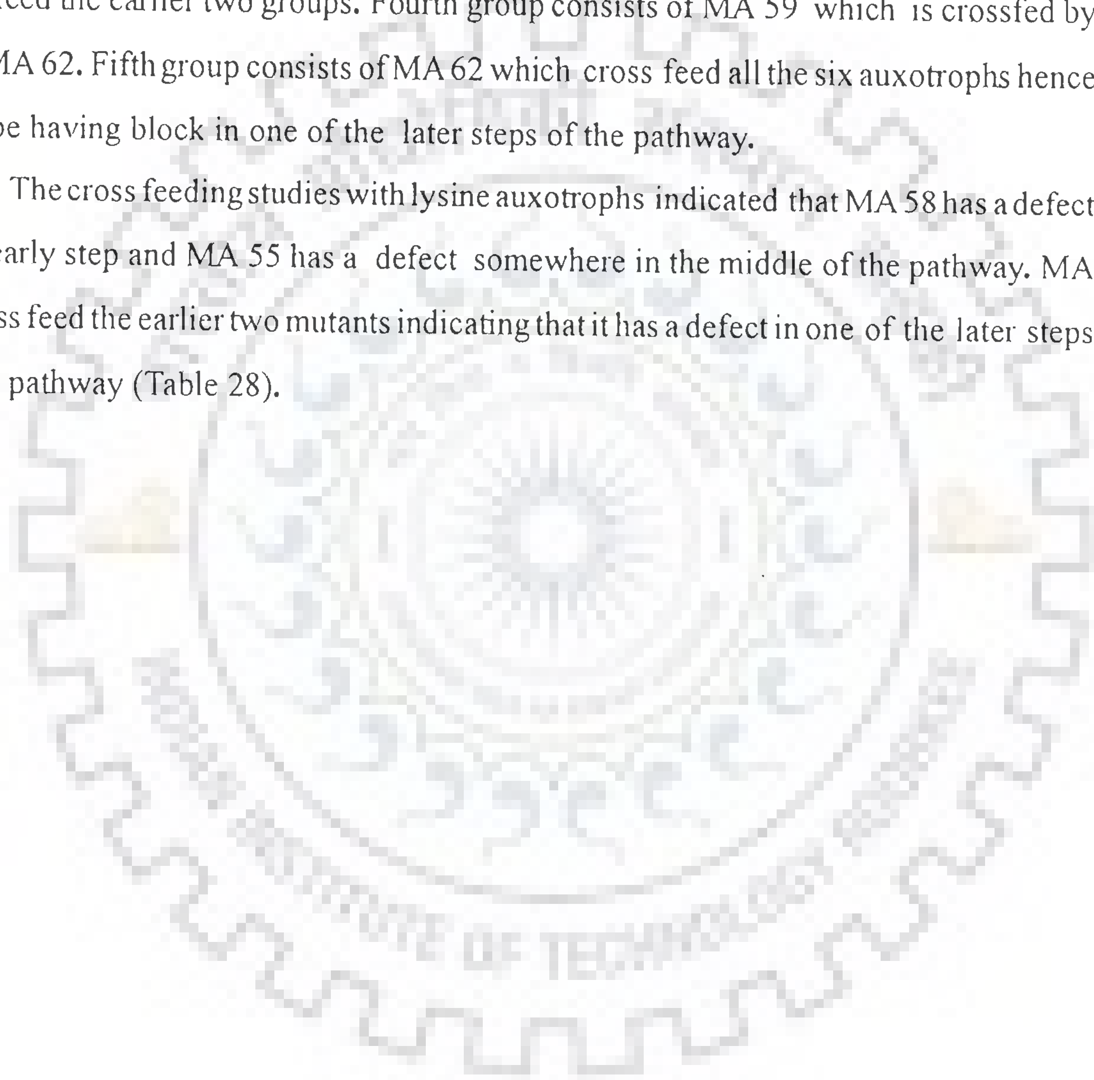
Abbreviations :

+ = Supports growth

- = Does not support growth

The cross feeding studies with threonine auxotrophs indicated that seven threonine auxotrophs can be divided into five groups (Table 27). The first group consists of MA 42 and MA 51 which appear to have a defect in early steps of the pathway. The second group consists of MA 56 which is crossfed by MA 46, MA 52, MA 59 and MA 62 but not by MA 42 and MA 51. The third group consists of MA 46 and MA 52 which cross feed the earlier two groups. Fourth group consists of MA 59 which is crossfed by only MA 62. Fifth group consists of MA 62 which cross feed all the six auxotrophs hence must be having block in one of the later steps of the pathway.

