GENETICS OF EXOPOLYSACCHARIDE SYNTHESIS IN *RHIZOBIUM* SPECIES STRAIN TAL1145 THAT NODULATES TREE LEGUMES

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DEDICATED TO MY PARENTS AND SIBLINGS

Whose continuous support and encouragement has brought me to this stage

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ABSTRACT

Rhizobia form nitrogen-fixing nodules on leguminous plants. Development of the nodules and nitrogen-fixing bacteroids is a complex process. The polysaccharides of rhizobia, especially exopolysaccharides (EPS) and lipopolysaccharides (LPS), have been shown to be involved in the symbiotic interactions with various legumes. This study involves the identification and characterization of the genes essential for LPS and EPS synthesis in *Rhizobium* sp. strain TAL1145 and the determination of the role of EPS and LPS in symbiosis with several legume hosts.

A Tn5*lacZ*-insertion mutant, NP84, of the strain TAL1145 defective in EPS and LPS synthesis was characterized. NP84 formed pseudonodules on *Phaseolus vulgaris* and *Gliricidia sepium*. On *Leucaena leucocephala* it formed nodules which appeared Fix^- at four weeks but Fix^+ at eight weeks of inoculation. Microscopic examination of the sections of the *Leucaena* nodules formed by NP84 showed fewer infected cells than those formed by TAL1145. A 3.8-kb DNA fragment of TAL1145 was identified and cloned which complemented NP84 for EPS, LPS and symbiotic defects. Sequencing of this fragment showed the presence of two genes involved in EPS and LPS synthesis. A novel gene involved in polymerization / transport of LPS and EPS was also identified.

Complementation of three spontaneous EPS-defective mutants using a genomic library of TAL1145 resulted in the isolation of five overlapping cosmid clones. Fifty seven EPS-defective mutants were isolated by site-directed mutagenesis using Tn3Hog*us*. The mutants were categorized into three classes on the basis of Calcofluor fluorescence. Class

I and class III mutants showed a severe symbiotic defects on *Leucaena, Gliricidia* and *Phaseolus* while class II mutants showed normal symbiotic phenotype on all three legumes. This is the first report to show normal nodule formation by EPS-defective mutants of rhizobia on both indeterminate- and determinate-nodulating hosts. Expression of *exo* genes was studied by using GUS fusions under both cultural and symbiotic conditions. Fluorometric quantification of GUS activity was used to determine the direction of transcription of *exo* genes. Sequence analysis of a 4.7-kb DNA fragment encompassing class I and class III mutants showed the presence of four genes with high homology to the *exo* genes *of R. meliloti.*

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CHAPTER I

INTRODUCTION

Rhizobia present in the soil interact with the roots of different legume hosts, induce nodule formation, invade these nodules through the formation of infection threads, differentiate into bacteroids and finally fix atmospheric nitrogen. Development of the nodule and the nitrogen-fixing bacteroids is a complex process that requires signal exchanges between rhizobia and the plant cells. The cell surface polysaccharides of rhizobia, particularly exopolysaccharides (EPS) and lipopolysaccharides (LPS) have been shown to be involved in the symbiotic process with various legumes (for reviews, see Gray and Rolfe 1990, Leigh and Coplin 1992).

In legumes, the region of growing root that is most susceptible to infection by *Rhizobium* is located behind the apical meristem at the site of emergence of root hairs. Mitotic activity is induced in the root cortex in this region within 12-24 h of *Rhizobium nod* gene activity and nodule primordium gives rise to an organized meristem consisting of a mass of small cells dividing in all directions (Brewin 1991). The sequence of *Rhizobium*-induced cell division and cell invasion varies for different legumes resulting in variation in nodule morphogenesis. In *Phaseolus, Glycine* and *Lotus* cortical cell division occurs just beneath the epidermal layer and the cells are invaded by rhizobia before they become meristematic. Therefore, rhizobia can spread by dividing in the cytoplasm of actively dividing host cells resulting in the formation of spherical or determinate nodules. Meristematic activity in these nodules is only a transient phase in these nodules. However, in *Medicago, Trifolium, Leucaena, Pisum* and *Vicia*, the nodule primordium arises in the

inner cortical layers present adjacent to the pericycle near the xylem pole. Some of these primordial cells stop dividing four days after *Rhizobium*-infection starts and are invaded by rhizobia released from the infection thread. Other cells in the proximal side at the same time become smaller and rich in cytoplasm. These cells as well as pericycle cells which are also stimulated to divide form the apical meristem of the developing nodule. The dividing apical meristematic cells grow outwards from the root. Cell-to-cell spread of rhizobia occurs by the infection thread developing in the cells that cease dividing resulting in cylindrical indeterminate nodule formation which occasionally bifurcate to form coralloid structure.

Generally, nodulation of indeterminate-nodulating hosts by *Rhizobium* mutants defective in EPS is severely affected while such mutants have little effect on nodulation and nitrogen fixation of determinate-nodulating hosts. *Rhizobium meliloti* mutants which fail to make succinoglycan induce nodules on alfalfa, but the nodules are devoid of bacteria and consequently fail to fix nitrogen (Finan et al. 1985, Leigh et al. 1985, Hynes et al. 1986, Long et al. 1988a, Muller et al. 1988). Similarly, some EPS-defective mutants of the broad-host-range *Rhizobium* NGR234 form callus-like pseudo-nodules on *Leucaena leucocephala* (Chen et al. 1985). The nodulation and nitrogen-fixing abilities of some of these mutants can be restored by the addition of EPS from NGR234 (Djordjevic et al. 1987). EPS-deficient mutants of *R. leguminosarum* bv. *viciae* do not form any nodules on peas but such mutants of *R. leguminosarum* bv. *phaseoli* form nitrogen-fixing nodules on *Phaseolus vulgaris* (Borthakur et al. 1986). Similarly, EPS-defective mutants of *R. loti* have been observed to form nitrogen-fixing nodules on a determinate-nodulating host, *Lotus pedunculatus* but form

only tumor-like growths on the indeterminate-nodulating host *Leucaena* (Hotter and Scott, 1991).

As in other Gram-negative bacteria, LPS is a unique component of the outer membrane of rhizobia and consists of a variable O-oligosaccharide linked to the highly conserved core oligosaccharide which is anchored to the membrane by glycolipids. Previous studies have shown the involvement of LPS in specific attachment of rhizobia to the host plant roots (Lagares 1988, Kato et al. 1979, for review see Noel 1992). In determinate-nodulating hosts like *P. vulgaris* and *Glycine max*, LPS mutants lacking O-antigen failed to form normal infection threads or bacteria were not released from the infection threads to invade the nodules (Cava et al. 1989, 1990, Maier and Brill 1978, Puvanesrajah et al. 1987, Stacey et al. 1991). The symbiotic phenotypes of LPS-defective mutants on indeterminate-nodulating hosts may vary among different legumes. LPS-defective mutants of *R. leguminosarum* bv. trifolii formed small white non-fixing nodules on *Trifolium hybridum* (Brink et al. 1990). Such mutants of *R. leguminosarum* bv. viciae also formed small ineffective nodules on *Vicia hirsuta*, *V. sativa* and *Pisum sativum* (de Maagd et al. 1989, Goosen-De Roo et al. 1991, Priefer 1989). On the other hand the LPS-defective mutants of *R. meliloti* are not defective in symbiosis (Clover et al. 1989).

Brink et al. (1990) suggested that LPS may be a signal molecule that is required at different times during nodule development in different hosts. It has also been proposed that LPS may protect rhizobia by suppressing the host defence mechanism (Noel 1992). Studies of *R. meliloti*-alfalfa symbiosis showed that the genes for EPS and LPS synthesis determine similar functions in the course of nodule development and thus EPS and LPS provide

equivalent information for the host plant (Putnoky et al. 1990). Williams et al. (1990) showed that *lpsZ*, a gene involved in LPS synthesis in *R. meliloti* Rm41 allowed the *exoB* and other *exo* mutants to form nitrogen-fixing nodules on various legume hosts. However, Reuhs et al. (1993) have shown that the presence of a modified form of the capsular-type polysaccharide, KPS, and not the LPS may substitute for the EPS in this strain.

Most studies on the genetics of EPS and LPS synthesis have been carried out in fast-growing *Rhizobium* spp. which nodulate the temperate field legumes such as peas, alfalfa and clovers. Genes involved in EPS and LPS synthesis have not been cloned and characterized in the tree legume-nodulating rhizobia. Although a few genes have been identified and characterized from the broad host range *Rhizobium* sp. strain NGR234, this strain is phylogenetically closely related to *R. meliloti* and *R. fredii*. Therefore, this strain is not a true representative of the tree legume rhizobia.

Rhizobium sp. strain TAL1145 forms an effective symbiosis with several tree legumes such as *L. leucocephala, Gliricidia sepium, Calliandra* spp. and a number of other legumes including *Phaseolus* beans. TAL1145 has been identified as a superior and competitive strain and is used for inoculant production for tree legumes at the Nitrogen fixation of Tropical and Arid Legumes (NifTAL) project, Maui. Phylogenetically it is different from other *Rhizobium* spp. including *R. tropici*. Therefore, this strain is ideal for studying the genetics of surface polysaccharide synthesis and determining their role in symbiosis. The following were the objectives of the present investigation :

1. Identification and characterization of genes for EPS and LPS synthesis in *Rhizobium* sp. strain TAL1145.

2. Determination of the role of EPS and LPS in the indeterminate- and determinate-nodule-forming hosts, mainly the tree legumes.

3. Chemical characterization of EPS and LPS of TAL1145 and of mutants defective in the synthesis of these components.

CHAPTER II

REVIEW OF LITERATURE

2.1 Introduction:

The family Rhizobiaceae of Gram negative bacteria includes the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* (Jordan, 1984 and Dreyfus et al. 1988) that form nitrogen-fixing root nodules on legumes and *Agrobacterium* spp. that are pathogenic to a number of dicotyledonous plant species. These bacteria produce various kinds of extracellular polysaccharides and other surface polysaccharides. Lipopolysaccharides (LPS) are integral components of bacterial outer membrane while extracellular polysaccharides (EPS) may be secreted as a loosely bound slime layer or exopolysaccharide or may form a tightly adhering capsule encapsulating bacteria. These polysaccharides play important roles in the life cycles of bacteria such as in holding minerals and nutrients near the bacterial cell surface; adherence to solid surfaces where nutrients are available; protection against desiccation, starvation, and various hydrophobic compounds and plant defense mechanisms; as signal molecules for interaction with the host; and a multifaceted role during symbiosis and pathogenesis (Costerton et al. 1987, Nikaido and Vaara 1987, Sutherland 1988, Whitfield 1988).

Being outermost rhizobial EPS and surface polysaccharides are first to come in direct contact with the external environment and the host. Therefore, their importance in survival and sustenance of *Rhizobium* in the soil can not be underestimated. This review highlights the chemical structure of EPS and the genetics of the biosynthesis of EPS in

different *Rhizobium* species. The roles of LPS and EPS in symbiosis with legume hosts as studied by different workers are also summarized. Special references to other plant bacteria are included wherever relevant.

2.2 Different polysaccharides produced by *Rhizobium:*

Various species of *Bradyrhizobium* and *Rhizobium,* especially the fast-growing strains, produce large quantities of EPS when grown in carbohydrate-rich medium resulting in the formation of mucoid and slimy colonies (Aman et al. 1981, Borthakur et al. 1985, Carlson 1982, Chen et al. 1985, Diebold and Noel 1989, Hotter and Scott 1991, Jansson et al. 1977, Ko and Gadya 1990, Leon et al. 1992, Sanders et al. 1978, Tully and Terry 1985 etc.). EPS-defective, non-mucoid mutants have been isolated by different workers on mannitol-containing media (Borthakur et al. 1988, Chen et al. 1986, Diebold and Noel 1989, Ko and Gadya 1990, Lopez-Lara et al. 1993, Napoli and Albersheim 1980 etc.).

Bacteria including some *Rhizobium* spp. exhibit blue-green fluorescence under long-wave UV light when grown on Calcofluor-containing media (Leigh et al. 1985). Acidic EPS or succinoglycan binds the dye, Calcofluor, that imparts fluorescence to bacteria. Nonmucoid mutants of *R. meliloti* and *R. loti* that are defective in EPS synthesis can be screened for their inability to fluoresce on Calcofluor-containing media (Hotter and Scott 1991, Leigh et al. 1985, Long et al. 1988a, Muller et al. 1988). However, EPS-defective mutants of certain *Rhizobium* spp. can not be isolated by this method. For example, only 2 of the 14 wild-type *R. fredii* strains that were examined produced Calcofluor-fluorescent EPS (Kim et al. 1989) and EPS-defective mutants of *R.*

leguminosarum showed more intense fluorescence than the wild type (Diebold and Noel 1989).

At any given time most *Rhizobium* strains produce only one kind of EPS (Gray et al. 1992). Zhan et al. (1989) reported the production of a second type of EPS, EPS II or EPS b, by *R. meliloti* strain Rm1021 when the *mucR* (*expR*) gene was mutated. At the same time Glazebrook and Walker (1989) also identified EPS II in an *expR* mutant of this strain which showed the same phenotype. Although acidic in nature like succinoglycan / EPS I, EPS II lacks Calcofluor fluorescence. Wild-type Rm1021 produces only EPS I indicating that EPS II, a galactoglucan, was produced cryptically in this strain. Functional replacement of succinoglycan by EPS II had occurred during symbiosis with *Medicago sativa* in *exo-expR* double mutants. In contrast to strain Rm1021, another *R. meliloti* strain YE-2 can simultaneously produce succinoglycan in the low molecular weight fraction and EPS b in the high molecular weight fraction (Zevenhuizen 1990).

Several species of *Rhizobium* produce EPS of two molecular ranges, a high molecular weight (HMW) component that elutes with the void volume and had a molecular mass in the order of one to five million daltons, and a low molecular weight (LMW) oligomer that elutes in the salt volume and had a molecular mass up to 10,000 daltons (Cangelosi et al. 1987, Diebold and Noel 1989, Gray et al. 1991, Leigh and Lee 1988, Lopez-Lara et al. 1993). Both of these are secreted in the supernatant and are made up of the same repeat units. Purification of the LMW fraction by chromatography showed the presence of monomers, trimers and tetramers of the repeat units with different

degrees of anionic characteristics (Gray et al. 1991). The anionic nature was imparted by the noncarbohydrate substituents.

In addition to the acidic exopolysaccharides, *Rhizobium* and *Agrobacterium* spp. also produce neutral homopolymers containing up to 20 residues of β-(1→2) linked glucose sugars (Dylan et al. 1990a and 1990b, Ielpi et al. 1990, Stanfield et al. 1988, York et al. 1980, Zevenhuizen et al. 1979). The sugars are often present in a cyclic configuration. Neutral β-1,2-glucans are present in the periplasm and are also exported from the bacterial cells. Breedveld et al. (1993a) observed strong correlation between enhanced secretion of cyclic glucans and inability of mutants to synthesize other polysaccharides under different culture conditions. *Bradyrhizobium japonicum* produces small oligomers of β-1,3- and β-1,6- linked glucose residues instead of β-1,2 -glucans which probably have a similar function. The *ndv* genes responsible for β-1,2-glucan synthesis show structural and functional homology to *chvA* and *chvB* genes of *Agrobacterium tumefaciens* that are known to be essential for oncogenic transformation of dicotyledonous plants (Dylan et al. 1986). In addition to β-1,2-glucans some *Rhizobium* spp. form elaborate cellulosic microfibrils that help in the attachment of rhizobia to the root hair and even non-root-hair epidermis (Mateos et al. 1995, Smit et al. 1987, Zevenhuizen et al. 1986).

Lipopolysaccharides (LPSs) are unique, integral components of the outer membrane of all Gram-negative bacteria. LPSs are complex molecules that can vary significantly in different *Rhizobium* spp. and even among different strains of a single species (Carlson 1982, Carlson et al. 1978, Zevenhuizen et al. 1980). Major components

of LPSs are; lipidA, a membrane anchor with a glucosamine backbone and characteristic β-hydroxy fatty acids, a core oligosaccharide attached to lipidA by a 3-deoxy-D-manno-2-octulosonic acid (Kdo) that is highly conserved within a species of *Rhizobium,* and a highly variable portion which carries major antigenic determinants of wild-type strains and is known as O-antigen. LPS from different *Rhizobium* spp. display a characteristic banding pattern on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). On the basis of their mobility the bands can be classified as slowly migrating LPS I that may show one to several bands and a faster moving LPS II. Both of these banding regions contain lipidA and the conserved core oligosaccharide but only LPS I contains O-antigen (Brink et al. 1990, Carlson et al. 1987, Carrion et al. 1990, Cava et al. 1990, deMaagd et al. 1989, Priefer 1989 and Tao et al. 1992). Antigenic variation in LPS I of *R. leguminosarum* has been observed under different environmental conditions as well as during symbiosis (Goosen-de-Roo 1991, Kannenberg and Brewin 1989, Kannenberg et al. 1994, Tao et al. 1992).

In addition to LPS a new class of cell-associated, acidic polysaccharides that are structural analogues of the K antigen (capsular polysaccharide) of *E. coli* has been identified in *R. meliloti* strain AK631 and *R. fredii* strain USDA205 (Reuhs et al. 1993). Two polysaccharides isolated from USDA205 were found to be polymers of a galactose and Kdo disaccharide subunit and hence were named as Kdo-containing polysaccharides (KdoPS or KPS) . Kdo is also a major component in the similar polysaccharides of *R. meliloti*. Recently, Reuhs et al. (1995) showed that the *lpsZ* gene is involved in size modulation and expression of KPS in *R. meliloti* strain AK631 although this gene is not

essential for KPS synthesis. Mutation in *lpsZ* resulted in production of HMW KPS that showed lower mobility in polyacrylmide gel electrophoresis. Also, LMW KPS was absent and the *lpsZ* strain displayed a Fix- phenotype on alfalfa.

Many *nod* genes including common *nodABC* genes of different *Rhizobium* spp. are involved in synthesis of extracellular Nod factors (Denarie et al. 1992, Fisher and Long 1992). Nod factors have been shown to share a common core which is a mono-N-acylated chitin oligomer. In *R. meliloti* the Nod factors are tetra- or pentamers of chitin sulfated at the reducing end and O-acetylated and N-acylated at the non-reducing end (Demont et al. 1993, 1994). The *Rhizobium* sp. strain NGR234 produces a family of NodNGR factors that include both sulfated and non-sulfated Nod factors. NGR234 produces mono-N-acylated chitin pentamers carrying a variety of substituents. The terminal non-reducing glucosamine is N-acylated and also N-methylated. The reducing N-acetyl-glucosamine residue is substituted at position 6 with 3-O-sulfated or 4-O-acetylated or non-substituted 2-O-methyl fucose. In Nod factors of *R. leguminosarum* bv. viciae the reducing glucosamine is not sulfated and the non-reducing end is N-acetylated and N-acylated by a highly unsaturated fatty acid (Spaink et al. 1991). These acylated glucosamine oligosaccharide- or lipo-oligosaccharide-signal molecules produced by different *Rhizobium* spp. elicit root hair deformation and mitotic divisions in root cortical cells. They are also involved in infection and formation of nodules on the respective hosts.

2.3 Structure of EPS in different *Rhizobium* spp. :

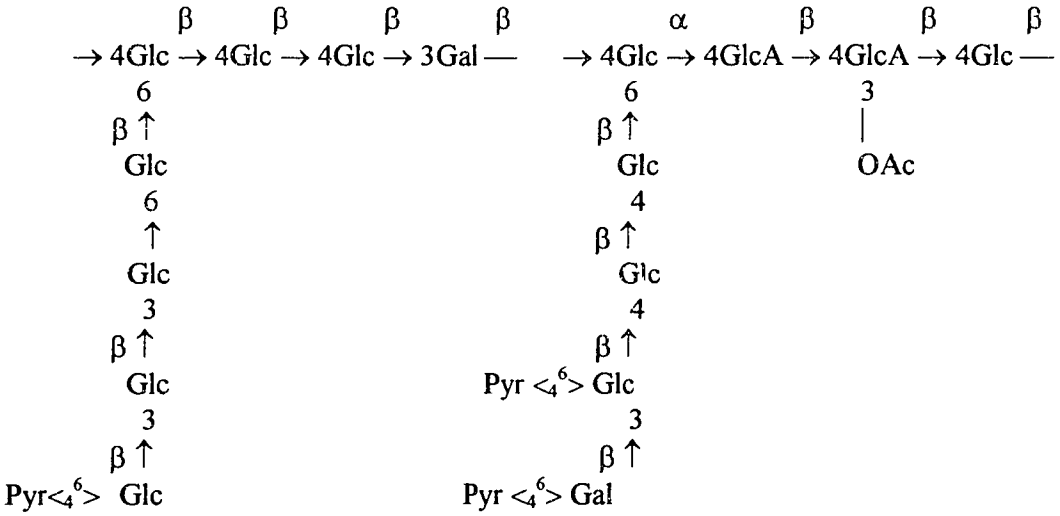
Complete structure of EPS has been determined in several *Rhizobium* spp. and plant pathogenic bacteria such as *Xanthomonas campestris*. The acidic EPSs are large

heteropolymers that consist of repeat units of 7,8 or 9 sugar residues linked by various α and β linkages. The repeat sugar units may be linear or branched and usually contain noncarbohydrate substituents of succinate, acetate, pyruvate and hydroxybutanoate which impart an acidic nature to these EPSs. The structure of repeat oligosaccharide units of some known *Rhizobium* strains is shown in Fig.2.1. The EPS structures of all strains of a particular *Rhizobium* sp. are not always identical. The EPS produced by *R. meliloti* strain 201 is different from both succinoglycan and EPS II or galactoglycan of the strain Rm1021 (Amemura et al. 1981, Yu et al. 1983). The strain 201 produces an acidic EPS molecule with three oligosaccharide repeat units containing mannose and glucosamine. These sugars are not present in the EPSs of Rm1021.