The cross feeding studies with lysine auxotrophs indicated that MA 58 has a defect in an early step and MA 55 has a defect somewhere in the middle of the pathway. MA 60 cross feed the earlier two mutants indicating that it has a defect in one of the later steps of the pathway (Table 28).



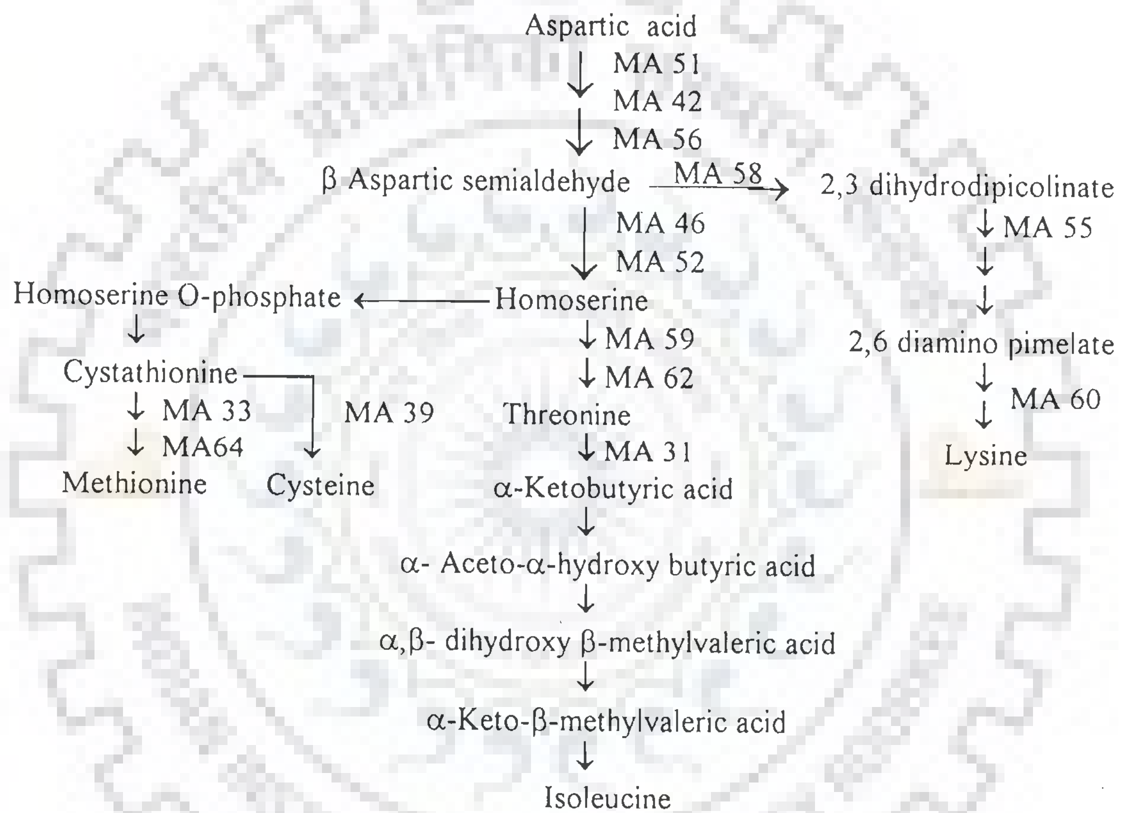


Fig. 9 : Location of mutational defects in threonine, methionine, cysteine, lysine and isoleucine biosynthetic pathways.