R. meliloti succinoglycan and *R. leguminosarum* and *R.* sp. strain NGR234 EPSs are polymers of octasaccharide repeat units; however, the length of the branched oligosaccharide component varies in these strains. Starting with galactose, the first five sugar residues and their linkages are identical in *R. meliloti* strain Rm1021 and *R.*sp. strain NGR234 (Zhan et al. 1990). Galactoglycan of *R. meliloti* is significantly different from the succinoglycan. Unlike succinoglycan it is a polymer of disaccharide units with acetate and pyruvate substituents. However, succinate is absent in this polysaccharide (Zhan et al. 1989). A side chain present in EPS I is absent in EPS II and EPS I has only β-linkages while EPS II has both α- and β-linkages. A pyruvate substituent is present on D-glucosyl unit of EPS I but it is on the D-galactosyl unit of EPS II. An acetyl substituent is present on the glucosyl unit of EPS II. The only common feature in the two polysaccharides is the

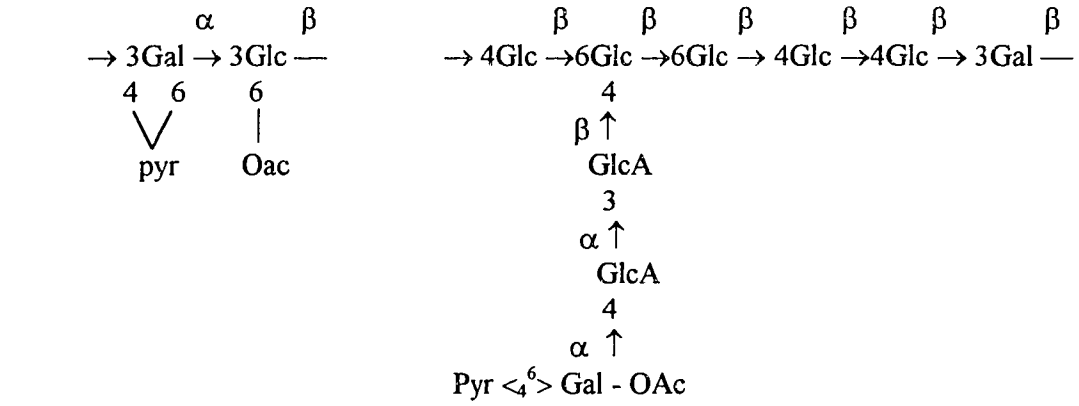
β-D-glucosyl-(1→3)-D-galactosyl linkage with each sugar of EPS II modified

(Chandrasekaran et al. 1994). Matulova et al. (1994) assigned succinate on the 3-linked



EPS I of *R. meliloti* ***R. leguminosarum* EPS**

(Aman et al. 1981, Jansson et al. 1977) (Jordan 1984)



EPS II of *R. meliloti* *R.* sp. strain NGR234 EPS

(Her et al. 1990) (Djordjevic et al. 1986)

Fig.2.1 Repeat unit structure of some representative acidic exopolysaccharides.

β-D-Glucose unit of side chain and acetate to the 4-linked β-D-glucose units in the backbone of succinoglycan in *R. meliloti* strain YE-2.

The basic structure of the EPS of different strains of *R. leguminosarum* has been found to be the same irrespective of the biovars (Canter Cremers et al. 1991, Hollingsworth et al. 1988, McNeil et al. 1986 and Robertsen et al. 1981). Slight variation in the amount and pattern of non-carbohydrate substituents seen in different biovars indicate that EPS probably does not play a major role in determining the host specificity in this species. The EPS produced by a wide-host-range legumes-nodulating *Rhizobium* strain GRH-2 was found to be similar to the EPS produced by *R. leguminosarum* bv. trifolii strain ANU843 (Lopez-Lara et al. 1993). Presence of a structurally related low molecular weight native heterooligosaccharide led these authors to suggest that a tightly conserved structure and function of EPS in both *Rhizobium* strains exists and they probably shared a common ancestor.

2.4 Effect of physico-chemical factors on biosynthesis of EPS in bacteria : The ecological importance of EPS in bacteria has not been fully realized because of limited information available regarding the effect of growth and environmental conditions on EPS production. Bacteria in soil generally live in colonies within a matrix mainly composed of EPS (Foster 1981). EPS has been suggested to protect bacteria from drying and from fluctuations in water potential (Hartel and Alexander 1986, Wilkinson 1958). Robertson and Firestone (1992) observed an increase in the amount of polysaccharide with

simultaneous decrease in total protein content following the desiccation of soil *Pseudomonas* spp.

Breedveld et al. (1993b) studied the effect of growth conditions on the production of capsular and extracellular polysaccharides in *R. leguminosarum* (*R. l.*). EPS is produced as a major component in batch and continuous cultures of *R. l.* bv. *trifolii* during logarithmic growth phase while capsular polysaccharides (CPS) are produced under excess carbon concentration at low dilution rate of the medium or in the stationary phase (Zevenhuizen et al. 1986). Higher substrate concentration resulted in higher cell mass, EPS production and β-1,2-glucan secretion while CPS production was inhibited (Breedveld et al. 1990a and 1993b). Breedveld et al. (1990b, 1991) observed a significant increase in succinoglycan synthesis and trehalose accumulation when NaCl was added to the medium to a final concentration of 0.2 M but the amount produced was comparatively lower at 0.6 M NaCl. However, the amount of LMW fraction decreased on adding NaCl to the medium. At a pH below 6 EPS production in both *R. meliloti* and *R. leguminosarum* was repressed (Breedveld et al. 1993b). Tully and Terry (1985) reported a decrease in EPS synthesis by *Bradyrhizobium japonicum* under anaerobic conditions as well as during symbiosis. However, they suggested that the reduction in EPS may be species and strain specific. Zevenhuizen (1986) also reported that incubations of *R. l.* bv. *trifolii* under forced aeration resulted in higher EPS production. Tully (1988) also showed production of low levels of acidic EPS by *B. japonicum* in the presence of the hydroaromatic acids, shikimate and quinate, which may be available as a carbon sources in the soil.

Apanna (1988a and 1988b) studied the effect of metal ions on the production of EPS. An increase in EPS synthesis by *R. meliloti* strain JJ-1 in medium supplemented with manganese was observed. The EPS showed changes in the composition with regard to both sugar and noncarbohydrate residues. An increase in the quantity of EPS as well as changes in hexose content and NMR spectra were observed in bacteria grown in iron and aluminium rich medium. In *R. leguminosarum* bv. *trifolii* sulfate deficiency was shown to enhance EPS production (Sutherland 1985). In *R. meliloti* limiting nitrogen, phosphate or sulfur stimulated succinoglycan synthesis but only phosphate limitation stimulated EPS II production (Doherty et al. 1988, Leigh et al. 1985, Zhan et al. 1991).

2.5 Genetics of biosynthesis of EPS in rhizobia:

The genetics of biosynthesis of EPS has been most extensively studied in *R. meliloti*; however, genes involved in EPS synthesis in other *Rhizobium* spp. have also been studied in some detail. Systematic studies on the genetics of EPS synthesis started when Muc- or EPS-defective mutants from fast-growing *R. leguminosarum* strains and *Rhizobium* sp. strain NGR234 were isolated (Chakravorty et al. 1982, Rolfe et al. 1981, Rolfe et al. 1984, Sanders et al. 1981). The mutants generally formed poorly developed and non-nitrogen fixing nodules on their respective hosts. Chen et al. (1985) isolated 90 random transposon-insertion, EPS-defective mutants of broad host-range *Rhizobium* sp. strain NGR234. The mutants were classified into nine groups based on their colony phenotypes ranging from non-mucoid or rough to overproducers of EPS. The *exo* gene cluster of NGR234 was found to contain 4 complementation groups, *exoB, exoD, exoY, and exoC* (Chen et al. 1988, Zhan et al. 1990). By site-directed transposon mutagenesis

Borthakur et al. (1986) isolated mutants of *R. leguminosarum* that were defective in EPS synthesis and formed non-mucoid colonies. A recombinant plasmid from the clone library of *Xanthomonas campestris* pathovar *campestris* which corrected the defect in EPS synthesis in a *X. campestris* mutant also restored partial mucoidy level to *R. leguminosarum pss* mutants. These transconjugants of *R. leguminosarum* also formed nitrogen-fixing nodules on peas.

Leigh et al. (1985) isolated 26 random transposon-insertion mutants of *R. meliloti* strain Rm1021 that formed nonfluorescent colonies on Calcofluor-containing media when observed under long range UV. The mutants failed to produce acidic EPS or succinoglycan. The mutants were classified into six groups on the basis of their complementation by 5 different recombinant plasmids as well as on the basis of their resistance to 9 bacteriophages of *R. meliloti*. Long et al. (1988) obtained more Tn5 insertion mutations in *R. meliloti* genes that affected the synthesis of Calcofluor-binding acidic exopolysaccharide. They identified 12 loci that mapped within a 22-kb region of DNA. The *exoA, exoB, exoQ, exoF, exoL, exoM,*and *exoP* mutants did not fluoresce, whereas the *exoG, exoJ* and *exoN* mutants showed dim fluorescence on Calcofluor-containing plates. The *exoK* mutants exhibited delayed fluorescent halo in this medium. Leigh et al. (1987) obtained several *exoH* mutants which formed haloless colonies on Calcofluor plates and produced EPS that lacked the succinyl substituents. Muller et al. (1988) obtained Calcofluor fluorescence-negative mutants of strain Rm2011 in which the EPS lacked the pyruvate substituent. In addition to the gene cluster involved in succinoglycan synthesis, another cluster of genes has been located on megaplasmid b in *R.*

meliloti known as *exp* or *muc* genes that are involved in synthesis of EPS II (Glazebrook et al. 1989, Zhan et al. 1989). At least six *exp* genes and the product of *exoB* are required for synthesis of EPS II (Glazebrook and Walker 1989). Mutation in *exoD*, a gene located on the chromosome in Rm1021, resulted in the production of less EPS when starved for nitrogen but the mutant produced more anthrone-positive material than the wild-type when grown in the presence of ammonia (Reed and Walker 1991). The *exoZ* strain showed dim fluorescence on a Calcofluor plate which became fully fluorescent after several days indicating this gene product is required but is not essential for EPS synthesis (Buendia et al. 1991).

The *exoB* and *exoC* mutants showed multiple carbohydrate defects in *R. meliloti* (Leigh and Lee 1988). The *exoC* mutation affects synthesis of LPS, β-1,2-glucans, succinoglycan and EPS II while the mutation in *exoB* affected LPS, succinoglycan and EPS II in *R. meliloti* (Leigh and Lee 1988, Glazebrook and Walker 1989). The gene *exoC* is located on the chromosome. ExoC is a phosphoglucomutase required for synthesis of UDP-glucose, a precursor for synthesis of all these polysaccharides (Leigh et al. 1993). Galactose is present in LPS, EPS I and EPS II of *R. meliloti* and of EPS, CPS and LPS in *R. l.* bv. viciae. Since galactokinase is absent in *R. l.* bv. viciae UDP-galactose can only form by epimerization of UDP-glucose. Cremers (1990) proposed that *exoB* encodes for UDP-glucose 4'-epimerase enzyme. This was further confirmed by the finding that *exoB* mutants of *R. l.* bv. viciae as well as *R. meliloti* lacked UDP-glucose 4'-epimerase activity. Also, the deduced amino acid sequence of *exoB* showed significant homology to *galE* gene of *E. coli*. The *exoB* gene is organized as a monocistronic unit in *R. meliloti*

(Buendia et al. 1991). The LPS of *B. japonicum* strain USDA110 lacks galactose. Therefore, the mutation in *exoB* only affected synthesis of EPS (Parniske et al. 1993).

In the past few years sequencing of a 24-kb *exo*-gene region of *R. meliloti* strain Rm2011 and 12 *exo*-genes of Rm1021, located in a cluster on the second megaplasmid have been completed (Becker et al. 1993a, 1993b and 1993c, Glucksmann et al. 1993, Muller et al. 1993). Although these strains are descendants of the strain SU47, they show some differences. A possible model for arrangement of Exo proteins and possible pathway for EPS synthesis has been proposed on the basis of an extensive analysis of structure and function of genes in *R. meliloti* (Leigh and Walker 1993, Reuber and Walker 1993). A brief summary of the conclusions based upon the sequence analysis is documented here.

The genes *exoH, exoK* and *exoL* were found to be monocistronic (Becker et al. 1993a). However, *exoK* forms a bicistronic transcriptional unit with *exoH* but is additionally transcribed at low levels by a promoter located within the *exoH* coding region. The presence of an N-terminal hydrophobic domain, as well as the significant similarity of this region with known signal peptide sequences indicated that ExoK may be an extracellular or periplasmic protein. The deduced amino acid sequence of ExoK showed high homology to β-1,3-1,4-glucanases of *Bacillus* and *Clostridium* spp. Since *exoK* mutant produced reduced amounts of EPS I and the presence of multicopies of *exoH-exoK* resulted in increased synthesis of low-molecular-weight EPS (Urzainqui and Walker 1992), Becker et al. (1993a) concluded that ExoK may be important in efficient EPS I synthesis and it is probably involved in the conversion of high-molecular weight to low-

molecular-weight EPS. This function is also compatible with the homology of ExoK to β-1,3-1,4-glucanases. The *exoH* mutants produce only traces of low-molecular-weight EPS. This suggests that *exoH* may also convert HMW to LMW EPS form. Restoration of an almost normal symbiotic phenotype to *exoH* by addition of LMW EPS supports this hypothesis. Presence of hydrophobic domains through the entire length of protein and charged amino acids and possible β turns between the hydrophobic domains indicate that ExoH is a transmembrane protein. The absence of hydrophobic domains in ExoL indicate it to be a cytoplasmic protein.

Five complete open reading frames following *exoL* were observed in the same orientation as *exoL* by Becker et al. (1993b). The *exoA, exoM, exoN* and *exoP* correspond to the respective complementation groups identified by Long et al. (1988) while a new gene, *exoO*, was identified between *exoM* and *exoN*. Presence of an open reading frame designated as *thi* that is involved in thiamine biosynthesis was found in the opposite orientation to other ORFs. The gene is unrelated to EPS synthesis and hence demarcates the boundary of *exo*-gene cluster in *R. meliloti*. Hydropathic profiles of ExoL, ExoM, ExoO and ExoN indicate that these proteins are located in the cytoplasm (Becker et al. 1993b); however, on the basis of probable membrane spanning regions, Gluckmann et al. (1993) suggested ExoM may be a membrane associated protein. ExoN was suggested to be a UDP-glucose pyrophophorylase because it shows homology to UDP-glucose pyrophosphorylase of *Acetobacter xylinum*.

The presence of three hydrophobic helices each in ExoA and ExoP indicated that these two are membrane associated proteins (Glucksmann et al. 1993). In addition,

significant homology of the N-terminal region of ExoP to signal peptides of prokaryotes suggested that this protein traverses through the membrane. Using translational fusions of *exoP* gene with *lacZ* and *phoA* genes Becker et al. (1995) showed that ExoP can be divided into an N-termianl domain which is present in the periplasmic region and the C-terminal cytoplasmic domain. The N-terminal domain showed similarities with proteins involved in polysaccharide length determination while the C-terminal domain had an ATP/GTP-binding motif. Leigh and Walker (1993) have placed ExoA on the cytoplasmic and ExoP on the periplasmic side of the membrane in their proposed model for the arrangement of Exo proteins. Entire ExoA, ExoM, and ExoP did not show homology to any known proteins while ExoO showed weak homology to NodC proteins of *R. meliloti* and *R. loti* that are suggested to be involved in the synthesis of the oligosaccharide backbone of Nod factors. Recently, Becker et al. (1995) identified a stretch of 33 amino acids of ExoP protein in the periplasmic domain that were homologous to the motif present in a number of proteins involved in the biosynthesis of polysaccharides in a variety of bacterial species. Some of these proteins have been shown to determine the degree of polymerization of O-antigen chains. Mutation in *exoP* containing only the N-terminal region caused a significant increase in the ratio of LMW to HMW succinoglycan. C-terminal domain of ExoP was localized in the cytoplasm and it displayed similarities to prokaryotic ATPases.

The divergently transcribed *exoX* and *exoY* genes of Rm2011 were found to be separated by 772-bp with no coding probability (Muller et al. 1993). The protein encoded by *exoX* showed 73% identity to ExoX of NGR234 (Gray et al. 1990). Although at the

primary sequence level the *exoX* gene of *Rhizobium* sp. strain NGR234 showed only 33% similarity with the equivalent *R. leguminosarum* bv. phaseoli gene, *psi*, both genes synthesize polypeptides of similar sizes and showed striking similarity in their hydrophobicity plots and in an 18-amino-acid domain.

Significant homology between the *exoY* gene products of Rm2011 and NGR234 was also reported by Muller et al. (1990). Earlier, Muller et al. (1988) and Reed et al. (1991) reported significant homology between ExoY of *R. meliloti* to the Pss2 protein (Borthakur et al. 1988) which is essential for EPS synthesis in *R. leguminosarum* bv. *phaseoli*. Hydrophobicity plots of these proteins suggested that ExoY is a membrane associated protein. Homology of ExoY with the C-terminal part of GumD of *Xanthomonas campestris,* which is a glucosyltransferase (Coplin and Cook 1990), and with the C-terminus of RfnP protein of *Salmonella typhimurium,* which is a galactosyltransferase that catalyzes the transfer of UDP-galactose to the C55 lipid carrier (Jiang et al. 1991), provided strong evidence that ExoY is a glycosyltransferase. Borthakur et al. (1986) could correct the EPS defect of the *pss*::Tn5 mutant by a cloned DNA fragment from *X. campestris* which probably contained the *gumD* gene. Ivashina et al. (1994) observed a 95.5% identity between the amino acid sequence of Pss4 of *R. l.* bv. *viciae* and Pss2. All six proteins, ExoY of *R.meliloti* and NGR234, Pss2 and Pss4 of *R. l.* bv. *phaseoli* and *R. l.* bv. *viciae*, GumD of *X. campestris* and RfbP of *S. typhimurium,* contain several conserved amino acid residues which were suggested to be components of the active sites of the enzymes.

The *exoF* gene located downstream of the *exoY* gene in Rm2011 encodes a protein that showed 83% identity with the protein encoded by ORF1 of NGR234. The hydrophobic N-terminal region of the *exoF* product is preceded by a positively charged structure which has a high probability of being a signal peptide. Therefore, ExoF was suggested to be a periplasmic protein. The *exoQ* is the third gene in the *exoYFQ* operon. The ExoQ protein did not show homology to any known proteins. On the basis of the hydropathic profile the *exoQ* gene product was suggested to be a transmembrane protein with 11 membrane spanning regions (Muller et al 1993). It was also supported by studies of Reuber et al. (1991) who reported active Tn*phoA* fusions with *exoF* and *exoQ* genes of strain Rm1021.

Five open reading frames were identified in the DNA fragment located between *exoX* and *exoH* of Rm2011 (Becker et al. 1993c). Two ORFs upstream of the *exoH* designated as *exoI* and *exoT*, and one downstream *exoX*, named as *exoU*, were in the same orientation as these genes while *exoW* and *exoV* were in the opposite orientation. On the basis of their hydrophobicity profiles ExoW, ExoV and ExoU were suggested to be cytoplasmic proteins (Becker et al. 1993c). However, Gluckmann et al. (1993) suggested that the ExoW protein is anchored to the membrane by its carboxy-terminal end similar to that of ExoM in Rm1021. These differences in *R. meliloti* strains Rm1021 and Rm2011, both of which were derived from strain SU47, indicate some variation in their genetic composition.

ExoT with its 13 hydrophobic domains probably is a transmembrane protein. Observations of Reuber et al. (1991) that *TnphoA* insertions in exoT displayed alkaline

phosphatase activity support this suggestion. The presence of a potential signal peptide in ExoI indicated that it may be a periplasmic protein. ExoU and ExoW revealed some homologies to the deduced amino acid sequence of ExoO. All three proteins indicated significant homologies to several polysaccharide synthase genes products of prokaryotic and eukaryotic origins. ExoV displayed weak homology to ExoU, ExoW, ExoL, ExoM, ExoA and ExoO proteins of *R. meliloti* suggesting that all these proteins are glucosyltransferases. All five genes, *exoU, exoV, exoW, exoT and exoI,* are monocistronic transcription units as indicated by *exo-lacZ* fusions. *R. meliloti* strains with mutations in *exoT,exoW, exoU* and *exoV* failed to synthesize EPS I indicating that all four genes are essential for succinoglycan synthesis. The *exoI* mutants produced 50% of the EPS produced by the wild type Rm2011. An altered form of EPS was synthesized by *exoI* mutants resembling the phenotype of *exoO* mutants (becker et al. (1993c).

Reuber and Walker (1993) conducted ingenious experiments to characterize the biosynthetic intermediates in different *exo* mutants. As mentioned earlier the *exoB* gene encodes UDP-glucose-4-epimerase which converts UDP-glucose to UDP-galactose which is the first sugar added to the lipid carrier (Buendia et al. 1991, Canter-Cremers et al. 1990, Tolmaski et al. 1980, 1982). Therefore, succinoglycan can not be initiated in *exoB* mutants. However, they observed that the incorporation of UDP-[^{14}C]galactose in the reaction mixture containing the permeabilized *exoB* cells increased the level of radioactivity in the succinoglycan intermediates as compared to wild type. In addition, Reuber and Walker (1993) took advantage of the fact that *exoR* is a negative regulator for succinoglycan synthesis and *exoR* mutants express *exo* genes at higher level (Doherty et al.

1988, Reed et al. 1991a, Reuber et al. 1991). This resulted in much higher incorporation of radioactivity into lipid linked intermediates than into the wild type. Labeled lipid-linked loligosaccharides from different *exo* mutants in *exoB-exoR* background were isolated by a chloroform-methanol-water mixture and analyzed by TLC after hydrolysis with trifluoroacetic acid and dephosphorylation by alkaline phosphatase.

Reuber and Walker (1993) proposed a pathway for succinoglycan synthesis, polymerization and export on the basis of information on the nature of the protein products of different genes as well as by the characterization of lipid-linked biosynthetic intermediates. Lack of incorporation of any radioactivity in *exoY* and *exoF* strains as well as the high homology observed between ExoY and GumD of *X. campestris* and RfbP of *S. typhimurium* indicated that the *exoY* gene product is a galactose transferase in *R. meliloti* while *exoF* is also needed for transfer of galactose to the lipid carrier. Accumulation of single sugar, disaccharide and trisaccharide residues linked to lipid carriers were observed in *exoA, exoL* and *exoM* mutants respectively which indicated that gene products of *exoA, exoL* and *exoM* function as glucose transferases that add first, second and third glucose residues to lipid-linked galactose in that order. Analysis of the lipid-linked intermediates in *exoM exoB exoR* strains showed accumulation of a compound that migrated like maltotriose as well as of a fast-migrating substance. Reuber and Walker (1993) showed that acetate group is present in the succinoglycan of Rm1021. Also, oligosaccharides with an acetyl substituent have been known to migrate faster than unmodified oligosaccharides. Therefore, the fast-migrating substance was suggested to be an acetylated trisaccharide.

Accumulation of a faster migrating species was also observed in addition to the tetrasaccharide component in *exoO* strains by thin layer chromatography showing that the ExoO protein adds a fifth sugar to its lipid linked intermediate. The component with fast mobility disappeared after treatment with KOH. These results further confirmed that the acetylation of the third sugar by ExoZ occurs in these mutants even before the octasaccharide subunit of EPS is completed. In the wild-type the trisaccharides probably convert to octasaccharide before acetylation occurs.

Based on similar observations ExoU and ExoW were suggested to be involved in the addition of sixth and seventh sugar residues respectively to the growing lipid-linked subunit. In the *exoU* strain a small peak with mobility slightly higher than the full-size wild-type intermediate was observed. The peak was suggested to indicate that some polymerization of the lipid-linked oligosaccharide occurred in these mutants to give a dimer of truncated subunit. This suggestion was consistent with the observation that *exoU* mutants produced a small amount of Calcofluor-binding material.

Previous studies showed that ExoH adds succinyl substituent to the seventh sugar of the octasaccharide subunit of succinoglycan (Leigh et al. 1987). The deduced amino acid sequence of ExoV showed a significant similarity to GumL which is a ketalase responsible for the transfer of pyruvate to the terminal sugar of the side chain of xantham gum. The *exoV* strains accumulated an unmodified octasaccharide subunit of succinoglycan that lacked a pyruvate indicating that the ExoV protein adds a pyruvate substituent to the eighth sugar of the octasaccharide subunit of EPS I. An unknown gene

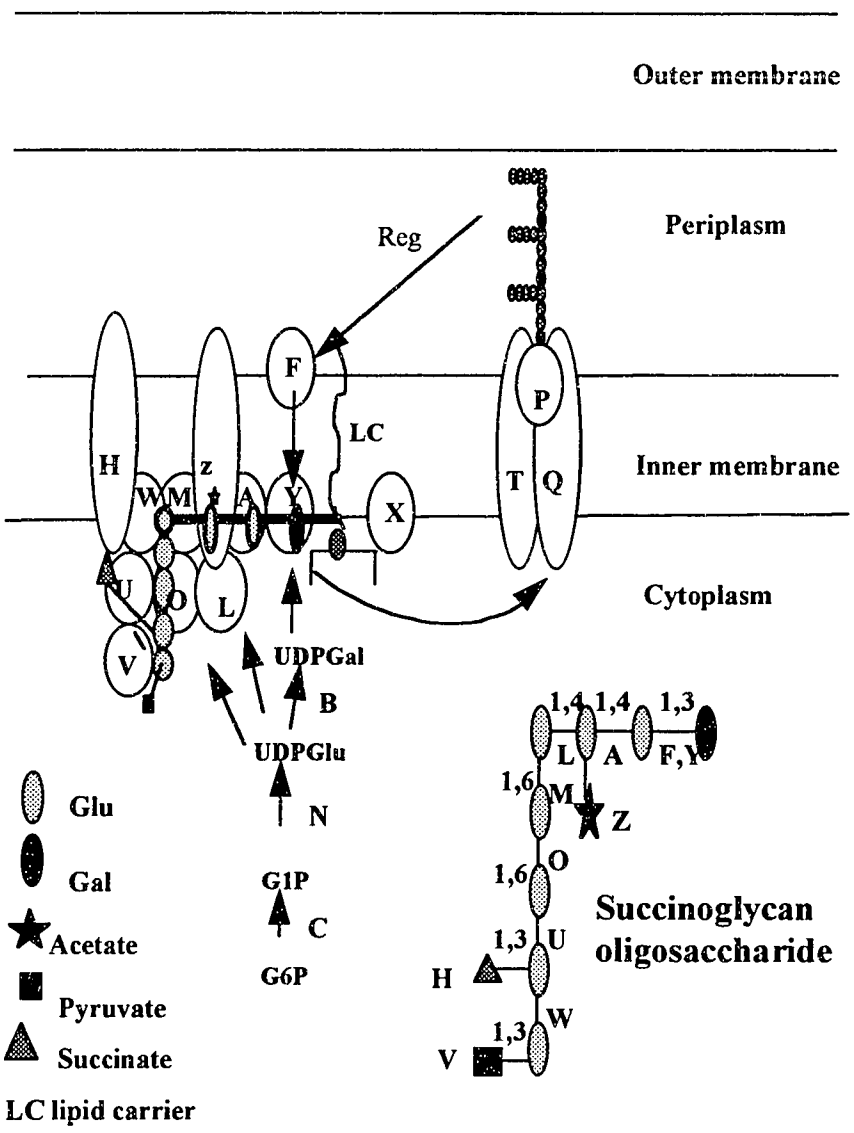


Fig. 2.2 A model showing genetics and biosynthesis of succinoglycan subunit in *R. meliloti* (Based upon Glazebrook and Walker 1993, Leigh and Walker 1993)

product was suggested to be involved in addition of the eight sugar residue to the growing subunit. The *exoP*, *exoQ* and *exoT* strains accumulated lipid-linked intermediates indistinguishable from wild type but failed to produce succinoglycan. In *exoQ* mutants a fast-migrating (probably a dimer of octasaccharide) was consistently absent. The exoP and exoT mutants also did not release any succinoglycan inspite of producing oligosacchrides indistinguishable from the wild-type suggesting a role for ExoP, ExoQ and ExoT proteins in polymerization of the octasaccharide or export of the completed polymer.

2.6 Regulation of EPS biosynthesis:

Regulation of EPS synthesis in rhizobia differs from other regulatory mechanisms in bacteria. EPS synthesis is regulated at the transcriptional, translational as well as post translational levels. A novel regulatory mechanism dependent on gene dosage was first discovered by Borthakur et al. (1985) in *R. l.* bv. *phaseoli* when a regulatory gene, *psi,* was identified which is not usually expressed under free living conditions. However, it was expressed constitutively when present in multicopies or in a *psr* background where it inhibited the expression of genes involved in exopolysaccharide synthesis. Inhibition of transcription of *psi* gene by *psr* gene under free-living conditions was noticed in *R.l. phaseoli* (Borthakur et al. 1987). The deduced polypeptide product of *psrA* showed it to be a member of transcription regulators and showed similarity to the helix-turn-helix DNA-binding domains of these transcription regulatory proteins (Mimmack et al. 1994). A gene equivalent to *psr* has not been reported in any other *Rhizobium* sp. In *Rhizobium* sp. strain NGR234 and *R. meliloti* strain Rm1021 multicopy *exoX* inhibited EPS synthesis

while *exoX* mutants produced higher amounts of EPS (Gray et al. 1990, Gray and Rolfe 1992, Zhan and Leigh 1990).

Multiple copies of *pss* gene in *R. l.* bv. *phaseoli* and *exoY* genes of NGR234 and Rm1021 overcame the multicopy effect of the *psi* gene (Borthakur et al. 1988, Gray et al. 1990, Reed et al. 1991 and Zhan and Leigh 1990). The effect of Psi A and ExoX was proposed to be post translational and they were suggested to form a regulatory complex on the cell surface with *pss* and *exoY* gene products in the respective species (Borthakur et al. 1988, Gray and Rolfe 1990, Latchford et al. 1991). However, these genes did not affect the synthesis of EPS II (Glazebrook et al. 1990).

Products of two unlinked genes, *exoR* and *exoS,* that are located on the chromosome of *R. meliloti* strain Rm1021 were found to be negative regulators of EPS I synthesis (Doherty et al. 1988). The *exoR* gene lacks homology with known regulatory genes. Increased production of normal size succinoglycan in the *exoR* strain in the presence or absence of ammonium chloride suggested that ExoR was modulated by the presence of ammonia in the medium. The *exoS* mutant produced increased levels of EPS I only in ammonia starvation conditions. Increased alkaline phosphatase activity in Tn*phoA* generated fusions with *exoA, exoF, exoP, exoQ* and *exoT* genes in *exoR* background was observed. Increased levels of transcription of several *exo* genes in *exoR* mutant confirmed the role of ExoR as a negative regulator of succinoglycan synthesis (Doherty et al. 1988, Reed et al. 1991 and Reuber et al. 1991). Reuber et al. (1991) found that mutation in *exoL, exoM, exoP, exoQ* and *exoT* in *exoR* background was lethal for rhizobia indicating accumulation of some intermediates that are toxic to bacterial cells. They suggested a

possibility that accumulation of lipid-linked intermediates results in unavailability of lipid carrier molecules for synthesis of certain essential cell components such as peptidoglycan and lipopolysaccharides. Mulligan and Long (1989) identified a symbiotic regulatory gene, *syrM*, which in conjunction with *syrA* was found to induce the expression of structural *exo* genes and suppress the Exo- phenotype of two positive regulatory *exo* genes in *R. meliloti* strain Rm1021.

Glazebrook and Walker (1989) identified a negative regulator gene, *expR*, for EPS II synthesis in *R. meliloti* since mutation in this gene resulted in increased transcription of six *exp* genes and overproduction of EPS II. The *mucR* was found to be a positive regulator for *exo* genes and a negative regulator for *exp* genes as indicated by reduction of EPS I and increase in the production of EPS II (Zhan et al. 1989). Recently, Keller et al. (1995) observed that the deduced amino acid sequence of MucR showed an 80% identity to the *Agrobacterium tumefaciens* Ros protein which is a negative regulator of *vir* genes and is essential for succinoglycan synthesis. Using transcription fusions of *exo-* and *exp-* genes with the *lacZ* marker gene they also found that MucR negatively regulated *exp* genes at the transcription level whereas *exo* genes were probably regulated post-transcriptionally. The *mucR* was also found to be autoregulatory.

2.7 Role of *Rhizobium* EPS in symbiosis with legume hosts :

Rhizobium-legume symbiosis is a multistep, complex process starting with signal exchange,root hair curling, infection thread formation, initiation of nodule formation, invasion of the nodule and finally nodule development and differentiation of bacteroids. Various studies have suggested that EPS synthesis is required for the establishment of

effective nodulation on indeterminate nodulating-legumes but not determinate nodule-forming hosts (Borthakur et al. 1986, Chakravorty et al. 1982, Chen et al. 1985, Diebold and Noel 1989, Finan et al. 1985, Hotter and Scott 1991, Kim et al. 1989, Ko and Gadya 1990, Leigh et al. 1985, Lopez-Lara et al. 1993, Napoli and Albersheim 1980 etc.). However, some exceptions do exist (Chen et al. 1985, Sanders et al. 1981, Vandenboch et al. 1985). Mutants producing altered EPS in *R.l.* bv. *phaseoli* formed small, white nodule-like structures on *Phaseolus* beans that were uninfected. Instead of normal peripheral vascular bundles and a central bacteroid zone, vascular tissue differentiated in the center of these nodule-like structures (Vandenbosch et al. 1985). Whether there was a pleiotropic effect of the mutation on other polysaccharide structures is not known.

2.7a Symbiotic phenotype of EPS-defective mutants :

The genetic evidence for involvement of EPS in symbiosis has been provided by studies with EPS-defective mutants of different *Rhizobium* spp. Chen et al. (1985) observed that alteration of EPS in various Tn5-insertions, Muc⁻ mutants of *Rhizobium* sp. strain NGR234, did not block infection and nodule initiation but severely affected nodule development. An extreme effect of Muc⁻ strains was the formation of callus-like structures on the indeterminate nodule-forming host *Leucaena leucocephala* while in the determinate nodule forming hosts Muc⁻ strains formed normal nodules to poorly developed, non-nitrogen fixing nodules. *R. l.* bv. *phaseoli pss*::Tn5 mutants induced nitrogen-fixing nodules on *Phaseolus vulgaris* while *R. l.* bv. *viciae pss*::Tn5 strain could curl root hairs but failed to nodulate *Pisum sativum* (Borthakur et al. 1986). Broad-host range *R. loti* strain NZ2037 EPS-defective mutants were ineffective on indeterminate host

L. leucocephala but formed fully effective nodules on determinate-nodule-forming *Lotus pedunculatus*. Similarly, tree legumes-nodulating *R.* sp. strain GRH2 Exo- mutant formed nitrogen-fixing nodules on *Acacia cyanophylla, A. melanoxylon* and nonlegume *Prosopis chilliness* although the number of infected cells in *A. cyanophylla* were much less as compared to the wild type. The mutant failed to nodulate indeterminate hosts *Vicia hirsuta* and *Trifolium* spp. Typical nodules were induced by *exo* mutants of *R. fredii* on the soybeans, *Glycine max* and *G. soja* (Kim et al. 1989, Ko and Gadya 1990).

Most Exo- mutants of the strain Rm1021 isolated by Leigh et al. (1985) and Finan et al. (1985) showed Fix- phenotype on *M. sativa*; however, delayed Fix+ phenotype was observed in two mutants while two more mutants formed normal Fix+ nodules. Ineffective nodules formed by Exo- mutants lacked shepherd's crooks, infection threads and bacteroids. Yang et al. (1992) observed shepherd's crooks and infection threads in Rm1021 Exo- mutants but infection threads aborted in the peripheral cells. They also observed the absence of a persistent meristematic zone in all pseudonodules induced by *exo* mutants. Niehaus et al. (1993) observed an activation of the alfalfa defense mechanism against EPS I defective mutants of *R. meliloti*. However, delayed infection of pseudonodules by EPS I-deficient bacteria occurred via aberrant infection threads and effective symbiosis was established. Therefore EPS was suggested to play a role in the suppression of the plant defense systems in addition to acting as a signal molecule to the plants and being a major component of the infection thread matrix. Caetano-Anolles (1990) found that prior inoculation of an alfalfa split root with wild-type strain completely inhibited formation of the pseudonodule by the *exoF* mutant on the other side of the root

and vice versa. This suggested that EPS is not required for feedback suppression of nodule formation.

Calcofluor-dark mutants, *exoA, exoB, exoQ, exoF, exoL, exoM* and *exoP*, of strain Rm1021 formed Fix- nodules on alfalfa while the nodules formed by Calcofluor dim mutants, *exoG, exoJ* and *exoN*, were Fix+. Mutants in *exoH* that formed haloless colonies on Calcofluor plates and produced nonsuccinylated EPS also formed empty Fix- nodules (Leigh et al. 1987) but *exoK* mutants had no nodulation defect (Long et al. 1988). Formation of shepherd's crooks was delayed and infection threads were absent in curled roots on inoculation of alfalfa roots with *exoB* and *exoH* mutants (Leigh et al. 1987, Norris et al. 1988). In addition, only one nodulin gene expression was observed in nodules elicited by *exoH* and *exoB* mutants. No infected cells were observed in the central zone of the nodules formed by *exoB* mutants. In the nodules that were formed by the *exoH* mutants release of some bacteria occurred in the host cytoplasm but they did not differentiate into bacteroids. Becker et al. (1995) noticed normal nodule formation on alfalfa by mutants with the truncated *exoP* gene. The *exoR* mutants were unable to invade alfalfa nodules but *exoS* mutants produced nitrogen-fixing nodules (Doherty et al. 1988 and Ozga et al. 1994). Some pink nodules observed in *exoR* strains were not overmucoid and had acquired a suppressor mutation in the *exoS* complementation region. The reason for this phenotype contradictory to the *exoS* phenotype was not explained. The *exoD* mutants that underproduced EPS I under nitrogen starvation conditions and in *exoR* background failed to invade the nodules and symbiotic deficiency of *exoD* strains could

not be suppressed by an *expR* mutation or by *lpsZ*+ lipopolysaccharides (Reed and Walker 1991).

The *exoB* mutants of *B. japonicum* that are defective only in EPS synthesis formed normal nodules on soybean, a determinate-nodule-forming host (Parniske et al. 1993). However, altered EPS caused a delay in nodulation indicating a possible role of EPS in the early stages of symbiosis. The mutants exhibited a much reduced competitiveness over the wild type (Bhagwat et al. 1991, Parniske et al. 1993) which is probably due to a delay in infection. Hahn and Hennecke (1988) also had shown a correlation between delay in nodulation and reduced competitiveness.

2.7b Suppression of symbiotic defects of *exo* mutants :

Different strategies have been used to overcome symbiotic deficiencies of *exo* mutants of rhizobia. Djordjevic et al. (1987) showed that addition of homologous EPS or oligosaccharide of ANU280, a derivative of the strain NGR234, or *R. trifolii* strain ANU843 to their Exo- mutants restored the Fix+ phenotype while heterologous saccharides failed to correct the symbiotic deficiencies. Restoration was successful with both determinate- and indeterminate-nodulating hosts. These workers also observed formation of nitrogen-fixing nodules on coinoculation of Exo+, Sym plasmid-cured and Exo- strains.

Klein et al. (1988) were unable to overcome the symbiotic defects of *R. meliloti exo* mutants by exogenous application of EPS but observed that in coinoculation experiments a helper strain should be *nod-exo*+, and not *nod*+*exo*+, to restore Fix+ phenotype to *nod*+*exo*- mutants. Close contact and cooperation of partners was found to

be essential to form Fix+ nodules on the host and both partners invade the alfalfa nodules and differentiate into bacteroids in mixed inoculation experiments (Kapp et al. 1990). Battisti et al. (1992) demonstrated that the exogenous application of a LMW succinoglycan fraction, containing a highly charged tetramer, and not the fractions containing smaller or less charged forms, overcame block in invasion by *exo* mutants. However, invasion was not as pronounced as Exo+ *R. meliloti.* Urzainqui and Walker (1992) independently observed exogenous suppression of symbiotic deficiencies of *exoA* and *exoH* mutants of *R. meliloti* by addition of LMW EPS during inoculation.

2.7c Expression of EPS-synthesis genes during nodulation :

Borthakur et al. (1985) found that *psi* mutants of *R. l.* bv. *phaseoli* induced nodules on *P. vulgaris* but the nodules failed to fix nitrogen. The authors suggested that under normal conditions EPS production is inhibited by *psi* gene product inside the nodules while production of EPS by bacteroids in *psi* strains caused the Fix- phenotype. However, mutations in *exoX* genes of *R. meliloti* and *R.* sp. strain NGR234, which are considered functionally equivalent to *psi* of *R. leguminosarum,* formed normal nitrogen-fixing nodules on their respective hosts (Reed et al. 1991 and Zhan et al. 1990).

Active transcription of the genes involved in EPS synthesis in *R. meliloti* strain Rm2011 in the bacteroid stage was reported by Keller et al. (1988). The *exoR* mutants that overproduced EPS I formed white, Fix- nodules from which bacteria could not be isolated (Doherty et al. 1988). Using *exo*::Tn*phoA* fusions in *exoR* background Reuber et al. (1991) observed high alkaline phophatase activity in the early symbiotic or the invasion zone, but not in the late symbiotic zone of nodules formed by *exoF, exoA* and *exoP*

mutants indicating little, if any, EPS I synthesis was needed at the later stages of nodulation. The authors suggested that *exoR* may be involved in the suppression of EPS I synthesis in later stages of symbiosis. The acquired suppressor mutations to *exoR* mutations produced pseudorevertants that formed pink nodules on alfalfa (Doherty et al. 1988). Therefore, Reed et al. (1991) and Zhan and Leigh (1990) suggested that moderation or attenuation of EPS I in nodules at later stages of development is advantageous for effective symbiosis.

Certain strains of *B. japonicum* deposited polysaccharides in the symbiosomes of the infected cells in the nodules (Streeter et al. 1992, 1993). The polysaccharide formed specifically in the nodules by the bacteroids (NPS) did not affect nitrogen fixation by the plant. NPS of one group of strains was different from EPS produced under in vitro conditions but the NPS composition of a second group of strains resembled their EPS. However, the structure of NPS has not been determined yet. Recently, Subba-Rao et al. (1995) showed the presence of symbiosome-packed infected cells at a later stage of differentiation in an aquatic legume, *Neptunia natans*. The bacteroids in symbiosomes were embedded in an unusual and elaborate electron dense fibrillar matrix which resembled the exopolysaccharide network surrounding rhizobia when they are grown in carbon-rich medium. The role of these polysaccharides in the nodules is not yet understood.

2.8 Role of *Rhizobium* LPS in symbiosis with legumes :

Mutants defective in LPS structure play different roles in symbiosis with determinate- and indeterminate-nodule-forming hosts. Mutants affected in abundance and

chain length of O antigen of LPS, i. e., altered LPS I, result in severely underdeveloped, non-nitrogen-fixing nodules on determinate-nodulating hosts such as soybeans, *Phaseolus* beans etc. (Cava et al. 1989, Maier and Brill 1978, Noel 1992, Noel et al. 1986, Puvanesarajah et al. 1987 and Stacey et al. 1991). Symbiotic defects in these mutants occurring during initial infection thread formation and invasion of cortical cells did not occur.