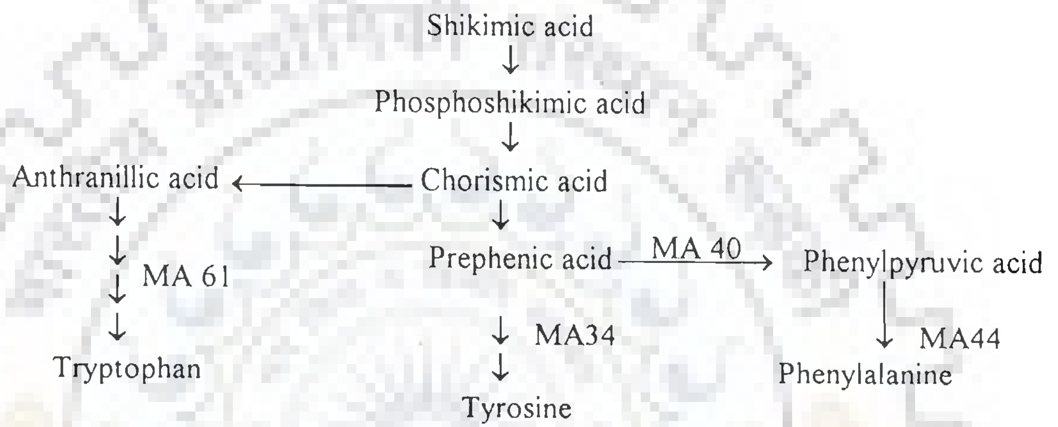


Fig. 10 : Location of mutational defects with tryptophan, tyrosine and phenylalanine pathways.

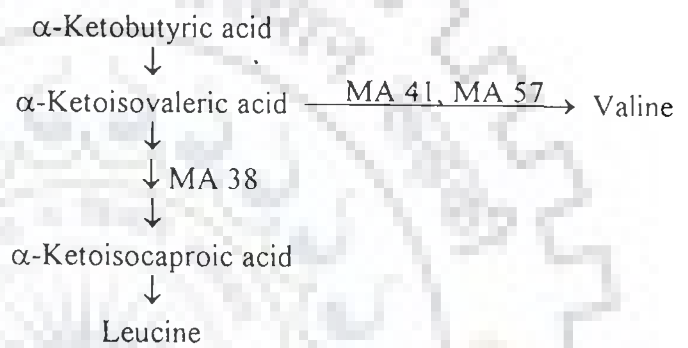


Fig. 11 : Location of mutational defects with leucine and valine pathways.

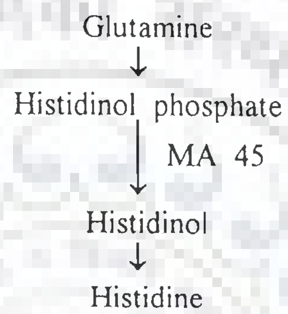


Fig. 12 : Location of mutational defect with histidine pathway.