Nodulation of indeterminate nodulating hosts, *Pisum sativum, Vicia hirsuta, V. faba, Lens culinaris, Trifolium* spp. etc., by LPS I defective *Rhizobium* mutants was affected at later stage of infection i.e., during release of the bacteria from the infection thread. As a result, some endocytosis of rhizobia and nitrogen fixation was observed (Brink et al. 1990, Carlson et al. 1987, deMaagd et al. 1989, Noel 1992, Priefer 1989). Clover et al. (1989) observed that LPS mutants of *R. meliloti* did not affect symbiosis with alfalfa which forms the indeterminate type of nodules. However, the authors did not report the extent of alteration in LPS structure in these mutants.

Brewin et al. (1993) observed three kinds of LPS mutants of *R. leguminosarum* on the basis of their symbiotic phenotype. Severe mutants resulted in empty nodules on peas, moderate mutants delayed nodule development and reduced nitrogen fixation while slightly disabled mutants showed minor changes in the symbiotic process. Unlike the *exo* mutants severely affected LPS mutants could not infect the host in co-inoculation experiments and only the Nod- partner in this experiment could be recovered from pea nodules.

Very little information is available about the genetics of LPS synthesis in *Rhizobium* spp. Cava et al. (1989) and Priefer (1989) reported the presence of LPS synthesis genes in a cluster in *R. l.* bv. *viciae*.

2.9 Functional and evolutionary relatedness of genes involved in EPS synthesis :

Borthakur et al. (1986) observed that mutation in *pss* genes which affected EPS synthesis in *R. leguminosarum* strains could be complemented for EPS and symbiotic defect by cloned DNA that contains EPS genes of *X. campestris*, a phytopathogen of crucifers. The gene(s) on the DNA fragment of *X. campestris* was not structurally homologous to *pss* gene but showed functional homology.

The *exoB* mutants of *R. meliloti* were similarly complemented by cloned DNA of *Azospirillum brasilense* since EPS identical to normal EPS was produced by the complemented mutant. Furthermore, the Fix$^-$ phenotype of the mutant was also corrected. However, transfer of cloned DNA of *A. brasilense* to *exoC* mutants of *R. meliloti* produced a novel, Calcofluor-binding EPS but these strains did not form efficient nodules (Michiels et al. 1988). Both *R. meliloti* and *Agrobacterium tumefaciens* produce succinoglycan as the major acidic EPS and secrete neutral, cyclic β-1,2-glucans. Plasmid clones isolated from a cosmid clone library of *A. tumefaciens* complemented Calcofluor-dark *exoB, exoC* and *exoD* mutants of *R. meliloti* but not other EPS-defective mutants. Invasion of nodules was partially restored in *exoB* and *exoC* mutants by cloned DNA of *A. tumefaciens* but nodule invasiveness was not restored in *exoD* mutants (Cangelosi et al. 1987). Also, interspecies complementation of *exoB* mutants between *R. l.* bv. *viciae* and

R. meliloti was also reported (Canter Cremers et al. 1990) indicating that the *exoB* and *exoC* genes were conserved during evolution.

Genes, *ndvA* and *ndvB,* required for β-1,2-glucan synthesis and effective nodulation in *R. meliloti* were reported to be analogous to the chromosomal virulence genes, *chvA and chvB*, of *A. tumefaciens* (Dylan et al. 1986 and Lelpi et al. 1990).

R. leguminosarum psi, R. meliloti exoX and *R. sp.* strain NGR234 *exoX* genes, functioned interchangeably in the inhibition of EPS synthesis in the three species. Thus, Zhan and Leigh (1990) suggested that these genes have equivalent functions. All three proteins have been suggested to function by complexing with proteins involved in EPS biosynthesis. Also, *exoF* of *R. meliloti, exoY* of NGR234 and *pss-2* of *R. leguminosarum* showed homology and appeared to be functionally equivalent. By genetic complementation experiments using *exo* mutants and cloned genes of NGR234 and *R. meliloti,* Zhan et al. (1990) showed that NGR234 *exoY* was equivalent to *R. meliloti exoF*; NGR234 *exoC* was equivalent to *R. meliloti exoB; exoD* of NGR234, which is probably polycistronic, was equivalent to *exoM, exoA* and *exoL* of *R. meliloti*. The *exo* region of NGR234 cloned in the plasmid R'3222 contains additional, unidentified genes which were able to complement *exoP, exoG* and *exoN* mutants of *R. meliloti*. The mutations in the *exoB* region of NGR234 could not be complemented by the *R. meliloti* DNA clone bank suggesting that this region was specific for EPS synthesis in NGR234. Heterologous EPS was produced when the NGR234 deletion mutant was complemented by *R. meliloti* clones. It was proposed that EPS polymerization was provided by NGR234 in other *exo* mutants and was lacking in the deletion mutant. These transconjugants produced

heterologous EPS and were able to invade plant cells and form non-nitrogen-fixing nodules on *L. leucocephala* (Gray et al. 1991).

2.10 Functional exchangeability among rhizobial polysaccharides :

Zhan et al. (1989) and Glazebrook and Walker (1989) independently observed production of a second exopolysaccharide by *R. meliloti* strain SU47 or its derivatives in the *mucR / expR* background. This polysaccharide was found to be structurally different from EPS I or succinoglycan and did not bind Calcofluor. The most important observation was the restoration of a normal symbiotic phenotype on *M. sativa* by EPS II in the *exo* mutants of *R. meliloti*. However, Glazebrook and Walker (1989) observed that EPS II did not substitute for EPS I on four other legumes which are effectively nodulated by Rm1021 producing normal EPS I. This finding suggested that hosts recognize some structural features of the EPS or their lower molecular weight forms during symbiosis. Among the various roles assigned to EPS during symbiosis, the LMW form of EPS is suggested to be a signal molecule to the plants during nodule development (Battisti et al. 1992, Djordjevic et al. 1987, Leigh et al. 1987). Glazebrook and Walker (1989) suggested that if LMW fractions act as signal molecule, alfalfa receptors can recognize oligosaccharides derived from both EPS I and EPS II while other hosts recognize signal from EPS I only.

Putnoky et al. (1990) showed that EPS and LPS in *R. meliloti* strain Rm41 can perform the same function during symbiosis. Both *exo* and *lps* mutants of this strain produced normal effective nodules on all natural hosts while *exo-lps* double mutants formed Fix- nodules. The *fix* region involved in LPS synthesis in this strain was shown to

contain a cluster of genes organized in four complementation groups that were earlier known as the nodule development region. This region was localized on the chromosome. Williams et al. (1990) identified a new gene in Rm41 named *lpsZ* on megaplasmid b which alters the structure and composition of LPS such that it can function in place of EPS during nodule development in *exo* mutants of both SU47 and Rm41 strains of *R. meliloti*. Thus, nodules formed by *exo* mutants of *R. meliloti* in *lpsZ* background are Fix+. Williams et al. (1990b) identified two more genes, *lpsX* and *lpsY*, in both Rm41 and SU47 which are also required for suppression of Fix- phenotype of the EPS- defective mutants of *R. meliloti*. These genes were found to be located on the chromosome. Whether *lpsX* and *lpsY* are genes within the *fix* region as identified by Putnoky et al. (1990) is not known.

Suppression of *ndv* mutants of R. meliloti strain Ll1 by cosmids containing *exo* genes suggested that β-1,2 -glucans and EPS probably interact intracellularly at the initial stage of nodule development.

CHAPTER : III

MATERIALS AND METHODS :

3.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Tables 3.1 and 3.2, respectively.

Table 3.1 : Bacterial strains

Strains	Relevant characteristics	Source of reference
Rhizobium		
TAL1145	Tree legumes-nodulating wild-type strain, EPS^{+}, Rif^{R}, Str^{R}	George et al. 1994
NP84	Tn*5lacZ* insertion mutant of TAL1145, EPS & LPS defective	Parveen and Borthakur 1994
NP85	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP86	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP87	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP88	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP89	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work

NP90	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP91	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP92	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP93	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP94	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP95	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP96	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP97	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP98	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP99	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP100	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP101	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP102	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP103	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work

NP105	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP106	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP107	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP108	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP109	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP110	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP111	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP113	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP114	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP116	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP117	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP118	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP119	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP120	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP125	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work

NP126	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP127	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP128	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP129	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP130	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP131	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP132	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP133	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP134	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP135	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP136	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP137	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP138	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work
NP139	Tn3Ho*gus*-insertion, EPS-defective mutant of TAL1145	This work

NP140	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP141	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP142	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP143	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP144	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP145	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP146	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP147	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP148	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
NP149	Tn3Hog*us*-insertion, EPS-defective mutant of TAL1145	This work
RUH123	Spontaneous, EPS-defective mutant	This work
RUH124	Spontaneous, EPS-defective mutant	This work
RUH125	Spontaneous, EPS-defective mutant	This work
NGR234	Wild type, broad-host-range cowpea *Rhizobium*	Chen et al. 1988
BR828, BR833, BR835, BR836, BR842, BR846, CFN299	*R.tropici* wild type strains of Type A	Martinez et al. 1991

BR847, BR850, BR852, BR857, BR858, BR859, BR863, BR864	*R. tropici* wild type strains of Type B	Martinez et al. 1991
F4B94	*R.leguminosarum* bv. *viciae*	NifTAL collection
WU290	*R.l.* bv. trifolii	NifTAL collection
TAL182	*R. etli* wild-type strain	Pooyan et al. 1994
SU47	*R. meliloti* wild type strain	Vincent 1941
USDA110	*Bradyrhizobium japonicum* wild type strain	USDA collection
USDA205	*R. fredii* wild type strain	USDA collection
E. coli		
DH5αmcr	*recA1*, *lacU*169,φ80d *lacZ*ΔM15	Bethesda Research Laboratories
HB101 (pHo*gus*+pSheI)	Tn3Ho*gus*, Kan^R, Cam^R, Transposase in trans on pSheI	
MC1061	*hsdR, mcrB, araD* 139 Δ (*araABC-leu*) 7679, Δ*lac*X74, *galU, galK, rpsL, thi*	Meissner et al. 1987

Table 3.2 : Plasmids constructed and used

Plasmids	**Relevant characteristics**	**Source of reference**
pUHR182	pLAFR3, TAL1145 cosmid complementing the mutant NP84	Parveen and Borthakur 1994
pUHR183	pLAFR3, TAL1145 cosmid complementing the mutant NP84 and overlapping pUHR182	This work

pUHR251	6.3-kb *Eco*RI fragment from pUHR182 cloned in pRK404, complements NP84	This work
pUHR252	4.2-kb *Eco*RI fragment from pUHR183 cloned in pRK404, complements NP84	This work
pUHR221	pLAFR3, TAL1145 cosmid complementing mutants	This work
pUHR222	pLAFR3, TAL1145 cosmid complementing mutants	This work
pUHR223	pLAFR3, TAL1145 cosmid complementing RUH123 and NP125	This work
pUHR224	pLAFR3, TAL1145 cosmid complementing RUH123	This work
pUHR225	pLAFR3, TAL1145 cosmid complementing RUH125	This work
pUHR230	2.2 kb *Eco*RI fragment of pUHR221 cloned in pRK404	This work
pUHR236	1.8 kb *Eco*RI fragment from pUHR221 cloned in pRK404	This work
pUHR237	4.6 kb *Eco*RI fragment from pUHR221 cloned in pLAFR3	This work
pUHR256	7.6 kb HindIII fragment of pUHR221 cloned in pRK404	This work
pUHR257	7.0 kb HindIII fragment of pUHR221 cloned in pRK404	This work
pUHR258	4.6 kb HindIII fragment of pUHR221 cloned in pRK404	This work
pD56	pLAFR1, *R.meliloti exoBF*-complementing plasmid	Long et al. 1988
pEX154	pLAFR1, *R. meliloti exoAH*-complementing plasmid	Long et al. 1988

pEX312	pLAFR1, *R. meliloti exo* AFH-complementing plasmid	Long et al. 1988
R'3222	Plasmid containing *Rhizobium* sp. strain NGR234 *exo* genes, Tet^R	Gray et al. 1991
pIJ1427	Plasmid containing *pss* gene of *R. leguminosarum* bv. phaseoli	Borthakur et al. 1986
pIJ1433	Plasmid containing *psi* gene of *R. leguminosarum* bv. phaseoli	Borthakur et al. 1985
pUHR115	Plasmid containing *nod* genes of TAL1145	George et al. 1994
pRK404	Tet^R IncP	Ditta et al. 1985
pRK2013	RK2 derivative Kan^R, Tra^+	Figurski and Helinski 1979
pPH1JI	IncP plasmid incompatible to pLAFR3, Gen^R, Spc^R	Beringer et al. 1978
pBR322	Tet^R Amp^R colE1	Balbas et al. 1986
pSUP102-Gm	pSUP102 derivative containing transposon Tn*5lacZ*	Simon et al. 1989

3.2 Bacterial growth media and culture conditions:

All media for growing bacteria were prepared in deionized water and the pH was adjusted to 7.0 using 1N NaOH or 1N HCl. Agar at a concentration of 15 g $liter^{-1}$ was added to make solid media for plates. Media were sterilized by autoclaving at 15 lbs. per square inch for 20 min on liquid cycle. For preparation of antibiotics-containing plates molten media in 250 ml capacity Pyrex bottles (150 ml per bottle) was allowed to cool to

approximately 45°C and the appropriate amount of antibiotic solution was added. After mixing thoroughly by swirling medium was poured into plates. Final concentration of antibiotics used are listed in Table 3.3. Throughout this study *Rhizobium* cultures were incubated at 28°C and *E. coli* at 37°C. Bacterial cultures were grown in broth in flasks on a rotary shaker at a speed of 175 rpm at suitable temperatures.

Luria-Bertani Medium (Miller, 1972) :

	Per liter
Bacto-tryptone	: 10.0 g
Yeast extract	: 5.0 g
NaCl	: 5.0 g

Tryptone-Yeast extract Medium (Beringer, 1974) :

	Per liter
Bacto-tryptone	: 10.0 g
Yeast extract	: 5.0 g
$CaCl_2$	: 0.2 g

Yeast extract-Mannitol Medium (Vincent, 1970) :

	Per liter
Mannitol	: 10.0 g
Yeast extract	: 0.5 g
K_2HPO_4	: 0.5 g
$MgSO_4.7H_2O$	: 0.2 g
NaCl	: 0.1 g

EPS-Cyclic β-(1,2)-glucan secretion medium (Breedveld et al. 1990a) :

	Per liter :
Mannitol	5.0 g
Glutamic acid	1.0 g
K_2HPO_4	1.0 g
$MgSO_4.\ 7H_20$	0.2 g
$CaCl_2.\ 2H_2O$	0.04 g
$FeCl_3.\ 6H_2O$	0.0025 g
$MnCl_2.\ 4H_2O$	0.001 g
$Na_2MoO_4.\ 2H_2O$	0.00001 g
$ZnSO_4.\ 7H_2O$	0.00001 g
$CuSO_4.\ 5H_2O$	0.00001 g
H_3BO_3	0.00001 g
$CoCl_2.\ 6H_2O$	0.00001 g
Biotin	0.00001 g
Thiamine	0.0001 g

pH was adjusted to 7.0 with NaOH.

***Rhizobium* Minimal Medium (modified Kingsley, 1986) :**

Solution A		Per liter
$MnSO_4.H_2O$	:	1.510 g
$ZnSO_4.7H_2O$	:	1.150 g
$CuCl_2.2H_2O$	:	0.170 g

$Na_2MoO_4.2H_2O$	:	0.048 g
$CoCl_2$	:	0.006 g

deionized water to make the volume to 1 liter.

Solution B		Per liter
Mannitol	:	10.0 g
Sodium glutamate	:	1.8 g
KNO_3	:	0.6 g
K_2HPO_4	:	0.032 g
KH_2PO_4	:	0.016 g
$MgSO_4.7H_2O$	:	0.250 g
$CaCl_2.2H_2O$	:	0.067 g
KCl	:	0.112 g
Fe-EDTA	:	0.010 g

deionized water to make the volume to 1 liter.

Vitamin solution		Per liter
Biotin	:	0.1 g
Thiamine	:	0.1 g
Ca-Pantothenate	:	0.1 g

Vitamin solution was sterilized by filtration through 0.45 μ filter from Millipore Corp. and used at 1 ml/l of medium.

Both of these minimal media were used for production of exopolysaccharide and for quantification of the secreted EPS.

3.3 Antibiotics :

Antibiotic resistance is the most commonly used marker in molecular biological work. Resistance to the antibiotics have been used throughout this study for the selection of appropriate plasmids and transposons. Various antibiotics used in this study, their concentrations and the solvents used to prepare their stock solutions are listed in Table 3.3. Appropriate amounts of antibiotics stock solutions were added to the broth medium or to solid media melted and cooled to 45°C.

Table 3.3 : Antibiotics

Antibiotics	Stock solution (mg/ml)	Final conc. (μg/ml)	Supplier
Rifampicin (Rif)	10 (methanol)	30	USB Corp.
Streptomycin sulfate (Str)	100 (deionized water)	100	Sigma
Kanamycin sulfate (Kan)	25 (deionized water)	25	USB Corp.
Tetracycline hydrochloride (Tet)	10 (ethanol)	10	Sigma
Gentamycin sulfate (Gen)	10 (deionized water)	10	Sigma
Ampicillin Sod. salt (Amp)	200 (deionized water)	200	Sigma
Spectinomycin dihydrochloride (Spc)	100 (deionized water)	100	Sigma
Chloramphenicol (Cam)	10 (ethanol)	10	USB Corp.

3.4 Transfer of plasmids between bacteria by conjugation:

Non-conjugative plasmids can use *tra* gene products of compatible conjugative plasmids for transfer to the recipient bacteria. The plasmids used in this study to clone DNA of *Rhizobium* were non-conjugative and lacked the ability of self transfer. Therefore, plasmids were transferred from *E. coli* to *Rhizobium* by the triparental patch mating method (Johnston et al. 1978). The *Rhizobium* strain was first patched on Tryptone-yeast extract (TY) medium containing 1% mannitol and incubated overnight at 28°C. The next day it was mixed with the *E. coli* plasmid donor strain from an overnight-grown plate culture and 30µl of freshly grown broth culture of *E. coli* strain containing the conjugative or helper plasmid pRK2013 or pRK2073. The plates were incubated overnight at 28°C and the bacteria in the patches were streaked or spread plated on selective media containing appropriate antibiotics. The transconjugants were observed within 5 to 7 days of incubation at 28°C.

3.5 Preparation of *E. coli* competent cells and transformation (Cohen et al. 1972)

Mandel and Higa (1970) first observed that bacteria treated with ice-cold $CaCl_2$ and then heated momentarily can be transfected by bacteriophage λ DNA. The same method which was used throughout this study was first used by Cohen et al. (1972) for transformation of bacteria with plasmid DNA. *E. coli* strain was inoculated in 5 ml LB broth and was grown at 37°C overnight. The next day 500 ml of LB medium was inoculated with this culture and incubated on a rotary shaker at 37°C for 2 to 3 hrs until an OD_{600} of 0.4-0.6 was obtained. The culture was harvested by centrifugation at 6,000xg at 4°C. The pellet was resuspended in 50 ml of cold 0.1 M $CaCl_2$ and kept over ice for 20-25

minutes. After centrifugation the supernatant was discarded and the pellet was resuspended in 5 ml of 0.1M $CaCl_2$ and glycerol (85 and 15% respectively) solution. The suspension was dispensed in sterilized microfuge tubes and the tubes were immediately frozen in isopropanol chilled with dry ice. The competent cells so obtained were stored at -70°C until needed.

Transformation was carried out using the modified method of Cohen et al. (1972). To 100 µl of competent cells 100 µl of chilled 0.1M $CaCl_2$ and 1 to 10 µl of plasmid DNA were added and the mixture was incubated over ice for 20 min. After giving a heat shock at 42°C for 1-2 minutes, the tubes were chilled on ice for 1 min. The mixture was transferred to 3 ml LB broth and incubated at 37°C for 1 hr with shaking. The cultures were spread on appropriate antibiotics-containing LB medium and the plates were incubated at 37°C. The transformants appeared within 24 hrs.

3.6 Isolation of genomic DNA of *Rhizobium*

3.6.1 Large scale preparation of genomic DNA of TAL1145 (Pooyan et al. 1994) :

The application of molecular biological techniques to analyze complex genomes requires preparation of pure high molecular weight DNA which can be easily digested with the restriction endonucleases. Different protocols can be used to obtain purified genomic DNA from bacteria. TAL1145 genomic DNA was used as control in different Southern hybridization experiments and was needed in larger quantity. Therefore, genomic DNA was prepared by the standard procedure of Ausubel et al. 1989 as modified by Pooyan et al. 1994.

Rhizobium culture was grown in 5 ml TY medium for two days at 28°C. One ml of this culture was used to inoculate 50 ml TY broth and the broth culture was grown for two days with shaking. Sodium-n-lauryl sarcosine (sarkosyl) /pronase E solution (10% Sarkosyl and 5mg/ml pronase E) was prepared in TE_{25} (10 mM Tris, pH8.0, and 25 mM EDTA) and incubated at 37°C for one hr to get rid of the nucleases. A two day old culture was centrifuged at 5,000x g for 10 min and the pellet was washed once with 20 ml of 1M NaCl and twice with 20 ml of TES buffer (10 mM Tris, pH 8.0, 25 mM EDTA and 150 mM NaCl). Fresh lysozyme solution (2 mg/ml in TE_{25}) was prepared while spinning down the cells. The cell pellet was suspended in 5 ml TE_{25} buffer and treated with 0.5 ml lysozyme solution at 37°C for 15 min. To this suspension 0.6 ml of sarkosyl-pronase mixture was added, mixed and incubated at 37°C for 2 hrs to break down the proteins completely. The viscous lysate so obtained was extracted with an equal volume of equilibrated phenol, pH 7.8 in SS34 tube. Equilibrated phenol was prepared by adding 300 ml of warm (to boiling) 25 mM NaCl, 10 g of Tris-base and 0.9 g of 8-hydroxyquinoline to good quality 500 g solid phenol kept at 60°C for 30 min. Phenol and Tris were allowed to completely dissolve at room temperature overnight and then phenol was kept in the refrigerator. After extraction with phenol the remaining proteins and phenol present in the aqueous layer were removed by treating with an equal volume of chloroform. The volume of aqueous layer obtained after chloroform extraction was determined and 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol were added. The tube was capped and inverted several times until contents were completely mixed and white fibrillar DNA was observed floating on the surface. DNA was spooled

with a closed end, hooked disposable Pasteur pipette, rinsed in 70% ethanol for one minute and then dried on the Pasteur pipette for 5-10 min. DNA was finally suspended in 1 ml TE buffer (10 mM Tris, pH8.0, and 1 mM EDTA).