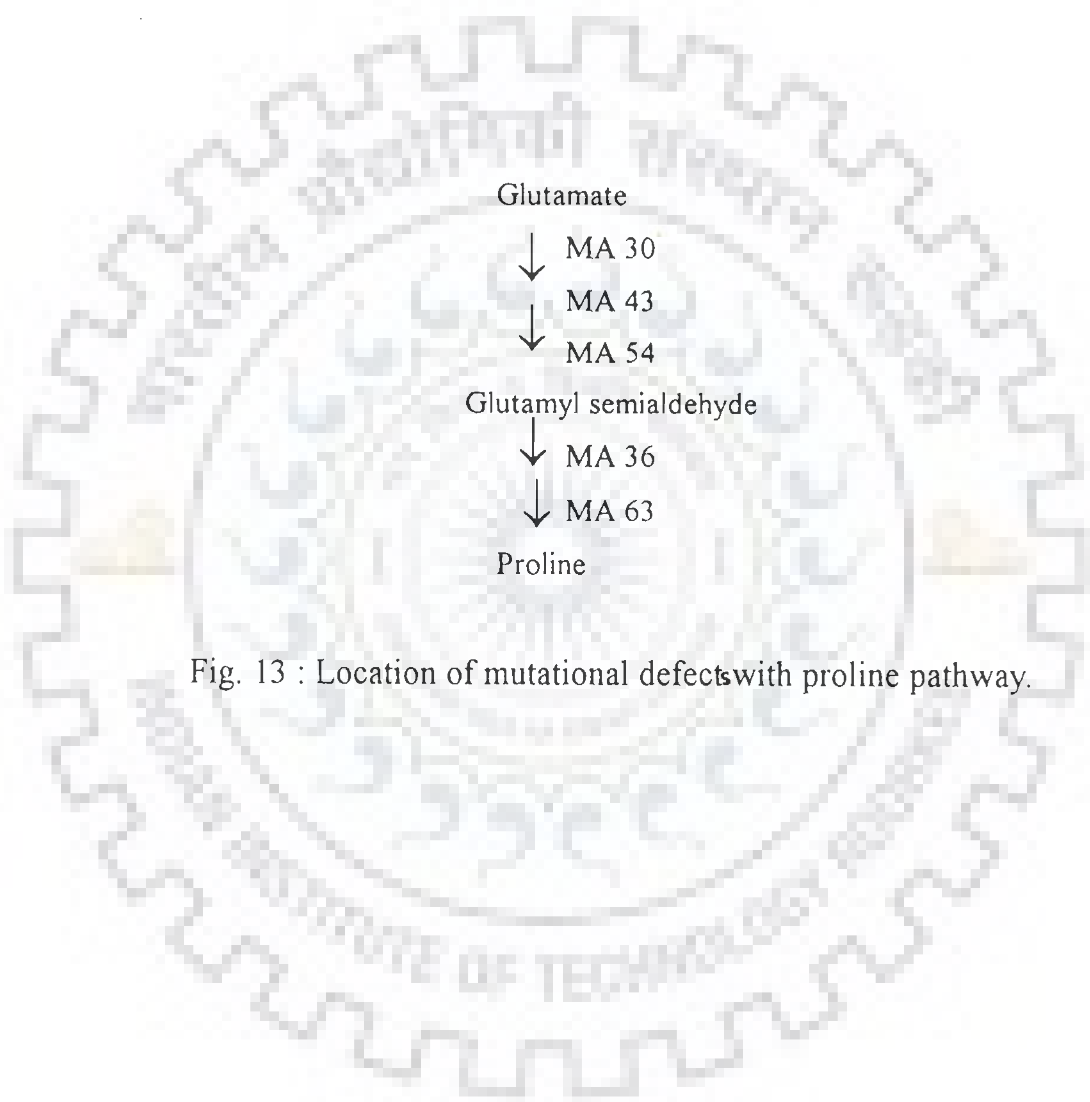


Fig. 13 : Location of mutational defects with proline pathway.

Table 27: Cross feeding assays of the threonine auxotrophs.

Recipient / Donor	MA 42	MA 46	MA 51	MA 52	MA 56	MA 59	MA 62
MA 42	-	-	-	-	-	-	-
MA 46	+	-	+	-	+	-	-
MA 51	-	-	-	-	-	-	-
MA 52	+	-	+	-	+	-	-
MA 56	+	-	+	-	-	-	-
MA 59	+	+	+	+	+	-	-
MA 62	+	+	+	+	+	+	-

Abbreviations :

+ = Supports growth

- = Does not support growth

Table 28: Cross feeding assyas of the lysine auxotrophs.

Recipient / Donor	MA 55	MA 58	MA 60
MA 55	-	+	-
MA 58	-	-	-
MA 60	+	+	-

Abbreviations :

\+ = Supports growth

\- = Does not support growth

DISCUSSION

In this work, thirty one auxotrophic derivatives of R. meliloti Rmd201 were isolated following transposon and nitrosoguanidine mutagenesis. Several experiments were conducted for detailed analysis and characterization of these auxotrophs. The various implications of these experimental results are discussed here.

5.1 ISOLATION OF AUXOTROPHIC MUTANTS

5.1.1 Transposon Induced Auxotrophic Mutants

Transposon Tn5 was chosen because it has been successfully used for generating insertion mutants in several members of Rizobiaceae to study the role of biosynthetic pathways in plant-microbe symbiotic interaction (105,122,158,206). Like in previous works with Agrobacterium and Rhizobium, Tn5 was the insertion element used to generate mutations in R. meliloti.

The optimization of mating time for Tn5 transfer frequency was essential for laying down the foundation for the isolation of Tn5 induced mutants in R. meliloti. A sixteen hour mating time was found suitable to get good frequency of transposon Tn5 derivatives. Insertion mutations due to Tn5 were recovered with a high frequency ($\geq 10^{-4}$). This was presumably because of expression of Tn5 born transposases (Fig.2) required for transposon insertion (201), easy selection of kanamycin resistant cells (258) and stability of insertions (162).

The successful introduction of Tn5 into R. meliloti, the loss of suicide plasmid and subsequent Tn5 insertion into R. meliloti genome were shown by the following points : (i) All Tn5 induced Km^r mutants were chloramphenicol sensitive which indirectly indicates loss of the suicide plasmid, (ii) Km^r auxotrophs were detected at a frequency of $\geq 10^{-4}$ that was many times greater than the rate of spontaneous mutations leading

to auxotrophy, and (iii) Prototrophic revertant could be detected at a frequency of $\geq 10^{-9}$ and were also shown to be Km^s, this result suggests that the Km^s revertants were caused by a precise excision of Tn5 insertion. The similar low frequency of precise excision of Tn5 has also been shown by other workers (158,162). Because mutations could be obtained readily, mutants were developed from a large number of independent matings between R. meliloti and E. coli WA803, donor of suicidal vector pGS9 (Fig. 3) harbouring Tn5. It was expected that mutations among 5000 Tn5 mutants would represent random insertions of Tn5 into R. meliloti genome (16).

When R. meliloti ZB201, a cysteine auxotroph was mutated using transposon insertions, only multiple mutations were obtained. It may be due to cysteine auxotrophy of ZB201. Cysteine may be interfering in some or other way in determining their exact nutritional requirement. In case of R. meliloti Rmd201 seventeen auxotrophic mutants for twelve amino acids, showing randomness of transposon Tn5 insertions were obtained.

5.1.2 NTG Induced Auxotrophic Mutants

Nitrosoguanidine, a potent mutagen was also used to isolate auxotrophic mutants of R. meliloti Rmd201. NTG mutagenised cultures were screened by two different methods and thus 30 auxotrophs could be isolated. Out of these, the nutritional requirement of sixteen auxotrophs could not be specified because these were unable to grow on any of the nutritional supplements tested. These may require more than one supplement or some complex factor found in bactonutrient. The nutrient requirement of fourteen auxotrophs could be specified (Table 7).

5.1.3 Genetic Linkage of Tn5 With Transposon Induced Auxotrophies

To verify that the insertion of Tn5 was indeed the cause of auxotrophic mutations (in case of Tn5 induced mutants), the linkage of the Tn5 conferred kanamycin resistance phenotype to the auxotrophic phenotype was determined (Table 10). The result establishes that the Tn5 inserted and the mutant phenotype are in close genetic linkage and suggests that the Tn5 insertion was the cause of mutation.

5.1.4 Reversion Studies

Reversion studies with amino acid auxotrophs indicated that all auxotrophs reverted spontaneously and in case of NTG induced mutants, their reversion frequencies could be enhanced with NTG treatment. The observations are commensurate with the fact that these mutations are a result of a point mutation.

5.2 METABOLIC BLOCK OF AMINO ACID AUXOTROPHIC MUTANTS

Two approaches were adopted to identify the metabolic blocks in the aminoacid biosynthetic pathways--Intermediate supplementation of auxotrophs and cross feeding of mutants.

5.2.1 Feeding of Metabolic Intermediates

When different intermediates of the amino acid biosynthetic pathway was supplied to auxotrophic mutants, it was found that some intermediates could not restore the growth of the amino acid auxotrophs. The lack of growth on any intermediate may be due to auxotrophic mutant's inability to transport this particular intermediate or the mutant is defective in the step(s) of biosynthetic pathway which is required for that intermediate's utilization. Thus by supplying intermediates, the probable defect in the amino acid biosynthetic pathways could be determined.

5.2.2 Cross Feeding Studies

Studies on the cross feeding of the mutants were done to identify whether the mutants which are requiring same amino acid for their growth are having same metabolic blocks or not. Results of cross feeding experiments show that five proline auxotrophs (viz., MA 30, MA 36, MA 43, MA 54 and MA 63) are having defects in at least two different steps of the pathway. Seven threonine auxotrophic mutants could be divided into five groups on the basis of these results. The cross feeding studies with three lysine auxotrophs indicated that these three mutants (viz., MA 55, MA 58 and MA 60) are having different metabolic blocks in the lysine biosynthetic pathway.