3.6.2 Mini-Prep of *Rhizobium* genomic DNA (Saunders, 1989) :

The *Rhizobium* strain was grown in 10 ml of TY broth for 2 days. After centrifugation at 5,000x g for 10 min. the cell pellet was suspended in 1 ml 1M NaCl and transferred to a microfuge tube. The pellet was centrifuged and washed twice with 1 M NaCl. The supernatant was aspirated completely and the pellet was resuspended in 200µl of 10 mg/ml lysozyme. After incubation at room temperature for 20 min the cells were lysed by addition of 400 µl of 5 M guanidine thiocyanate, pH 7.0, with 0.1 M EDTA. The lysate was mixed by inversion and 300 µl of 7.5 M ammonium acetate was added and mixed. The cloudy lysate was vortexed with an equal volume (1 ml) of chloroform : isoamyl alcohol (24 : 1) mixture. The emulsion so obtained was centrifuged at 16,000x g for 5 min and 700 µl of the aqueous phase was transferred to another tube. DNA was precipitated by addition of 0.54 volume of isopropyl alcohol followed by incubation at room temperature for 10 min. The DNA pellet was obtained by centrifugation at 16,000x g for 10 min. After discarding the supernatant the DNA pellet was washed with 76% ethanol in 10 mM ammonium acetate. After air drying, the DNA pellet was resuspended in 200 µl TE buffer.

3.7 Extraction and purification of plasmid DNA :

3.7.1 Isolation of recombinant plasmid from *Rhizobium* by alkaline-prep method (Grosveld et al. 1981) :

Plasmids are stably inherited extrachromosomal replicons. They have been widely used as vectors to clone and transfer DNA fragments into various hosts and to study the expression of genes present on cloned fragments. Various techniques have been used to isolate the recombinant plasmids and analyze the cloned fragment.

Rhizobium transconjugants grown in 10 ml TY broth or on plates were harvested and washed twice with 1 M NaCl to remove polysaccharides. The cell pellet was then resuspended in 200 µl GTE buffer (50 mM glucose, 25 mM Tris, pH8.0, and 10 mM EDTA). The cells were lysed with 400 µl of 0.2 M NaOH and 1% SDS mixture (solution II) by incubation at room temperature for 5 min. After adding 300 µl of 5M potassium acetate and 3M formic acid mixture (solution III) the suspension was mixed thoroughly but gently and kept over ice for 5 min. After centrifugation for 10 min at 16,000x g the clear supernatant was transferred to another microfuge tube. The plasmid DNA was precipitated with 0.1 volume 3 M Sodium acetate and 0.54 volume isopropyl alcohol at room temperature for 5 min. The DNA pellet obtained after centrifugation was washed once with 70% ethanol, dried and resuspended in 20 µl TE buffer.

3.7.2 Isolation of plasmid from *E. coli* (Holmes and Quigley 1981) :

A loopful of *E. coli* culture grown on an appropriate antibiotic-containing plate was suspended in 300 µl STET buffer (8% sucrose, 0.5% Triton 100, 25 mM EDTA and 50 mM Tris, pH 8.0) to which was added 30 µl of 10 mg/ml lysozyme. After incubation at room temperature for 5 min the tube was placed in a boiling-water bath for 45 seconds. The bacterial lysate was centrifuged at 16,000x g in a microfuge for 10 min and the debris was removed using a micropipette. The DNA was precipitated with an equal volume of

isopropanol at room temperature for 5 min and the pellet was recovered by centrifugation. The supernatant was removed by aspiration and the DNA pellet was washed with 70% ethanol to remove salts. After drying DNA was resuspended in 20-30 µl TE buffer by gentle vortexing.

3.7.3 Large-scale preparation of plasmid DNA (Clewell and Helinski 1969) :

Plasmids can be isolated from *E. coli* cultures by the alkaline-lysis method as mentioned earlier for *Rhizobium*. Highly purified plasmid DNA can be obtained by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. The purification procedure exploits the relatively small size and covalently closed nature of plasmids. Linear and nicked DNA bind ethidium bromide to saturation while binding of ethidium bromide to circular DNA is limited due to topological constraints. Therefore, supercoiled plasmid DNA remains more dense and forms a discreet fluorescent band on ultracentrifugation which can be selectively removed using syringe and needle.

An *E. coli* culture containing the plasmid was grown overnight at 37°C in 500 ml LB broth with appropriate antibiotics. The cells were harvested by centrifugation in polystyrene bottles and the pellet was resuspended in 20 ml of GTE buffer. To this suspension 40 ml of solution II containing 0.2 M NaOH and 1% SDS was added and mixed by gentle swirling. After 5 min incubation at room temperature 30 ml of solution III (5M potassium acetate and 3 M formic acid) was added, mixed and the cell lysate was kept over ice for 5 min. The debris was separated by centrifugation at 8,000x g at 4°C for 10 min. Clear supernatant was filtered through cheesecloth and the DNA was precipitated with 0.54 volume of isopropanol at room temperature for 5 min. After centrifugation at

8,000x g for 10 min the supernatant was discarded and bottles were inverted on paper towels to drain away the last drops of supernatant. The DNA pellet was allowed to air dry and resuspended in TE to a total volume of 4.1 ml. In this DNA solution 4.9 g CsCl was dissolved and 0.7 ml of 10 mg/ml ethidium bromide solution (in water) was added before immediately mixing and centrifugation at 4°C at 8,000x g for 10 min. The clear red solution was transferred to a Beckman Quick-Seal ultracentrifuge tube and two tubes for opposite slots in the centrifuge rotor were accurately balanced (within a weight difference of 10 mg) and then sealed. The tubes were centrifuged in a Beckman centrifuge Vti65 rotor at 55,000 rpm for 12-16 hrs or Vti80 rotor at 75,000 rpm for 5 hrs. Two bands were usually observed, a thin upper band consisting of chromosomal and nicked plasmid DNA and a lower band of closed circular plasmid DNA. While viewing under a high wavelength range UV lamp a 18-gauge needle was inserted into the top of the ultracentrifuge tube to allow the air to enter. Another hypodermic needle attached to a Luer-lock syringe was inserted under the lower band with beeved side up. DNA was collected slowly into the syringe and transferred to a sterilized disposable tube.

To the solution of DNA an equal volume of 1-butanol saturated with water was added. After vortexing, the solution the two layers were allowed to separate. The upper butanol layer containing ethidium bromide was discarded. The extraction process was repeated until no pink color was observed in the butanol phase. The DNA solution was transferred to a dialysis bag (molecular weight limit of 8,000) and spin dialyzed against several changes of TE buffer to remove CsCl. The plasmid DNA was precipitated with 0.1 vol. 3M sodium acetate and 2.5 vol. cold ethanol. The DNA pellet recovered by

centrifugation was washed with 70% ethanol, dried and dissolved in 500 µl (low copy number plasmids) to 1 ml of TE buffer. The DNA was stored at -20°C.

3.7.4 Isolation of large indigenous plasmids of TAL1145 by in situ lysis :

Two ml of 10 hr old TAL1145 grown in TY broth at 28°C with continuous shaking was centrifuged in a microfuge tubes at 5,000 rpm for 3 min. The supernatant was discarded and the cell pellet was washed twice with 1 M NaCl. A 0.6% agarose gel was prepared. The cell pellet was finally washed with 1 ml of 0.3% Sarkosyl solution. The pellet was resuspended in 100 µl of lysozyme solution-containing 20 µg/ml lysozyme, 15% sucrose and 1% SDS and then loaded immediately on the gel and left undisturbed for 5 min. The suspension was then overlayered with 100 µl dye solution-containing 10% sucrose, 1% SDS and dye (10 mg bromophenol blue /ml). Gel electrophoresis was conducted at 20 volts for one hr followed by 95 volts for 6 hrs a in cold room.

3.8 Digestion of DNA with restriction enzymes and agarose gel electrophoresis:

3.8.1 Restriction enzyme cleavage :

Restriction enzyme systems probably evolved as mechanisms to degrade foreign DNA while leaving the self-DNA unaffected. Self-DNA is protected from cleavage by these enzymes by alterations of DNA with associated modification enzymes. Type II restriction enzymes recognize a specific nucleotide sequence usually 4-8 nucleotides long and under proper buffer and temperature conditions cleave the DNA producing blunt or cohesive ends.

Table 3.4 Restriction and modification enzymes:

Restriction/modification enzymes	Recognition sequence Cleavage sites	Suppliers
*Bam*H I	G▼GATCC	Promega Corporation
*Bst*E II	G▼GTNACC	United States Biochem. Corp.
*Eco*R I	G▼AATTC	Promega Corporation
*Eco*R V	G▼ATATC	Promega Corporation
*Hin*d III	A▼AGCTT	Promega Corporation
Kpn I	GGTAC▼C	United States Biochem. Corp.
Pst I	CTGCA▼G	Promega Corporation
Sac I	GAGCT▼C	Promega Corporation
Sal I	G▼TCGAC	Promega Corporation
Sma I	CCC▼GGG	New England Biolabs
Sph I	GCATG▼C	Promega Corporation
Xba I	T▼CTAGA	United States Biochem. Corp.
Xho I	Pu▼GATPy	Gibco Bethedsa Research Laboratories
Klenow	_	Promega Corporation
Taq I DNA Polymerase	_	Promega Corporation
Shrimp Alkaline Phosphatase	_	United States Biochem. Corp.

The various enzymes used in this study, their suppliers, recognition sequence and cleavage sites are listed in Table 3.4. Incubation conditions for enzyme reactions were as mentioned by the suppliers. Special conditions are mentioned wherever necessary. Enzymes were stored in -20°C. Assay conditions were used as recommended by the suppliers. In general, 2-3 units of enzyme were used to digest 1 μg of DNA for 1 hr and 20 μl of digested DNA was loaded for gel electrophoresis. Phenol and chloroform extraction of DNA was carried out for further manipulation of DNA after digestion with a restriction enzyme. DNA was precipitated with 0.1 vol. 3M sodium acetate and 2.5 volume cold ethanol and the DNA pellet was washed with 70% ethanol, dried and redissolved in TE for further modifications.

3.8.2 Agarose gel electrophoresis :

DNA and RNA can be separated by gel electrophoresis on the basis of their molecular weights. Polyacrylamide can be used for separation of very small DNA fragments. Agarose gel electrophoresis was used to determine size of DNA fragments throughout this study using *Hin*dIII digested λ phage DNA as the standard size marker.

Agarose gels of 0.7-1.0% concentration were prepared in TAE buffer containing 40 mM Tris-acetate and 1 mM EDTA by using a microwave oven. Different sizes of gel trays with 1 or 2 mm thick combs were used to cast the gel. After gels were set the gel trays were transferred to a gel tank filled with TAE buffer and DNA samples mixed with gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll in water) were loaded in the slots. The gel tank was connected to power supply by electric leads so that DNA migrated towards the anode. Ethidium bromide-DNA complexes emit

more red-orange light under UV than unbound ethidium bromide resulting in easy detection of DNA bands. However, ethidium bromide moves towards the cathode during electrophoresis and longer electrophoresis interferes with detection of smaller bands. Therefore, gels were stained after electrophoresis by soaking in ethidium bromide solution (approximately 10 μg/ml) for 15-20 min and were observed over the UV transilluminator.

3.9 Cloning of DNA fragments in plasmid vectors :

Shot-gun or directed cloning of DNA fragments in suitable vectors was done by methods as described below.

3.9.1 Preparation of plasmid vectors :

Cloning of DNA in a plasmid vector involves in vitro joining of plasmid and foreign DNA cleaved with same enzyme to obtain a recombinant plasmid. However, to increase the efficiency of cloning, it is essential to prevent recircularization of plasmid vector DNA. Directional cloning of DNA is possible when two different enzymes for digestion of plasmid vector can be used which are compatible with cohesive termini of foreign DNA. Recircularization of plasmid DNA can also be prevented by removal of the 5'-phosphate group from the linearized plasmid. Both of these methods were used in this study.

The plasmid used for cloning DNA fragments was first digested completely with an appropriate restriction enzyme. An aliquot of 0.5 μg of the linearized plasmid was checked by gel electrophoresis for complete digestion. Completely digested plasmid DNA was extracted with phenol and chloroform and precipitated and washed as mentioned earlier. After dissolving DNA in TE buffer, 1x shrimp alkaline phosphatase buffer with

one unit of shrimp alkaline phosphatase (SAP) per μg DNA were added and the reaction mixture was incubated at 37°C for 45 min to 1 hr. SAP was inactivated by incubating at 70°C for 10 min. The DNA was extracted with phenol and then with chloroform, precipitated with 0.1 vol of 3M sodium acetate and 2.5 vol cold ethanol and centrifuged. The DNA pellet was washed with 70% ethanol, dried and resuspended in TE buffer. Cloning of smaller DNA fragments in the plasmid vectors so prepared from cosmid clones was performed by one of the two methods mentioned below.

3.9.2 Shot-gun cloning :

Ligation of a fragment of DNA to linearized dephosphorylated plasmid vector involves formation of two new phosphodiester bonds between the 5'-phosphate residue of a DNA fragment and 3'-hydroxyl moieties of plasmid vectors by the bacteriophage T_4 DNA ligase enzyme. The hybrid molecules so obtained carry two single-strand nicks that are repaired when transferred to the competent cells.

For shot-gun cloning the cosmid clone was first digested completely with an appropriate enzyme. The ligation reaction mixture was set by mixing equimolar amounts of vector and digested cosmid DNA, 10 mM DTT, 1 mM ATP, 1x ligase buffer, 1 unit of T_4 ligase and water to make a final volume of 10 μl. The ligation control reaction with plasmid vector alone was also included. For blunt end ligations 10 times the concentrations of enzyme and ATP were used. The reaction mixture was incubated overnight at 18°C and recombinant DNA was used to transform DH5αmcr competent cells. Individual clones were analyzed by isolating plasmid DNA by the boiling-prep method and restriction enzyme digestion was followed by agarose gel electrophoresis.

Identification of bacterial colonies that contained recombinant pUC plasmids was facilitated by selection for lack of the α-complementation of the β-galactosidase gene (*lacZ*). Plasmids of the pUC series as well as pRK404 carry short regulatory sequence and N-terminal sequence of the *lacZ* gene while *E. coli* DH5αmcr contains a deletion of the operator proximal region of this gene. The bacterial encoded carboxy-terminal region and plasmid encoded N-terminal region of the *lacZ* gene result in Lac+ colonies due to α-complementation which can be easily identified as blue colonies on LB plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The colonies containing the recombinant plasmids remain white. On the LB plates containing appropriate antibiotics, 40 µl of X-gal solution (20 mg/ml in dimethylformamide) and 5 µl of IPTG (200 mg/ml in water) were spread and the plate was allowed to dry. Transformants were then plated on this plate to select colonies with recombinant plasmid.

3.9.3 Isolation of DNA fragment from low melting agarose gel and cloning :

Gels of highly pure low melting temperature agarose were used for isolation of DNA fragments for cloning. This agarose melts at 40°C and DNA fragments can be extracted without damage. Preparative gels with wide a comb (approximately 3-4 cm width)using 1.5% agarose were prepared and electrophoresis was done in the cold room to ensure that the gels did not melt during the run. A slice of the gel was cut longitudinally through the λ DNA size marker lane alongwith a 4 mm wide sample DNA lane. After staining with ethidium bromide the gel slice was placed together with the remaining gel and observed under long wave length UV. Using this method the DNA bands could be observed throughout the gel by reflection and radiation damage to the

DNA was prevented. The DNA bands of interest were cut out with a sharp scalpel blade and transferred to sterilized glass tubes. After melting the agarose at 65°C about 3 vol of TE buffer was added to each tube. The tubes were kept at 65°C for 5 min, cooled and an equal volume of equilibrated phenol was added. After gentle vortexing the aqueous phase was recovered by centrifugation. The aqueous phase was re-extracted with phenol followed by an extraction with chloroform. DNA was precipitated and washed as mentioned earlier and dissolved in TE buffer. Ligation with suitable vector was done as mentioned in the shot-gun cloning method (section 3.9.2).

3.10 Southern blotting of DNA to nylon membrane (Southern 1975) :

DNA fragments were transferred from the gel to the nylon membrane by a capillary transfer method throughout this study. In this method liquid is passed throughout the gel by the capillary action of a stack of absorbent paper towels placed over the gel.

After staining with ethidium bromide the gels were photographed using a polaroid camera. The unused areas were trimmed and the gel was treated with 0.25 M HCl with gentle shaking for depurination and nicking of large fragments of DNA. The gel was rinsed twice with deionized water and then treated with 0.4 M NaOH for denaturing double stranded DNA. After rinsing with water the gel was soaked in neutralization buffer (1M Tris-HCl, pH 7.5, and 1.5 M NaCl) for 30 min at room temperature when the nonradioactive method for DNA-DNA hybridization was used. Blotting was done using 0.4 M NaOH to transfer the DNA to GenScreen Plus membrane and with 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) to transfer the DNA to MagnaCharge membrane for

detection by nonradioactive probes. For blotting of DNA a glass plate was placed horizontally over the edges of a tray. A 3 MM Whatman filter paper was placed on the glass plate so that it covered the plate horizontally and its two ends dipped into the solution in the glass tray. The gel was placed on wet Whatman 3 MM paper upside down and the paper around the gel was covered with parafilm. A wet Gene Screen Plus or Magna Charge nylon membrane cut to the size of the gel was placed over the gel slowly to avoid trapping of air bubbles between the gel and the nylon membrane. Two wet filter papers were placed over the nylon membrane and a dry paper was placed on the top. A 5 cm thick layer of paper towels, a glass plate and an approximately 1 kg weight were placed on the Whatman paper and DNA was allowed to transfer overnight. Next day the weight, glass plate, paper towels and the Whatman paper layers were removed and the nylon membrane with the gel on top was placed on a filter paper. The slot positions in the gel were marked on the membrane with a ball-point pen. The gel was removed and the nylon membrane was rinsed with 2 x SSC + 100 mM Tris, pH7.5. The membrane was baked for 1 hr at 80°C (optional for Gene Screen Plus but compulsory for MagnaCharge nylon membrane) in a vacuum oven.

3.11 Southern hybridization of immobilized DNA with ^{32}P-CTP labeled DNA :

DNA transferred to the nylon membrane can be hybridized with oligonucleotide probes as small as 17 nucleotides long. Hybridization with radioactive probes was done under high stringency conditions (65°C) unless specified. Only homologous DNA hybridizes at high temperature while DNA duplexes which have a few nucleotide

mismatches are unstable and the probe dissociates from the immobilized DNA on the membrane.

3.11.1 Preparation of DNA probes by the random primer method :

For radioactive labeling of DNA fragments with high specific activity a mixture of all components except radiolabeled deoxynucleotide triphosphate and enzyme was prepared and stored at -70°C in 11.6 µl aliquots.

Tris-$MgCl_2$ (TM) buffer :

		Per ml
1 M Tris (pH 7.5)	:	250 µl
1M $MgCl_2$	:	25 µl
β-mercaptoethanol	:	4 µl
deionized water	:	721 µl

Deoxynucleotide mix (final concentration of 200 µM of each) :

		Per ml
10 mM dATP	:	20 µl
10 mM dGTP	:	20 µl
10 mM dTTP	:	20 µl
TM buffer	:	940 µl

Hexanucleotide mix :

Oligonucleotide mixture was dissolved in 1mM Tris and 1 mM EDTA, pH7.5 to a final concentration of 4.5 mg/ml (or 90 OD_{260}).

Hepes buffer : 1 M, pH 6.6

The DNA labelling mixture (2.32 ml) was prepared by mixing 1 M Hepes buffer, deoxynucleotide mix, Hexanucleotide mix, and bovine serum albumin (50 mg/ml) in a ratio of 25:25:7:1. The mixture (LS-BSA) was dispensed in 11.6 µl aliquots and stored immediately at -70°C.

For preparation of the probe water was added to 200 ng of DNA to bring the volume to 13.4 µl and boiled for 10 min. This was immediately chilled over ice and to this was added 11.6 µl LS-BSA, 5 µl α -^{32}P CTP and 1 µl (approximately 10 units) klenow enzyme. Incubation was at room temperature in a radioactive area.

3.11.2 DNA-DNA hybridization :

Prehybridization of the membranes before probe hybridization blocks non-specific nucleic acid-binding of the probe to the membrane. The prehybridization solutions mentioned below have been found to be efficient in blocking and produce no background.

Prehybridization solution :

Final concentration of the components were :

Dextran sulfate	:	10%
NaCl	:	1 M
SDS	:	1%

Water to adjust the volume.

For a 3x5 inch size nylon membrane a 20 ml prehybridization solution was used. To 20 ml of this solution 200 µl of 10 mg/ml salmon sperm DNA, boiled for 10 min and cooled over ice was added to prevent nonspecific probe binding to the membrane.

Prehybridization of the membrane was done in sealed plastic bags in awater bath or in bottles in a hybridization chamber from 2-5 hrs.

To the DNA probe 50 µl salmon sperm DNA and 150 µl water were added and the probe was boiled for 10 min. This was immediately cooled over ice and transferred to the prehybridization solution. Southern hybridization was done overnight at 65°C.

The next day the probe was transferred to a suitable container or stored at -70°C for reuse and the membrane was washed three times with 2 x SSC and 1% SDS solution. The membrane was wrapped in Saran Wrap. The wrapped nylon membrane was exposed to the X-ray film, Fuji or Kodak, in light-proof cassettes with an intensifying screen to obtain an autoradiographic image. Exposure was done at -70°C for 5 hrs to 48 h depending on the signal of ^{32}P. The X ray film was developed using Kodak developer and fixer as recommended by the suppliers.

3.12 Nonradioactive method of Southern hybridization :

Southern hybridization using nonradioactive probes is a sensitive and safe method for detection of DNA-DNA hybridization. Also, the exposure time is short as compared to that required for radioactive probes. The Genius™ System of Boehringer-Mannheim Chemicals was used for Southern hybridization of DNA by the nonradioactive method. Lumi-Phos 530 containing Lumigen™ PPD and an enhancer for chemiluminescent detection of alkaline phosphatase was used as a substrate in hybridization experiments. All solutions were filtered to minmize background.

3.12.1 Random primed DNA labelling with digoxigenin (DIG):

DNA was labelled with digoxigenin-dUTP by the random priming method. For preparation of the probe 1-3μg of DNA diluted with water to make the volume to 15 μl was boiled for 10 min and chilled on ice for denaturation. To the denatured DNA in a microfuge tube on ice was added 2 μl of 10X hexanucleotide mixture and 2 μl of DIG labelling dNTP mixture (10X). The tube was kept at room temperature for 5 min and 1 μl of klenow enzyme was added to a final concentration of 100U/ml. Labelling of DNA was allowed to occur at 37°C overnight.

3.12.2 Prehybridization and hybridization :

Hybridization buffer + 50% formamide :

5 X SSC

Deionized formamide	:	50.0%
Sodium lauroylsarcosine	:	0.1% using 10% stock solution
SDS	:	0.02% using 20% stock solution
Blocking reagent	:	2.0%

Washing solution 2X :

SSC	:	2X
SDS	:	0.1%

Washing solution 0.5X :

SSC	:	0.5X
SDS	:	0.1%

The membrane blot was placed in a hybridization tube and 20 ml of hybridization solution was added. Prehybridization was done in a hybridization chamber with continuous

rotation for 2 h at 42°C for genomic DNA and heterologous plasmid DNA and 50°C for homologous plasmid DNA blots. The DNA probe labelled with DIG was boiled for 10 min and immediately chilled over ice to denature DNA. The probe was diluted in a hybridization solution. The prehybridization solution was discarded and the hybridization solution containing the labelled probe was added. Hybridization was done overnight at the temperatures mentioned above. The hybridization solutions containing labelled DNA probes were stored at -20°C for future use. For reuse this probe was denatured at 68°C for 10 min.

The membrane was washed twice with 2X wash solution for 5 min each followed by two washings with 0.5X wash solution for 15 min each at the hybridization temperature.

3.12.3 Detection of DIG labelled DNA :

Chemiluminescent detection of DIG-labelled nucleic acids is a three step process i.e., blocking of the membrane, treatment with the anti-digoxigenin Fab fragment of antibody conjugated to alkaline phosphatase and third, reaction with the substrate Lumi-Phos 530 to assay for alkaline phosphatase activity. Finally the membranes were exposed to X-ray film to record the chemiluminescent signal.

Maleic acid buffer (Buffer 1) :

Maleic acid	0.1M
NaCl	0.15M

pH 7.5

Blocking reagent stock solution:

Skimmed nonfat dry milk 10% in buffer 1

Blocking solution :

Diluted blocking reagent stock solution to 1% in buffer 1

Washing buffer :

Added 0.3% (w/v) Tween 20 to the autoclaved buffer 1

Probe stripping solution :

NaOH 0.4 N

SDS 0.1%

After post-hybridization washing the membranes were treated with Blocking reagent for 1 h at room temperature to prevent nonspecific binding of antibody to the membrane. The anti-DIG antibody-alkaline phosphatase was diluted 1:10,000 in blocking solution. The blocking solution was discarded and the membrane was incubated with the antibody working solution prepared as above with gentle shaking. After incubation the antibody solution was discarded and the membrane was washed with washing buffer two times for 15 min each. After pouring off the washing buffer the membrane was equilibrated in detection buffer for 2 min. The membrane was placed between two clear acetate sheets (overhead projector transparencies). The top sheet was lifted gently and 0.2 ml of Lumi-Phos 530 was added in a line parallel to the membrane. The top sheet was lowered slowly to avoid trapping of air bubbles between the membrane and the acetate sheets. The membrane with the acetate sheets was incubated at 37°C for 15 min and then exposed to X-ray film for 1h and developed as described in the Southern hybridization by the radioactive-probe method.

3.13 Isolation of mutants:

Transposons are currently the most widely used in the mutation of genes. The advantages of using a transposon over other methods of mutagenesis are that transposon insertion results in non-leaky phenotypes with the complete disruption of the genes, selectable markers on the transposon, such as resistance to antibiotics, facilitates isolation of mutants and the site of the insertion of the transposon and hence the location of the gene can be determined by restriction endonuclease digestion and Southern hybridization. Various derivatives of transposons have been constructed by different workers that generate gene fusions with promoterless reporter genes such as *lacZ, phoA, luxAB, uidA* or *gusA, merA, arsA* and *arsB* etc. by insertional mutagenesis. Two such transposons, Tn5*lacZ* and Tn3Ho*gus* were used in this study for isolation of the mutants.

3.13.1 Tn5*lacZ* mutagenesis (Simon et al. 1989) :

The mobilizable plasmid pSUP102-Gm, a derivative of pACY184, carrying truncated *lacZ* in Tn5-B20 was transferred to *Rhizobium* strain TAL1145 by patch mating. The transconjugants were selected on YEM plates containing Rifampicin, Streptomycin and Kanamycin. The plasmid pSUP102-Gm can not replicate in *Rhizobium*, therefore, incorporation of kanamycin in the selection media ensured selection of random transposon mutants of TAL1145. One EPS-defective mutant, NP54, that forms small opaque colonies on YEM when compared with the large mucoid colonies of TAL1145 was selected.

3.13.2 Tn3HoGUS mutagenesis :

Three EPS-defective spontaneous mutants of TAL1145 were obtained on YEM. The mutants formed very small, dry colonies on this medium. Patch mating of the mutants with the TAL1145 cosmid clone library in *E. coli* strain HB101 was done using pRK2013 as helper as mentioned earlier. The mixture was grown overnight and then both streaked and spread plated on YEM Rif Str Kan Tet medium. After 5 days incubation at 28°C complemented mutants showing a wild type phenotype were selected. Six overlapping cosmid clones complementing these mutants were isolated. Two cosmid clones, pUHR221 and pUHR222 were used further for site-directed mutagenesis.

Competent cells of *E. coli* strain HB101 were prepared that contain Tn3Ho*gus* on a plasmid by using a transposase enzyme present *in trans* on the plasmid pShe1. These cells were transformed with the cosmids pUHR221 and pUHR222 and transformants were selected on LB plates containing tetracycline and kanamycin. All transformants so obtained were pooled and triparental mating was done with TAL1145 using pRK2013 as the helper. The mating mixture was plated on YEM Rif Str Kan Tet plates. The plasmids pUHR221 and pUHR222 containing transposon *Tn3Hogus* were thus transferred to TAL1145. All colonies so obtained were pooled and marker exchange between transposon containing cloned DNA in pLAFR3 and wild type genomic DNA of TAL1145 was enforced by transfer of plasmid pPH1JI by conjugation. The plasmid pPH1JI carries the gene for gentamycin resistance and is incompatible with pLAFR3. Therefore, plating of the mating mixture on YEM agar containing kanamycin and gentamycin facilitated selection of transposon inserted mutants. Only the mutants showing EPS-defective phenotypes were selected.

3.13.3 Interposon mutagenesis :

A 1.2 kb *Pst* I containing a part of the gene was cloned in the plasmid pBR322. The plasmid was transferred to TAL1145 by triparental patch mating on a TYM plate using pRK2013 as helper. The plasmid pBR322 can not replicate in *Rhizobium.* Therefore, the mutants resulting from single homologous recombination between cloned *Pst* I fragment and genomic DNA of TAL1145 only grew on YEM agar containing rifampicin, streptomycin and tetracycline.

3.14 Characterization of mutants for exopolysaccharide:

Exopolysaccharide-producing rhizobia form shiny, mucoid colonies on YEM medium while colonies of the mutants defective in normal synthesis of EPS are usually dull and dry on this medium. Therefore, the appearannce of colonies on this medium can be an indication of mutation in the gene(s) responsible for synthesis, polymerization or export of EPS. Various *Rhizobium* species have been shown to produce acidic EPS which is commonly known as succinoglycan. These β-linked EPSs emit blue-green fluorescence under UV on medium containing Calcofluor dye. Thus, mutants of *Agrobacterium*, *Rhizobium* and *Azospirillum* that fail to produce normal EPS I have been routinely selected on this medium by different workers. All EPS-defective mutants were screened on YEM containing 0.02% Calcofluor. The plates were observed under UV for fluorescence.

3.14.1 Quantification of EPS :

TAL1145 and NP84 were grown in 10 x GMS (Breedveld 1990) for the production of higher amounts of EPS but reduced levels of capsular polysaccharide. The

culture was grown at 28°C for seven days with continuous shaking. Cells were separated from the supernatant by centrifugation at 30,000 x g for 30 min. EPS present in the supernatant was precipitated with 2 volumes of ethyl alcohol by incubation at 4°C for 16-18 h. The EPS was separated by centrifugation in preweighed tubes. An average of three replicates was taken. Uninoculated medium was used as a control, its obtained reading was substracted from all values obtained for wild-type and mutant strains.

For EPS preparations TAL1145 as well as the mutants NP86, NP88, NP91, NP92, NP95, NP97, NP98, NP118, NP120, NP125 and NP146 were grown on YEM plates for 72 hrs at 28°C. The cells were harvested and resuspended in 1M NaCl. The suspension was used as the inoculum. Each culture was grown for 5 days in *Rhizobium* minimal medium and EPS was quantified after precipitation with 2 vol cold ethanol as mentioned for NP84.

3.14.2 Size fractionation of EPS :

Different workers (Cangelosi et al. 1987, Gray et al. 1991, Leigh and Lee 1988, and Zevenhuizen and Neerven 1983) have shown the presence of two major EPS peaks on size fractionation through BioGel Agarose A5-m columns. The high molecular weight peak was in the molecular weight range of several million daltons while the molecular weights of the other peaks samples were ≤ 10,000. *Rhizobium* sp. strain TAL1145 also showed the presence of high and low molecular weight fractions. A standard procedure for isolation of two fractions was used in this study.

After growing the culture in YEM broth for seven days the culture was centrifuged at 10,000x g for 30 min. The supernatant was then concentrated to 20% volume in a

rotary evaporator and was filtered through a 0.45 μ Milipore Corp. filter to remove the remaining bacterial cells. The concentrated supernatant was then lyophilized.

A Bio-Gel A-5m 100-200 mesh agarose column (40 by 4 cm) was equilibrated with 10 mM Phosphate buffer containing 100 mM NaCl. The lyophilized culture supernatant (20 mg for wild type and 2 g for the mutants) suspended in 10 ml sodium phosphate buffer was loaded on the column. EPS was eluted by 10 mM sodium phosphate buffer with a flow rate of approximately 0.5 ml/min. Fractions of 6-8 ml were collected and their hexose content was determined by an anthrone sulfuric acid method.

3.14.3 Estimation of total sugars by the anthrone-sulfuric acid method (Trevelyan and Harrison 1952):

Conc. H_2SO_4 (500 ml) was added to 200 ml of water. Anthrone (0.2 g) was dissolved in 100 ml of diluted acid by thorough shaking. Any particulate substance floating on the surface was removed with a pipette. This anthrone reagent was prepared fresh each time. To the screw-capped 1-inch diameter Pyrex tubes 5 ml of anthrone reagent was transferred. The tubes were cooled on ice and 1 ml of solution containing 10-100μg of glucose or fractions collected from Biogel-A5m columns were layered on top of the anthrone reagent. When all of the tubes were prepared in this manner they were removed from the ice and all the contents were mixed by rapid swirling. The tubes were tightly capped and kept in a vigorously boiling waterbath for 10 min. The tubes were cooled and green color absorbance readings were taken using a spectrophotometer set at 620 nm wave length. The sugars present in the EPS were analyzed by the method described by Albersheim et al. (1967).

3.15 Characterization of mutants for lipopolysaccharide :

Lipopolysaccharides are an integral component of cell walls of Gram negative bacteria. LPSs consist of a glycolipid membrane anchor, lipid A, a highly conserved core oligosaccharide linked to lipid A by a 3-deoxy-d-manno-2-octulosonic acid (Kdo) and highly variable oligosaccharide chains or O antigens. Polyacrylamide gel electrophoresis analysis of LPS of Gram negative bacteria results in banding patterns that indicate O antigens of specific lengths.

3.15.1 Preparation of LPS (Johnson and Perry 1976) :

Rhizobia were grown in 100 ml TY broth for 3 days at 28°C and *Salmonella typhimurium* in LB broth overnight at 37°C. The cells were separated by centrifugation at 5,000x g for 5 min and washed three times with TE buffer. The cell pellet was resuspended in 1 ml of suspension buffer containing 10 mM Tris, pH8.0, 5 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. After sonication for 5 min hen egg white lysozyme was added to the cell suspension to a final concentration of 10 mg/ml and the mixture was stirred with magnetic stirrer at 4°C for 16 h. The volume of the lysed cells was adjusted to 3 ml with suspension buffer and incubated at 37°C for 30 min. After adding deoxyribonuclease and ribonuclease to a final concentration of 100 µg/ml the tube was incubated at 37°C for 1 h. Enzymes were inactivated by incubation at 60°C for 15 min and Proteinase K was added to a final concentration of 1 mg/ml. The suspension was incubated for 2 h at 37°C and the hot phenol extraction procedure was followed.

An equal volume of equilibrated phenol preheated to 70°C was added to the suspension and vortexed for 5 min. The upper aqueous layer was separated from phenol

by centrifugation at 10,000 x g for 10 min. The aqueous layer was transferred to another tube and the phenol layer, removed by aspiration, was discarded. The interfacial layer was resuspended in 1 ml water and phenol extraction was repeated. The aqueous phases were then pooled and dialyzed against deionized water until no smell of phenol remained. The crude LPS so obtained was then lyophilized.

3.15.2 Sodium dodecyl sulfate-deoxycholate polyacrylamide gel electrophoresis of LPS (Reuhs et al. 1993) :

The SDS-DOC-PAGE method mentioned here is slightly different from that described by Reuhs et al. (1993).

Stock solutions :

Solution A : 30% Acrylamide (w/v), 0.8% Bisacrylamide (w/v)

Solution B : 22.71 g Tris base/75 ml deionized water, pH8.8. Brought volume to 100 ml

Solution C : 7.69 g Tris base/75 ml deionized water, pH6.8. Brought volume to 100 ml

Solution D : 2.5% Deoxycholic acid (Sodium salt)

Resolving gel (18%) :

SDS (1 g) was dissolved in 6 ml solution A and 2 ml each of solutions B and D were added . After adding 17.5 µl of 10% ammonium persulfate and 8.75 µl TEMED the solution was immediately mixed and poured. Spacers were used for making a 0.5 mm thick gel.

Stacking gel (4%) :

To 0.33 ml of solution A added 0.5 ml of solution C and 1.67 ml water. To this 12.5 µl ammonium persulfate (10%) and 6.75 µl TEMED was added and then poured over resolving gel and a comb was immediately inserted into the gel. Combs with 10 slots for small gels and 12 slots for large gels were used. After the stacking gel was set, a pre-run was done at 30 mA for 10 min for two small or one large gel prior to the loading of the samples.

Sample buffer : Bromophenol blue (5 mg) was dissolved in 4 ml of solution C to which 2 ml of glycerol was added . The volume was made to 20 ml with water.

After a pre-run, the samples were loaded into the gels and the run was conducted at 30 mA for two gels for about an hour at room temperature. Larger gels were run for 5-7 h at the same current setting.

Alcian blue/Silver stain staining (Corzo et al. 1991):

The gels were immediately immersed in 50 ml of 0.5% alcian blue solution prepared in 2% acetic acid. After 30 min the alcian blue solution was discarded and the gels were washed with deionized water and then oxidized by 100 ml of 0.7% sodium metaperiodate for 10 min followed by 7-8 washings with 100 ml deionized water, 5 min each. The gels were stained in 100 ml silver stain solution (10% of Bio-Rad silver stain concentrate) for 10 min and then rinsed with water for 1 min. The gels were soaked in 50 ml Bio-Rad developer (5.75 g/180 ml water) until a dark precipitate formed, after which the solution was discarded and then developed the gel further in the remaining developer. The reaction was stopped by 5% acetic acid for about 5 min and then the gels were rinsed with water 2-3 times. The gels were finally dried and photographed.

3.15.3 NMR of LPS :

^1H nuclear magnetic resonance spectra were recorded at 500 Mhz in D_2O using a General Electric GN Omega 500 spectrometer at the NMR facility of the department of Chemistry, University of Hawaii. The free decay signal was sampled at a block size of about 16,000 over 5-kHz spectral width. Typically, 2,000 transients were recorded with an acquisition time of 3.28 sec.

3.16 Complementation of mutants :

Restoration of the wild type phenotype by transfer of cloned fragments confirms the presence of gene(s) in a particular region as well as helps to delimit the boundaries of the genes of interest that can be further confirmed by sequencing. Therefore, attempts were made to complement the mutants with smaller cloned DNA fragments from respective complementing cosmids as well as by using cosmids with transposon insertions. *Eco*RI fragments from pUHR182 and pUHR183 cloned in pRK404 were used to complement NP84. *Eco*RI and *Hin*dIII fragments from pUHR221 and pUHR222 cloned in pRK404 were tested for complementation of mutants from NP85 to NP146.

3.17 Expression of genes involved in EPS synthesis using gene fusions :

Different genes in bacteria are usually transcribed with different frequencies depending upon their promoter strengths as well as on the interaction of the promoter with various regulatory proteins. The best way to study the regulation of gene expression is by the use of gene fusions. Gene fusions are the DNA constructions in which the coding sequence of one gene is transcribed and/or translated by the controlling sequences of another gene. The process of transcription fusion involves the fusion of a promoterless

reporter gene with the promoter of the gene of interest or vice versa in which the non-fusion polypeptide is synthesized, i.e., protein synthesis starts with the methionine of the desired polypeptide. Various transposons are now available which contain promoterless genes and cause such transcriptional fusions when they are inserted in the right orientation into the gene of interest. Thus, such transposon insertions interrupt the gene of interest and hence functionally inactivate the target gene. Location and orientation of transposon insertion can be determined by Southern hybridization. Also, the activity of the promoter of the interrupted gene can be studied by observing the expression of the promoterless reporter gene present on the transposon. Two such transposon constructs, Tn5-*lacZ* and Tn3Ho*gus* that contain promoterless β-galactosidase and β-glucuronidase genes of *E. coli,* respectively, were used in this study.

3.17.1 Determination of GUS activity on Petri plates :

The β-glucuronidase enzyme from *E. coli* produces a blue indigo dye precipitate from the colorless substrate 5-Bromo-4-chloro-3-indolyl β-D-Glucuronide (X-gluc). Hydrolysis of X-gluc by β-glucuronidase produces glucuronic acid and an indoxyl derivative. Indigo dye precipitate forms by oxidative dimerization of this indoxyl derivative.

GUS activity in the mutants was determined on YEM agar using X-gluc from Sigma Chemicals as the substrate. A stock solution (5mg/ml) of X-gluc prepared in dimethyl formamide was diluted to 10% in 50 mM sodium phosphate buffer, pH7.0. YEM agar plates were prepared and 50 µl of diluted X-gluc solution was spread on each

plate. The mutants were streaked and GUS activity was checked on these plates after incubation at 28°C for 5-7 days.

3.17.2 Quantitation of GUS activity by fluorometry (Jefferson 1987):

Fluorometric assay using the fluorogenic substrate 4-methyl umbelliferyl glucuronide (MUG) is the most sensitive method for quantitation of GUS activity. It offers very high signal-to-noise ratio and therefore, measures an absolute value over negligible background. Hydrolysis of MUG by β-glucuronidase produces the fluorochrome 4-methyl umbelliferone (MU) along with glucuronic acid that has a peak excitation at about 365 nm wavelength and a peak emission at 455 nm.

The fluorogenic assay was performed in microfuge tubes. To check the linearilty of the GUS activity several time points were taken.

GUS extraction buffer (1 liter)	**Stock solutions**	**Volumes used**
50 mM sodium phosphate, pH 7.0	1M Na_2HPO_4, pH7.0	50 ml
10 mM β-mercaptoethanol	original 14.4 M	0.7 ml
10 mM EDTA (sodium salt)	0.5 M Na_2EDTA, pH8.0	20 ml
0.1% sodium lauryl sarcosine	10% sarkosyl	10 ml
0.1% Triton X-100	10% Triton	10 ml

Volume was brought to 1 liter by deionized water.

MUG Assay buffer : Fifty ml of assay buffer (1 mM) was prepared by dissolving 22 mg of the substrate 4-methyl umbelliferyl β-D-glucuronide in the extraction buffer.

Stop buffer : Alkaline solution not only stops the reaction, but also make the product 4 methylumbelliferon fully fluorescent. Sodium carbonate (0.2 M) solution was used for this purpose.