5.3 DNA CLONES COMPLEMENTING AMINO ACID MUTATIONS

To determine the nature of amino acid gene(s) responsible for the observed pleiotropy, clones complementing the different auxotrophs were isolated from the gene bank of R. meliloti. The gene bank, consisting of DNA fragments of an average size of 20 Kb cloned into the BglII site of the plasmid pRK290 was screened. Clones could be isolated except for MA 39, MA 42, MA 51, MA 52 and MA 64. Inspite of several reptitions of the triparental crosses, no clones complementing them could be isolated. A possibility could be that such auxotrophs were antagonistic to the plasmid pRK290. However, since pRK290 plasmid containing excongjugants were recovered in these strains, this does not seem to be so. A second possibility could be that the genomic library is deficient in clones required to complement these mutants. However, this is unlikely, since the library is a partial BglII digest and the possibility that genes of some pathways are not represented is remote. Noel et al. (173), had a similar experience while screening for clones from their library of R. leguminosarum for purine auxotrophy.

In all auxotrophs (except MA 39, MA 42, MA 51, MA 52 and MA 64) carrying clones, it was conclusively shown by plasmid curing that it is the pRK290 with the cloned DNA fragment from R. meliloti genome, which is responsible for the wild type phenotype.

The plasmid cured cells had properties similar to the auxotrophic mutants where as the ones with the clones were in all aspects, like the wild type, Rmd201.

5.4 AMINO ACID AUXOTROPHY AND SYMBIOSIS

When thirty one auxotrophic mutants were tested for their symbiotic ability, ten amino acid auxotrophs were found to be symbiotically affected. Out of these ten amino acid auxotrophs, seven were found to have less symbiotic ability than the parental prototrophic strain of Rhizobium meliloti Rmd201, two were even more efficient in symbiosis than the parent strain and one auxotroph (i.e. MA 43) was unable to nodulate alfalfa plants. Other workers have also reported the affect of some auxotrophic

mutations on symbiotic ability.

Out of two aspartic acid auxotrophs, one mutant MA 32 was found to have less symbiotic ability than the parent strain. When aspartic acid was supplied to plant growth medium, the symbiotic defect could not be restored. Symbiotically defective aspartic acid auxotroph has also been reported by Kim et al. (122), but in this case aspartic acid supplementation could restore the symbiotic defect.

Five proline auxotrophs were found to be varying with respect to their symbiotic ability. One proline mutant MA 36 was having less symbiotic ability than wild type strain and on the other hand MA 43 was defective in nodulation. The symbiotic defect of both the mutants was restored on proline supplementation. Rest of three proline mutants were symbiotically competent.

Leucine auxotroph MA 38 was having altered symbiotic ability and this auxotroph was symbiotically competent when leucine was externally supplied to the plant medium. Several workers (56,119,210,251) have also isolated symbiotically defective leucine auxotrophic mutants. Truchet et al. (251), showed that addition of leucine to the medium around plants infected with this mutant led to the development of effective nodules. But it did not restore effectiveness to leucine auxotrophs isolated by Kerppola and Kahn (119).

One phenylalanine mutant MA 40 out of two phenylalanine auxotrophic mutants and one valine mutant MA 57 out of two valine auxotrophs were having altered symbiotic ability and their symbiotic ability could be restored on supplementation.

One histidine auxotroph MA 45 isolated in this study was having less symbiotic ability than the parent strain Rmd201. Histidine auxotrophs isolated by several workers found to be varying with respect to their symbiotic ability. One histidine auxotroph was found defective in nodulation by Kohli & Vashishat (130). However, in other studies histidine auxotrophs of *R. meliloti* were reported to form symbiotically defective nodules (75,153,154,250). In all these histidine auxotrophs, the defect could be restored on histidine supplementation to medium. Sadowsky et al. (206) isolated four

histidine mutants of Bradyrhizobium japonicum. Two of the histidine mutants were found to be symbiotically defective and did not from nodules. When histidine was supplied externally both the nodulation deficient mutants formed effective symbiosis.

One of the threonine auxotroph MA 62, out of seven threonine was found to be symbiotically less effective than the parent strain. This affect could not be restored on threonine supplementation to plant growth medium.

Methionine (119,173,210),tyrosine (105,119) and cysteine (173) auxotrophs have also been found symbiotically defective by several workers. But auxotrophs requiring these amino acids were found symbiotically competent in this study.

The symbiotic phenotype of all the mutants isolated in this study appear closely related to auxotrophy since reversion to prototrophy restores symbiotic competence. When plant growth medium was supplemented with their auxotrophic requirements, the symbiotic defect of all the eight auxotrophs except for threonine⁻ and aspartic acid⁻ auxotrophs could be restored. This shows that in auxotrophs for proline, leucine, phenylalanine, histidine and valine, probably the need was for particular amino acid for development of symbiosis and this was not available to the bacteria by the host plant in sufficient quantities. In such a situation, since the need could be met with, by providing the required amino acid externally, effective symbiosis could be restored. While in case of aspartic acid and threonine auxotrophs, it appears either externally supplied nutrient is not available to the auxotrophs or particular intermediate or enzyme of these biosynthetic pathway is involved in symbiosis. This involvement of functional ennzyme is supported by the fact that in several species, including bacteria, the polypeptides governing the various steps of biosynthetic pathways are multifunctional (98). It is likely that these polypeptides also catalyze reactions of pathways involved in symbiosis.

The two auxotrophs for lysine and tryptophan respectively, were found to be more efficient than the wild type with respect to their symbiotic ability. The revertants of these auxotrophs were having symbiotic ability equal to the parent stran, showing that the point muntation is responsible for auxotrophy and symbiotic effect. However, in both the

cases, external supplemtation did not affect their symbiotic ability. This indicates that particular intermediate or enzyme involved in these pathways may have negative role in symbiosis.

The difference in symbiotic ability of auxotrophs between the results of this study and previous studies may be due to the step affected by the mutations, or it may be due to variations between bacterial strains and alfalfa cultivars.

In this study, some of the proline, phenylalanine, valine and threonine auxotrophs were symbiotically effective whereas some were defective. This result is similar to those found by Sadowsky et al. (206) and Federov and Zaretskaya (75), in which histidine and methionine auxotrophs could either be effective or ineffective.

These studies with auxotrophs having altered symbiotic ability show a strong relationship between Rhizobium amino acid metabolism and nitrogen fixation. The precise reasons for this observation are far from known. However, there may be several reasons for this ineffectiveness :

1. This effect may be caused by a lack of some metabolites for bacterial metabolism. These defects could not be overcome by a plant supply because of a lack of synthesis of the suitable compounds by the host or because of too low a rate of synthesis, or an insufficient permeability of plant membrane envelop or bacterial membrane to these compounds.
2. Biosynthetic pathway is directly linked to nitrogen fixation.
3. The intermediate formed as a consequence of a particular step of the pathway or the enzyme controlling the particular step is crucial for normal symbiosis.

5.5 CELL SURFACE PROPERTIES OF AMINO ACID AUXOTROPHS

5.5.1 Cell Surface characteristics

5.5.1.1 Exopolysaccharide Production : MA 36 and MA 40 were the only auxotrophs isolated that were unable to produce exopolysaccharides at levels equivalent to the wild type, Rmd201. This could either be due to the pleiotropic nature of the mutation or due

to the polar effect of Tn5 insertion. The revertants of these two auxotrophs were not only able to grow on RMM, but simultaneously had also acquired the Exo^+ phenotype. The frequency of the revertants for the mutants were about 10^{-9} revertants per cell, which is too high to account for the simultaneous reversion of the two independent mutations, thus confirming that the phenotypes of these two mutants are the consequence of the single point mutation.

5.5.1.2 Reaction With Congo Red : Cellulose fibrils are present on the rhizobial cell surface. These are involved in the attachment of rhizobia to the root hairs of legumes (232). These cellulose fibrils present on the rhizobial cell surface can be stained with congo red (129). All the strains except MA 38 and MA 45 could be stained with congo red, confirming the presence of cellulose fibrils (Cel^+). Since the mutants, MA 38 and MA 45 did not take up the stain, they obviously lacked the cellulose fibrils (Cel^-). The revertants were Cel^+ and there were no partial revertant. The Cel^- phenotype, therefore, was the consequence of the same mutation which made these strain auxotrophs for leucine and histidine respectively.

5.5.1.3 Beta (1-3) glucan synthesis : Aniline blue is a dye that specifically binds to beta (1-3) glucans (166). Strain Rmd201 is deficient in beta (1-3) glucans as it fails to take up the dye. Two auxotrophic mutants MA 38 and MA 45 were found to be producing beta (1-3) glucans. The revertants of these auxotrophs had lost the capacity to produce beta (1-3) glucans, as they did not take up the dye. There were no partial revertants with intermediate phenotype. It is therefore, inferred tht pleiotropy of amino acid auxotrophy extends to beta (1-3) glucan production too.