Preparation of cell extract :

The cultures were grown on YEM plates for 5 days at 37°C so that the purity of the culture could be checked. The cells were suspended in 1M NaCl and harvested by centrifugation. After one more washing with 1 M NaCl to remove exopolysaccharide the cells were resuspended in the extraction buffer. Samples were diluted to the same cell density by measuring the OD_{600} using a spectrophotometer. To 10 ml of cell suspension 1ml of lysozyme solution (10 mg/ml) was added and incubated at room temperature for 20 min. Samples were sonicated for 5 min each and stored in aliquots at -70°C until use.

Fluorogenic assay :

One ml of MUG assay buffer was dispensed in Eppendorf tubes and incubated in a 37°C in a waterbath for 30 min to attain the appropriate reaction temperature. During this time stop buffer (0.9 ml) was distributed in properly labelled Eppendorf tubes kept at room temperature. To each tube with substrate 50 µl of cell extract was added and the tubes were returned immediately to the waterbath. The assays were done in duplicate for each sample. Five time points were taken for each sample. The time points for the mutants showing high GUS activity on plates were 6, 12, 18, 24, 30 and 120 min and for mutants with low enzyme activity were 6, 30, 60, 90 and 120 min. After each time interval 100 µl of reaction mixture was transferred to Eppendorf tubes containing the stop buffer. Readings were taken with the fluorometer as mentioned below.

Calibration of Fluorometer :

MU Standard and stock solutions : 1 mM stock solution was prepared by dissolving 19.8 mg of 4-methyl umbelliferone in 100 ml deionized water.

100 μM : Mixed 100 μl of stock solution with 900 μl stop buffer

10 μM : Added 100 μl of the above solution to 900 ml of stop buffer

100 nm : Added 0.1 ml of 10 μM solution to 9.9 ml stop buffer

An absorption spectrum of MU was taken and its excitation peak was observed at 370 nm and emission peak at 445 nm. Therefore, these wavelengths were selected for the fluorometric assay of GUS activity. The fluorometer was set to a value of approximately 100,000 for a 5 μM standard MU solution. A standard curve was prepared using a two fold dilution of 10 μM MU standard solution. GUS activity was calculated using the formula :

$$\text{Units of GUS activity / ml / h} = \frac{\text{(Sample reading -Blank reading) x 0.03 nM*}}{\text{Volume of sample used (in ml) x time of incubation}}$$

* One fluorescence unit was calculated to be equivalent to 0.03 nM MU from the standard.

3.17.3 Assay of β-galactosidase activity (Rossen et al 1985 after Miller 1972):

β-galactosidase activity in the wild type *Rhizobium* strain TAL1145 and the mutant NP84 was determined using o-nitrophenyl-β-D-galactoside (ONPG) as the substrate. The enzyme β-galactosidase hydrolyzes ONPG into galactose and o-nitrophenol. O-nitrophenol is a yellow compound having a specific absorption peak at 420 nm.

A 10 ml broth culture of *Rhizobium* in TY medium grown at 28°C for 3 days was centrifuged at 5,000x g. The cells were washed once with 1 M NaCl to remove traces of medium and resuspended in 1 ml of Z buffer (0.06 M Na_2HPO_4. $7H_2O$, 0.04 M NaH_2PO_4. H_2O, 0.01 M KCl, 0.001 M $MgSO_4$. $7H_2O$ and 0.05 M β-mercaptoethanol), pH7.0. Samples were diluted in Z buffer to obtain an OD_{600} of 0.5. Two drops each of chloroform and 0.1% SDS were added to 1 ml cell suspension in a microfuge tube and then vortexed for 10 sec to disrupt the cell wall and permeabilize the membranes of the bacteria. After incubation in a 28°C waterbath for 10 min 200 µl ONPG (4 mg/ml) was added to each sample and the time (t_0) was recorded. After incubation at 28°C the enzyme activity was stopped by adding 250 µl of 2 M Na_2CO_3 and the final time (t_f) was recorded. The cell debris was removed by centrifugation and the OD_{420} of the supernatant was recorded using a spectrophotometer. β-galactosidase activity was determined using the formula :

$$\text{Units of activity} = \frac{OD_{420}}{OD_{600} \times (t_f - t_0)} \times 1000$$

3.17.4 Assay of protein by Lowry's method :

Lowry's reagent :

Sodium/potassium tartarate stock solution (1%)	:	1.0 ml
$CuSO_4$ stock solution (1%)	:	1.0 ml
Na_2CO_3 stock solution (2%)	:	98.0 ml

To 0-80 µl BSA standard solution 3 ml of Lowry's reagent was added and volume was adjusted to 3.5 ml. For bacterial samples 50 µl of sample was used. The mixture was

incubated at room temperature for 10 min and 0.5 ml of 1:1 folin reagent and distilled water mixture was then added. After 30-60 min the absorbance readings were taken using a spectrophotometer at 660 nm.

$$\text{Gram of protein} = \frac{\text{BSA conc. (mg/ml) x equivalent volume of unknown}}{\text{Vol of unknown used (ml) x Conc. of cell homogenate (mg/ml)}}$$

3.18 Plant experiments :

Nodulation assays of the EPS-defective mutants on three legume hosts, *Leucaena*, *Gliricidia* and *Phaseolus* beans were conducted in growth pouches using a nitrogen-free nutrient solution (Hoagland and Arnon 1938) as described by George et al. (1994). Symbiotic effectiveness and competitiveness of the mutants were checked in Leonard jars (Vincent 1970). *Phaseolus* beans (variety Brazil 2), *Gliricidia sepium* and *Leucaena leucocephala* (variety K8) seeds were surface sterilized by a 5-10 min immersion in sulfuric acid followed by rigorous rinsing with sterile deionized water. The seeds were germinated aseptically on water agar for 24-48 h and then tranferred to growth pouches and Leonard jars. All nodulation experiments included uninoculated controls.

Hoagland and Arnon medium (1938) :

		Per liter
K_2SO_4	:	0.435 g
$MgSO_4.7H_2O$	:	0.424 g
$CaHPO_4.2H_2O$	:	0.127 g
$CaSO_4.2H_20$	:	0.253 g
FeEDTA	:	0.02 g

i.e.,$FeCl_3$, 7.5 g; EDTA, 12.5 g

pH was adjusted to 7.0 and autoclaved.

Solution B (Micronutrient solution) :

		Per liter
H_3BO_3	:	2.86 g
$MnCl_2$	:	1.81 g
$ZnSO_4$	:	0.12 g
$CuSO_4$	:	0.08 g
$Na_2MoO_4.2H_2O$	:	0.027 g
$CoCl_2.6H_2O$	:	0.026 g

Autoclaved solution B separately and added 1ml/liter of solution A.

Hoagland solution was regularly added to the plants. The plants were grown in the growth chamber and were harvested after 4-8 weeks. The plants were inoculated with a minimum of 10^6 cfu of rhizobia/ml. For competition experiments the plants were inoculated using three inoculant ratios, 1:1, 1:9 and 9:1 of TAL1145 and the mutant. The plants were harvested after 6 weeks following inoculation for nodule typing. Nodules were excised from the roots, surface sterilized with 20% bleach and washed 5-6 times with sterile distilled water. Individual nodules were crushed in 0.1 ml of sterile 0.25 M NaCl in 96 well microtiter plates with a multiple inoculator. Nodule occupancy was determined on the basis of antibiotic resistance markers of the exnodule rhizobia by replica plating on YEM agar containing appropriate antibiotics.

3.19 Light microscopic examination of nodule sections :

Thick and semithin sections of nodules formed by the mutants as compared to wild type were examined light microscopically to study the bacteroid zone development as affected by a defect in EPS and/or LPS synthesis. Also, using the *GUS* gene fusions, expression of the interrupted genes involved in EPS synthesis in TAL1145 was studied.

3.19.1 Observation of GUS activity in thick sections (Sharma and Signer 1990):

Fixation of nodules was done in 2% formaldehyde. For preparation of this fixative paraformaldehyde (6 g) was dissolved in 100 ml water containing three drops of NaOH by continuous stirring on a hot plate. The solution was removed from the hot plate before it started boiling. The pH was adjusted to 7.0 and the volume was adjusted to 300 ml. The nodules excised from the root with a small portion of root attached were trimmed longitudinally at two ends for better penetration of the fixative. After fixing in 2% formaldehyde at room temperature for 1 hr the nodules were washed with 50 mM sodium phosphate buffer, pH7.0, 3-4 times. X-gluc solution was prepared by diluting 25 µl of the stock solution (25 µg / 250 µl dimethylformamide) in 5 ml of 50 mM sodium phosphate buffer, pH 7.0. The background GUS activity in plant tissues occurs at mildly acidic pH, 5.5-6.0 which may intensify due to the presence of peroxidases that may have leaked leaked from the vacuoles during sectioning. It was minimized or completely abolished by using the buffered solutions at pH7.0. Thick sections of the nodules were cut using a Du Pont Instruments / Sorvall TC-2 tissue sectioner and the sections were immersed in X-gluc solution in microtiter plates or on slides at 28°C for 24 h. Sections were covered with a coverslip if slides were used for GUS enzymatic reaction. After rinsing with water and

then by 70% ethanol, the sections were mounted using the slide mount. Nodule sections were observed and photographed under the microscope.

3.19.2 Preparation and observation of semithin sections for GUS activity:

The basic techniques for fixation and embedding are given by Hayat, 1986 for transmission electron microscopy.

0.2 M Cacodylate buffer :

Sodium cacodylate, $Na(CH_3)_2AsO_2.3H_2O$: 10.7 g

Dissolved in 225 ml deionized water, adjusted the pH to 6.8 and diluted to a final volume of 250 ml with deionized water.

Karnovsky's fixative:

Formaldehyde	:	2.0 %
Glutaraldehyde	:	2.5 %
Cacodylate buffer, pH 6.8	:	0.1 M

Paraformaldehyde (4.36 g) was dissolved in 80 ml of deionized water with 3 drops of 10 N NaOH by heating and continuous stirring in a covered container. The solution was not allowed to boil. The solution was then allowed to cool to room temperature and 10 ml of 50% glutaraldehyde (Ted Pella, Incorporation) as well as 100 ml of 0.2 M cacodylate buffer were added. The volume was adjusted to 200 ml with deionized water.

Spurr resin-medium hardness (Spurr, 1969) :

Vinyl Cyclohexene dioxide (ERL 4206)	:	50.0 g
Diglycidyl ether of polypropylene glycol (DER 736)	:	30.0 g
Nonenyl succinic anhydride (NSA)	:	130.0 g

Dimethylamino ethanol (DMAE) : 2.0 ml

The components were added in the order listed above. After addition of each component the mixture was thoroughly mixed. DMAE being very volatile was added in the fume hood.

Nodules formed by the mutants on *Leucaena* were cut in 2 mm thick longitudinal pieces and immediately submerged in X-gluc solution made in sodium phosphate buffer, pH 7.0. The sample number written with pencil on a small piece of reference card was placed in each vial. The substrate solution was infilterated into the nodule tissue using vacuum and the nodule pieces were then incubated at 37 °C for 48 hrs to allow for complete enzymatic reaction to occur. The nodule pieces were washed once with sodium phosphate buffer and twice with cacodylate buffer followed by 24 h of fixation in Karnovsky's fixative at 4°C.

The nodules were washed thrice with cacodylate buffer and dehydration was done using 10% to 90% acetone at an interval of 10 min each. Dehydration with 95% acetone twice and with 100% acetone three times in the desiccator was followed by three changes with 1:1 acetone and propylene oxide (100% each). Infilteration of the specimens was done three times through propylene oxide at 10 min intervals. Finally, just enough propylene oxide was left to cover the samples and the level of propylene oxide was marked outside the vials with a permanent glass marker. Infilteration by Spurr resin : propylene oxide was done in a step wise manner. Three drops of a Spurr resin : propylene oxide mixture (1:1) were added at an interval of 15 min until the level of solution reached double of that of the marked propylene oxide level. The caps of the vials were replaced by

aluminium foil and the vials were kept in a slightly opened desiccator in the fume hood for two days to allow the propylene oxide to evaporate slowly. The specimens from the viscous resin were picked up with the wooden sticks and transferred to beam capsules or to the moulds containing fresh Spurr resin. The specimens orientation was carefully adjusted with the wooden stick and more resin was added (not more than 5 mm thick). Evacuation was done in a vacuum oven at room temperature for 15 min to allow the specimens to sink to the bottom of the resin which was followed by polymerization in an oven at 60°C for 24 to 48 hrs. The plastic beam capsule was removed from the polymerized resin and the specimen was ready for trimming and sectioning. Cubicle blocks were cut from the molds around the specimen and mounted on 1.5 cm long pieces of plastic rod for sectioning.

The blocks were trimmed by hand in a pyramid form for sectioning with a side angle of approximately 50°. Sections of 2.5 to 4 μ thickness were cut using a Sorvall microtome and observed under the light microscope.

3.20 Electron microscopic studies :

Thin sections of nodules formed on *L. leucocephala* were examined under the electron microscope to determine changes in the bacteroid zone development and the bacteroids' differentiation in the nodules formed by EPS-defective mutants as compared to the wild type. Nodules were excised from the roots, fixed in Karnovsky's fixative and washed with cacodylate buffer three times. Buffer, enough to cover the specimens, was left in each vial and an equal volume of 2% osmium tetraoxide solution in cacodylate buffer was added. Post fixation in OsO_4 was done for 2½ hrs at room temperature in the

fume hood followed by three washings with cacodylate buffer. Dehydration was carried out as mentioned for light microscopy except an ethanol instead of an acetone series was used. Infilteration through propylene oxide and spurr resin, embedding in Spurr resin and block cutting and trimming were done as mentioned above. Semithin (1μ) sections of the specimens were cut with a Sorvall microtome using glass knives, stained with Toluidine blue and examined under the light microscope. This facilitated preparation of the block face as well as the selection of the desired area for sectioning for electron microscopic examination. The blocks were further trimmed so that each side was not more than 0.5 mm. Thin sections were cut with a diamond knife using an RMC6000 microtome and section ribbons were collected on copper grids cleaned as mentioned below.

Cleaning of grids: Grids were covered with boiled and filtered water and a little detergent was added with the tip of a wooden stick. The grids were then sonicated for 10 min and rinsed with water several times over a Whatman paper cone on a conical funnel. The grids were transferred to a beaker containing 100% acetone and sonicated again for 10 min. After washing several times with boiled and filtered water on a paper cone the grids were allowed to fall on a Whatman filter paper disc in a Petri plate by inverting the cone. The grids were kept in covered Petri plate until use.

Lead citrate-uranyl acetate staining for electron microscopy : For all staining steps deionized water filtered through a Milipore (0.45μ) filter and boiled to remove carbon dioxide was used.

0.5% Lead citrate solution : A pellet of NaOH was added to 50 ml of water and lead citrate (0.25 g) was dissolved in this NaOH solution. After filtration through a Milipore

(0.2 μ) filter the stain was ready for instant use. Fresh lead citrate solution was prepared each time.

5% methanolic uranyl acetate: Uranyl acetate was added to absolute methanol and sonicated until completely dissolved. After filtration through 0.2 μ Millipore filter the stain was ready for use.

Staining procedure :

The grids with the sections were transferred to drops of uranyl acetate solution on dental wax with the sections side down and stained for 15 min at room temperature. After rinsing once with 70% ethanol the grids were washed three times with water, 1min each. In a four compartment Petri plate 8-10 crystals of NaOH were transferred in two opposite sections to absorb carbon dioxide and hence avoid lead carbonate precipitate formation. In one section of the plate lead citrate drops and in the opposite section drops of 0.05 N NaOH were placed. The grids were submerged in lead citrate solution for 15 min and then rinsed in NaOH in the opposite section of the Petri plate. The grids were then rinsed in 0.05 N NaOH in three small Petri plates, 15 sec each followed by three rinses in water. After drying on filter paper the grids were stored in the grid boxes. The nodule sections were examined and photographed under a Philips EM201 electron microscope. Kodak 4489 EM negatives were used for photography. Processing of negatives was done according to the supplier's instructions.

3.21 Sequencing of DNA :

DNA sequencing by Sanger's method (Sanger et al. 1977,1980) involves synthesis of a complementary DNA strand by a DNA polymerase in vitro. Synthesis is initiated at a

site where primer anneals to the template. The growing chain is terminated selectively by incorporation of 2'-3'-dideoxynucleoside triphosphate randomly at the 3'-end which does not support further DNA synthesis because it now lacks a 3'-hydroxyl group. *Taq* polymerase or sequenase enzymes are currently used for the polymerization reactions. Labelled single stranded oligonucleotides so obtained are then fractionated by high resolution denaturing polyacrylamide sequencing gels.

Generally, three methods are used for generating smaller DNA fragments to obtain DNA sequencing information. Subcloning of larger fragments by the shot-gun method using different restriction enzymes is a labor intensive method, results in duplication of fragments and usually leaves gaps between sequenced DNA. Primer walking is more systematic but needs sequential synthesis of desired primers and is more expensive and time consuming. Sequencing of DNA by generating nested deletions is the most convenient and efficient method and it provides information more quickly also. The two methods used in the present study to obtain nested deletions for sequencing are given below.

3.21.1 Preparation of deletion clones by the p-DELTA method (Gibco Bethesda Research Laboratories' Deletion Factory™ System) :

In this method nested deletions are obtained in vivo by intramolecular transposition of genetically engineered transposon, γδ (Berg et al. 1992, Wang et al. 1994). Deleted clones can be screened by using appropriate selectable markers on the plasmid pDELTA 1. This is the method of choice if the subcloned DNA has restriction sites for enzymes recommended to be used with an exonuclease deletion system.

A 3.8-kb *Eco*RI fragment from pUHR183 was cloned in pDELTA 1. *E. coli* strain DH5αmcr was transformed with this DNA and colonies were screened on LB agar containing X-gal and IPTG. Plasmid DNA was isolated by the boiling prep method and analysed by agarose gel electrophoresis after digestion with *Eco*RI. The selected cloned DNA was used to transform 100 µl of DF1 competent cells and the transformed bacteria were plated on LB containing chloramphenicol, tetracycline and kanamycin. The plates were incubated at 37°C overnight. Ten colonies were picked to inoculate LB containing kanamycin and chloramphenicol and 10 more colonies to inoculate LB containing tetracycline and chloramphenicol. Both tubes were incubated at 37°C overnight with continuous shaking. The next day, DNA was isolated from each culture by the alkaline prep method and labelled as 'cam-kan' and 'cam-tet', respectively. A small aliquot (2 µl) of each DNA was used to transform DH10B competent cells. The transformation reaction from cam-tet DNA was spread onto an LB plates containing tetracycline and 5% sucrose and from cam-kan DNA onto LB plate containing kanamycin and streptomycin. The plates were incubated at 37°C overnight. Next day, randomly selected colonies from the LB-tet-suc plate were grown in LB containing sucrose and tetracycline and from the LB-kan-str plate on LB containing these two antibiotics. After 24 hrs of growth at 37°C the plasmid DNA was isolated from each culture. DNA was digested with *Eco*RI and analysed by agarose gel electrophoresis. Deletion clones were selected which showed the presence of only one band since the other *Eco*RI site was deleted. The nested deletion clones were selected in a range of approximately 4-8 kb. The size of the actual deletion was determined by subtracting 3.1 kb from the tetracycline subclones (kanamycin-

streptomycin region of plasmid deleted) and 2.7 kb from the kanamycin subclones (tetracycline-sucrose region of plasmid deleted). The deleted clones were arranged in decreasing size range with an increment of approximately 300 nucleotides and a map was constructed in either orientation by using two sets of deletion subclones. Highly purified plasmid DNA were obtained from subclones by the CsCl-Ethidium bromide density gradient centrifugation method for sequencing.

3.21.2 Preparation of deletion clones by the Erase-a-Base system (Henikoff 1984): An Erase-a Base kit from Promega Corporation based upon the procedure developed by Henikoff (1984) was used to obtain nested deletions in 2.2, 1.8 and 4.4 kb *Eco*RI fragments from pUHR221 subcloned in pUC18 for sequencing. In this method exonuclease III is used to specifically digest insert DNA from a 5' protruding or blunt end restriction site. The adjacent sequencing primer binding site is protected from digestion by a 4-base 3' overhang restriction site or by an α-phosphorothiolate-filled end. The uniform rate of digestion by the enzyme allows deletions of predetermined lengths by simply removing timed aliquots from the reaction mixture to S1 nuclease which removes the single-stranded tails. S1 nuclease is active in ExoIII buffer. After S1 cleavage blunt-end DNA is obtained by klenow DNA polymerase using deoxynucleoside triphosphates and the ends are ligated to obtain a circularized plasmid.

In this study all three DNA fragments were cloned in both orientations in pUC18. Plasmids containing the 1.8 kb *Eco*RI fragment were digested with *Sph*I followed by the second enzyme, *Sma*I. Complete digestion of the plasmid was checked by the electrophoresis of a small amount of the sample. The digested DNA was extracted with

phenol:chloroform mixture, precipitated with cold ethanol and the procedure for deletion was followed as recommended by the suppliers.

The plasmid containing the 2.2 kb *Eco*RI fragment was digested with *Kpn*I and *Xho*I in one and by *kpn*I and *BstE*II in the other orientation and then the deletion by the Erase-a-Base system was followed. The gap between primer binding and the *Xho*I and *BstE*II sites in the clone were filled by using a deletion with *Sma*I and *Sal*I followed by religation. The deletion process using the Erase-a-Base kit was followed as recommended by the Promega Corporation instruction manual.

3.21.3 Automated sequencing :

Automated sequencing was done by the Biotechnology, Molecular Biology Instrumentation and Training Facility at the University of Hawaii. The PRISM™ ready reaction Dyedeoxy™ terminator cycle sequencing kit from Perkin-Elmer was used to prepare samples for sequence analysis on the ABI Model 373A DNA sequencer. The kit provides four dye-labelled dideoxy nucleotides : G, A, T and C DyeDeoxy terminators. During thermal cycling the dye label is incorporated into the DNA along with the terminating base by the more thermally stable enzyme, AmpliTaq® DNA Polymerase. All four termination reactions are carried out in one tube. Use of dITP instead of dGTP in the terminator mix minimizes band compressions while use of AmpliTaq allows the reactions to be run at high temperature to minimize secondary structure problems as well as to avoid nonspecific primer binding.