5.1.1.4 Beta (1-2) Glucan and LPS Production : Beta (1-2) glucans constitute a substantial part of the exopolysaccharides and are needed for normal nodule development. These molecules are also part of the flagellum needed for motility of the bacteria. All the auxotrophic mutants were found to be motile as determined from the colony diameter after two days growth on swarm plates.

Lipopolysaccharides (LPS) when present in the cell membrane make the beacteria

resistant to sodium deoxy cholate (DOC). All the auxotrophs except MA 43 were resistant to DOC, thus having normal production of LPS. Revertants of MA 43 were equally resistant to DOC. This indicates that LPS production was effected by proline auxotrophic mutation in this strain.

5.5.2 Reason For Change of The Cell Surface

It was seen that mutation for amino acid requirement had induced a series of changes of the cell surface molecules and there had also been an instance of the synthesis of new molecules, beta (1-3) glucans.

A functional redundancy in rhizobial exopolysaccharides has been earlier hypothesised (88), according to which the rhizobia have the capacity to produce more than one kind of exopolysaccharide. These are capable of replacing each other functionally. Some of these, under a set of conditions, may be normally repressed, while others may be expressed. EPS-III of R. meliloti is one such exopolysaccharide which may be induced by a chromosomal mutation (88).

A situation similar to the one observed by Glazebrook and Walker (88) is seen with congo red and aniline blue staining. It, though, involves cell surface molecules different from the exopolysaccharides, reported earlier. The staining of the rhizobial auxotrophs by these dyes is complementary (Table 16). It is possible, as was the observation of Glazebrook and Walker (88), that when the cellulose fibrils are being produced, the production of beta (1-3) glucans is repressed. Alternatively, the absence of cellulose fibrils removes the repression of beta (1-3) glucans and hence, they are produced.

5.6 PLEIOTROPY OF AMINO ACID AUXOTROPHIC MUTANTS

Most of the auxotrophs isolated in this study had pleiotropic phenotype (Table 29). The pleiotropic effect of leucine and thiamine auxotrophs has also been reported by Sathyanarayana (208). Revertants of all these auxotrophs behaved like the wild type strain indicating that the single point mutation was responsible for the pleiotropic phenotype. All the different phenotypes can only be resulted from a point mutation in

one gene, if the concerned gene was regulatory in nature. Thus the product of that gene is interfering in one or other way with a large number of other functions. In case of transposon induced auxotrophic mutants, pleiotropy may be due to the polar nature of the insertion mutations. This latter explanation for transposon induced mutants envisages that in a particular auxotroph the mutated gene for amino acid biosynthesis and the genes of other altered characters were part of one and the same operon and in that operon the amino acid gene was promoter proximal.

Table 29 : Pleiotropy of amino acid auxotrophic mutants.

Strain	Phenotype
MA 31	Isoleucine$^-$, Maltose$^-$, Osmosensitive.
MA 32	Aspartic acid$^-$, Symbiotically less effective* .
MA 34	Tyrosine$^-$, Arabinose$^-$, Tempertaure sensitive.
MA 36	Proline$^-$, Symbiotically less effctive* , Exo$^-$.
MA 37	Glutamic acid$^-$, Galactose$^-$.
MA 38	Leucine$^-$, Osmosensitive, Symbiotically less effective* , ~~Exo$^-$~~ , Cel$^-$, β(1-3) glucan$^+$.
MA 40	Phenylalanine$^-$, Symbiotically less effective*, Exo$^-$
MA 41	Valine$^-$, Xylose$^-$.
MA 43	Proline$^-$, Nod$^-$, LPS$^-$.
MA 45	Histidine$^-$, Symbiotically less effective*, Cel$^-$, β(1-3) glucan$^+$.
MA 53	Asparagine$^-$ & Tyrosine$^-$, Arabinose$^-$.
MA 55	Lysine$^-$, Symbiotically more efficient* .
MA 57	Valine$^-$, Xylose$^-$, Symbiotically less effective*.
MA 58	Lysine$^-$, Arabinose$^-$.
MA 61	Tryptophan$^-$, Symbiotically more effcient* .
MA 62	Threonine$^-$, Symbiotically less effective* .
MA 63	Proline$^-$,Lactose$^-$.

ABBREVIATIONS :

Cel = Cellulose fibrils

Exo = Exopolysaccharides

LPS = Lipopolysaccharides

* = in comparison to wild type strain Rmd201.

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