Double stranded plasmid DNA template was prepared by the CsCl-EtBr density gradient centrifugation method. A mixture of 1.0 μg DNA template and 3.2 pmol primer

was adjusted to a total volume of 10.5 µl with high quality water in a 0.6 ml microfuge tube. A terminator mix (9.5 µl) was added resulting in a final reaction volume of 20 µl and 25 cycles of sequencing was done in a thermal cycler (Model 480). After spin column purification the extension products were dried and 5 µl deionized formamide, 1 µl 50 mM EDTA, pH8.0 and dextran blue as loading dye were added to the samples. The samples were heated at 90°C in an oilbath for 2 min and then loaded immediately onto an ABI Model 373 DNA sequencer according to the instructions of the suppliers.

3.21.4 Sequence analysis :

The programs FASTA, TFASTA, and BLAST (Genetic Computer Group package; National Center for Biotechnology Information), based on the method of Wilburn and Lipman (1983) were used to search data banks for sequences with homology to the different open reading frames. The program BESTFIT in the GCG package, which uses the "local homology" algorithm of Smith and Waterman (1981) was used to perform sequence comparison. The percentage of identity and similarilty were derived by using BESTFIT and the following parameters : a gap weight of 3.0 and a gap length weight of 0.10. A degree of similarilty above 45% and a degree of identity above30 % were taken as significant.

CHAPTER : IV

IDENTIFICATION OF A GENE INVOLVED IN LPS AND EPS SYNTHESIS IN *RHIZOBIUM* SP. STRAIN TAL1145

4.1 Introduction :

The cell surface polysaccharides of rhizobia, particularly exopolysaccharides (EPS) and lipopolysaccharides (LPS) have been shown to be involved in the symbiotic process with various legumes (for reviews, see Gray and Rolfe 1990, Leigh and Coplin 1992). Generally, *Rhizobium* mutants defective in EPS have severe effects on the nodulation of indeterminate-nodulating hosts while such mutants have little effect on nodulation and nitrogen fixation of determinate-nodulating hosts. Nodules devoid of bacteria or callus-like pseudonodules are induced by EPS-defective mutants of *Rhizobium meliloti,* NGR234, *R. leguminosarum* bv. viciae and *R. loti* on respective indeterminate nodule-forming hosts such as, *Medicago sativa,Leucaena leucocephala, Pisum sativum* etc., that consequently fail to fix nitrogen. However, the same mutants of some of these species induced normal, nitrogen-fixing nodules on their determinate hosts, *Phaseolus vulgaris, Lotus pedunculatus* etc.

LPS is a unique component of the outer membrane of rhizobia as in all other Gram-negative bacteria and consists of a variable O-oligosaccharide linked to the highly conserved core oligosaccharide which is anchored to the membrane by glycolipids. Previous studies have shown the involvement of LPS in specific attachment of rhizobia to the host plant roots (Lagares 1988, Kato et al. 1979, for review see Noel 1992). In determinate-nodulating hosts like *P. vulgaris* and *Glycine max*, LPS mutants lacking O-antigen failed to form normal

infection threads or the bacteria were not released from the infection threads to invade the nodules (Cava et al. 1989, 1990, Maier and Brill 1978, Puvanesrajah et al. 1987, Stacey et al. 1991). The symbiotic phenotypes of LPS-defective mutants on indeterminate-nodulating hosts may vary among different legumes. LPS-defective mutants of *R. leguminosarum* bv. trifolii formed small white non-fixing nodules on *Trifolium hybridum* (Brink et al. 1990). Such mutants of *R. leguminosarum* bv. viciae also formed small ineffective nodules on *Vicia hirsuta*, *V. sativa* and *Pisum sativum* (Priefer 1989, de Maagd et al. 1989, Goosen-De Roo et al. 1991). On the other hand the LPS-defective mutants of *R. meliloti* are not defective in symbiosis (Clover et al. 1989).

Brink et al. (1990) suggested that LPS may be a signal molecule that is required at different times during nodule development in different hosts. It has also been proposed that LPS may protect rhizobia by suppressing the host defense mechanisms (Noel 1992). Studies of *R. meliloti*-alfalfa symbiosis showed that the genes for EPS and LPS synthesis determine similar functions in the course of nodule development and thus EPS and LPS provide equivalent information for the host plant (Putnoky et al. 1990). In *R. meliloti* the *exoB* and *exoC* mutants are defective in both EPS and LPS synthesis (Leigh and Lee 1988). Diebold and Noel (1989) described two *R. leguminosarum* bv. *phaseoli* mutants that were defective in both EPS and LPS synthesis and nodule development on beans.

A mutant of the *Leucaena*-nodulating *Rhizobium* sp. strain TAL1145 is described here which is defective in both EPS and LPS synthesis and forms nodules on *Leucaena leucocephala* that are delayed in bacteroid development and nitrogen fixation. TAL1145 forms effective nodules on *Leucaena* and other tree legumes such as *Gliricidia sepium* and

Calliandra spp. (Turk and Keyser, 1992). Besides, it also nodulates *P. vulgaris* (George et al. 1984). The mutant NP84 was constructed by a Tn*5lacZ* insertion into a 6.3-kb *Eco*RI fragment of TAL1145 (Parveen and Borthakur 1994). This mutant was tested for infection, nodule formation and bacteroid development on both indeterminate-nodule-forming (*Leucaena)* and determinate-nodule-forming (*Gliricidia)* tree legumes. A herbaceous determinate-nodule-forming host, *P. vulgaris* was also included in this study. An *Eco*RI fragment of 3.8-kb size was also cloned containing the wild-type copy of the gene which showed high homology with DNA from the *Leucaena*-nodulating strains but did not show any detectable level of hybridization with DNA from the *R. meliloti* and *R. leguminosarum* strains tested.

RESULTS

4.2 Complementation of EPS-defective mutant NP54 :

Previously, a clone library of *Rhizobium* sp. strain TAL1145 DNA was constructed in *E. coli* (George et al. 1994) by ligating genomic DNA fragments of 20 to 30 kb in size into the wide host-range cosmid vector pLAFR3 (Staskawicz *et al.* 1987). The library was mated *en masse* with the strain NP54 as the recipient and tetracycline-resistant (specified by pLAFR3) transconjugants were selected on YEM agar. Of these, approximately 10 colonies per 1500 colonies were mucoid, suggesting that the defect in EPS synthesis was complemented in these transconjugants. Plasmid DNA was isolated from five mucoid transconjugants and transformed into *E. coli*. Two overlapping plasmids, pUHR182 and pUHR183, containing approximately 22-24 kb of cloned DNA from TAL1145 were obtained. When pUHR182 or pUHR183, were transferred to the mutant NP54, all transconjugants were mucoid on YEM.

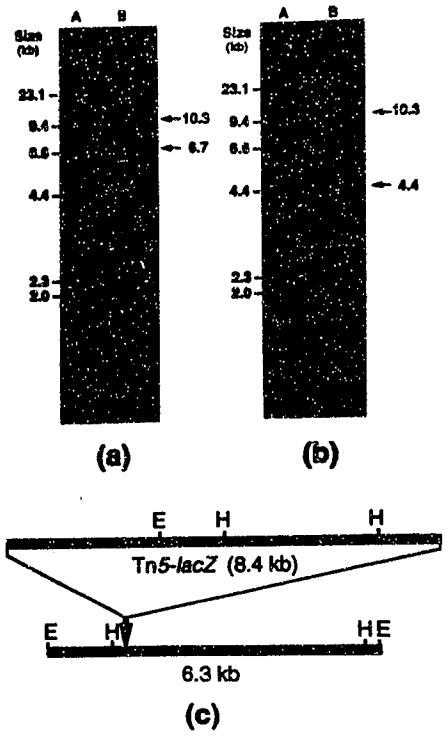


Fig. 4.1 Southern blot analyses of *Eco*RI-digested genomic DNA of TAL1145 (lane A) and mutant NP54 (lane B) using (a) ^{32}P-labeled internal fragments of Tn*5* and (b) plasmid pUHR182 as probes. (c) Map of 6.3-kb *Eco*RI fragment from pUHR182 with Tn*5-lacZ* insertion.

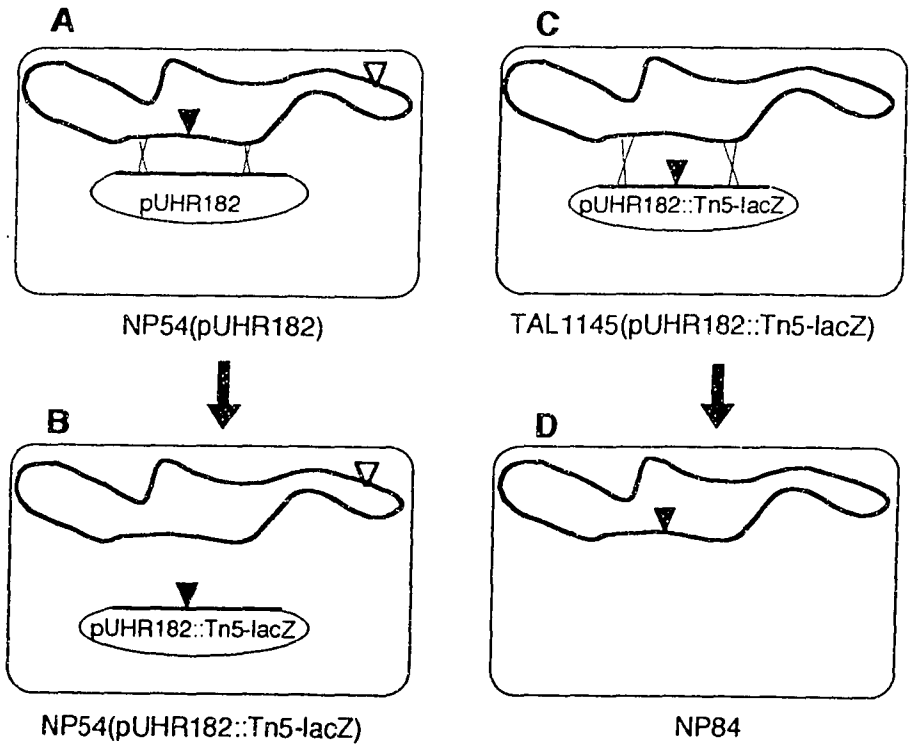


Fig. 4.2 Construction of the single Tn5-*lacZ* insertion mutant NP84 from the double-insertion mutant NP54. The Tn5-*lacZ* insertion for the EPS- phenotype of NP54 is shown with a solid triangle and the second Tn5-*lacZ* insertion is represented by an open triangle. The Tn5-*lacZ* insertion in the transconjugant NP54(pUHR182) (a) was transferred by double homologous recombination to plasmid pUHR182 (b). In the transconjugant TAL1145 (pUHR182::Tn5-*lacZ*), the transposon from pUHR182::Tn5-*lacZ* was transferred by double homologous recombination to the genome of TAL1145 (c) and subsequent loss of pUHR182 resulted in the mutant NP84 (d) the plasmids pUHR182 and pUHR183 contain the gene involved in EPS synthesis which was mutated in NP54.

4.3 Analysis of NP54 by Southern hybridization :

Southern analysis of *Eco*RI digested genomic DNA of NP54 with Tn*5* as a probe showed hybridization with two fragments, 10.3 kb and 6.7 kb in size, showing insertion of Tn*5*-*lacZ* at two sites (Fig. 4.1a). Southern hybridization of TAL1145 and NP54 genomic DNA using pUHR182 as a probe showed Tn*5*-*lacZ* insertion in NP54 in a 6.3 kb *Eco*RI fragment since the 6.3 kb band in TAL1145 was replaced by two bands of 10.3 kb and 4.4 kb in size in NP54 (Fig. 4.1b). The 10.3-kb fragment is the same as that hybridized with Tn*5* in Fig. 4.1a. These are compatible with the expected size of the fragments since Tn*5*-*lacZ* has an internal *Eco*RI site (Fig. 4.1c). These results show that mutant NP54 contains two copies of the transposon, one of which is located in a 6.3-kb *Eco*RI fragment which is homologous to the 6.3 kb *Eco*RI fragment in the plasmid pUHR182.

4.4 Tn5-*lacZ* insertion in plasmid pUHR182 by homologous recombination:

In the transconjugant NP54(pUHR182) transfer of Tn*5*-*lacZ* from genome of TAL1145 to plasmid pUHR182 at a low frequency was expected due to double homologous recombination (Fig. 4.2). Therefore, plasmid DNA was isolated from NP54(pUHR182) by the alkaline-prep method and was used to transform *E. coli*. Transformants were plated on LB agar containing tetracycline (10 mg/ml) and kanamycin (25 mg/ml) to select for derivatives of pUHR182. Plasmid DNA was isolated from one transformant and analyzed for location of the Tn*5*-*lacZ* insertion. Restriction analysis of pUHR182::Tn*5*-*lacZ* and pUHR182 with different enzymes and Southern hybridization

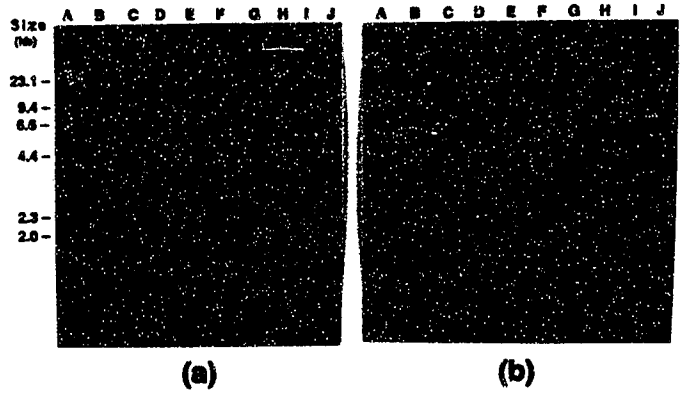


Fig.4.3 (a) Ethidium bromide stained agarose gel showing restriction of pUHR182 (lanes A, C, E, G and I) and pUHR182::Tn*5-lacZ* (lanes B, D, F, H and J) with *Eco*RI (lanes A and B), *Hin*dIII (lanes C and D), *Pst*I (lanes E and F), *Sac*I (lanes G and H) and *Eco*RV (lanes I and J). (b) Southern analysis of the gel in Fig. 3a using ^{32}P-labeled Tn5 internal PstI fragments as a probe.

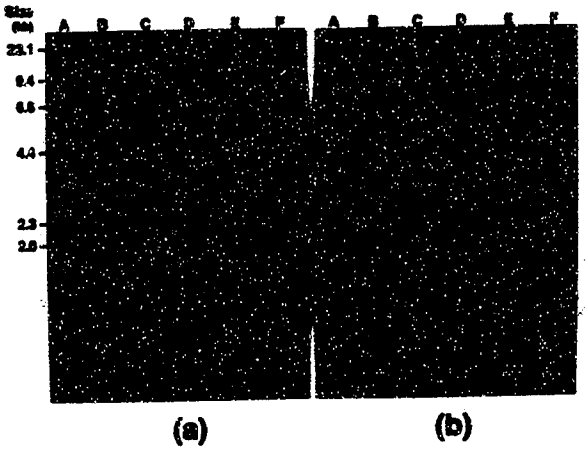


Fig. 4.4 Southern blot analyses of genomic DNA of TAL1145, double transposon insertion mutant NP54 and single Tn*5-lacZ* insertion mutant NP84 restricted with *Eco*RI (lanes A, B and C) and *Hin*dIII (lanes D, E and F) using ^{32}P-labeled internal fragments of (a) Tn*5* and (b) pUHR182 as probes.

showed transposon insertion in 6.3-kb *Eco*RI, 4.9-kb *Hin*dII, 1.2-kb *Pst*I and 7.8-kb *Sac*I fragments (Fig. 4.3).

4.5 Construction of single transposon-insertion mutant NP84 :

Plasmid pUHR182::Tn*5-lacZ* was transferred from *E. coli* to TAL1145 by conjugation and Tn*5-lacZ* was marker exchanged with the TAL1145 genome. Three EPS-defective colonies of homogenotes were selected and purified. The homogenotes were found to be sensitive to tetracycline indicating that double homologous recombination had occurred in these mutants. Southern hybridization of the genomic DNA of these mutants with Tn*5* as a probe showed a double insertion in two mutants (data not shown), however, one mutant NP84 had a single transposon insertion (Fig.4.4a). One insertion in each of these three mutants was found to be in the same site which is homologous to the position of transposon insertion in the plasmid pUHR182::Tn*5-lacZ*. The identical position of a transposon insertion in NP84 and NP54 was confirmed by hybridization with pUHR182 as a probe (Fig. 4.4b). The mutant NP84 formed small, opaque, EPS-defective colonies on YEM and the symbiotic phenotype on both hosts was the same as NP54. Complementation of NP84 with pUHR182 resulted in normal EPS synthesis and restoration of the wild-type symbiotic phenotype on beans.

4.6 NP84 is complemented by a 3.8-kb DNA fragment from TAL1145 :

Complementation of the mutant NP84 using the cosmid clone library of TAL1145 resulted in isolation of a cosmid, pUHR183 that is overlapping to the plasmid pUHR182. Cosmid pUHR183 was found to contain the same*Eco*RI fragments as pUHR182 except that the 6.3-kb *Eco*RI fragment in pUHR182 was replaced by a 3.8-kb *Eco*RI fragment.

Restriction mapping and further Southern analysis showed that the 3.8-kb fragment represents a part of the 6.3-kb *Eco*RI fragment (Fig. 4.14). One *Eco*RI site in this fragment comes from the multiple cloning site of the cosmid vector pLAFR3. Cosmid pUHR183 contained the 4.2-kb fragment as one of the end fragments in the insert. The 6.3-kb fragment of pUHR182 and the 4.2-kb fragment of pUHR183 were cloned in the broad-host-range cloning vector pRK404 to obtain plasmids pUHR251 and pUHR252, respectively. The transconjugants of NP84 containing either pUHR251 or pUHR252 also produced EPS phenotype like TAL1145 and formed normal nitrogen-fixing nodules on *P. vulgaris*.

4.7 Mutant NP84 is defective in both EPS and LPS synthesis :

The colonies of the mutant NP84 showed bluish fluorescence on Calcafluor-containing YEM agar similar to TAL1145. The smaller colony size of NP84 compared to TAL1145 was not due to slower growth rate of the mutant, since both strains showed similar growth rates when grown in YEM broth (data not shown). In Breedveld's medium NP84 produced less than half the amount of EPS produced by TAL1145 (Table 4.1). Size fractionation of the EPS made by TAL1145 and NP84 showed that EPS of both strains contains both high and low molecular weight fractions (Fig. 4.5). Thus, the EPS produced by the two strains could not be distinguished by size fractionation. Analysis of the sugar composition of the mutant and the wild type also showed that there was no detectable difference in the sugar composition of the EPS of the mutant (Fig. 4.6).

Mutant NP84 was found to be defective in LPS also. Crude LPS extracted from TAL1145, NP84 and three transconjugants of NP84 containing the plasmids pUHR182, pUHR251 and pUHR252 were analyzed by SDS-DOC-PAGE as shown in Fig. 4.7. The LPS

of TAL1145 showed two major banding regions, LPS I and LPS II. The high molecular weight LPS I showed four bands indicating different O-antigens while one major band was observed in the LPS II region. A series of minor bands (LPS III) were observed between LPS I and LPS II and above LPS I (LPS IV and LPS V). LPS I was severely affected in NP84 and all the LPS bands except LPS II and two central bands in the LPS I region were absent in this mutant. The LPS bands for the three transconjugants of NP84 were similar to those for TAL1145.

4.8 ^{1}H Nuclear magnetic resonance analysis of LPS and EPS :

Crude preparations of LPS of TAL1145 and NP84 were examined by ^{1}H NMR to determine differences that account for the LPS profile of the mutant on SDS-DOC-PAGE. Spectra of both TAL1145 and NP84 (Fig. 4.8a and 4.8b respectively) showed a small peak at 0.95 ppm probably due to terminal methyl groups of fatty acid chains and a larger signal at 1.3 ppm due to fatty acid methylenes. On the basis of relative intensities of fatty acids at 1.3 ppm and of carbohydrate ring protons between 3.0-4.0 ppm, it was concluded that in NP84 the fatty acid / carbohydrate ratio may be lower than in the wild type.

Four relatively strong peaks were observed at 3.45, 3.5, 3.75 and 3.85 ppm in TAL1145 LPS which may be methyl protons of methylated sugars. However, two peaks, at 3.45 and 3.65 ppm, were absent in the LPS of NP84. The carbohydrate β-anomeric protons around 5.0 ppm and possibly α-anomeric protons at 5.1 and 5.2 ppm observed in TAL1145 were missing in the LPS of the mutant. These changes substantiate the observation in SDS-DOC-PAGE that the O-antigen of LPS of NP84 is different from that of the wild type.

Table 4.1 : Quantification of EPS produced by TAL1145 and NP84 and the effect of inoculation by these strains on the growth of *L leucocephala* plants

Strain	Dry weight of EPS/ml (mg)	Dry weight of shoot of *Leucaena* plants in mg** (Mean ± SD)	
		4 weeks	8 weeks
TAL1145	11.84	251 ± 48	488 ± 72
NP84	5.71	132 ± 48	477 ± 72
NP84(pUHR182)	10.3	244 ± 36	ND
Control		114 ± 02	142 ± 10

* EPS in culture supernatant precipitated using 3 volumes of ethanol and dried. Mean of three replicates shown.

** Eight replicates were used for each treatment.

ND : Not determined

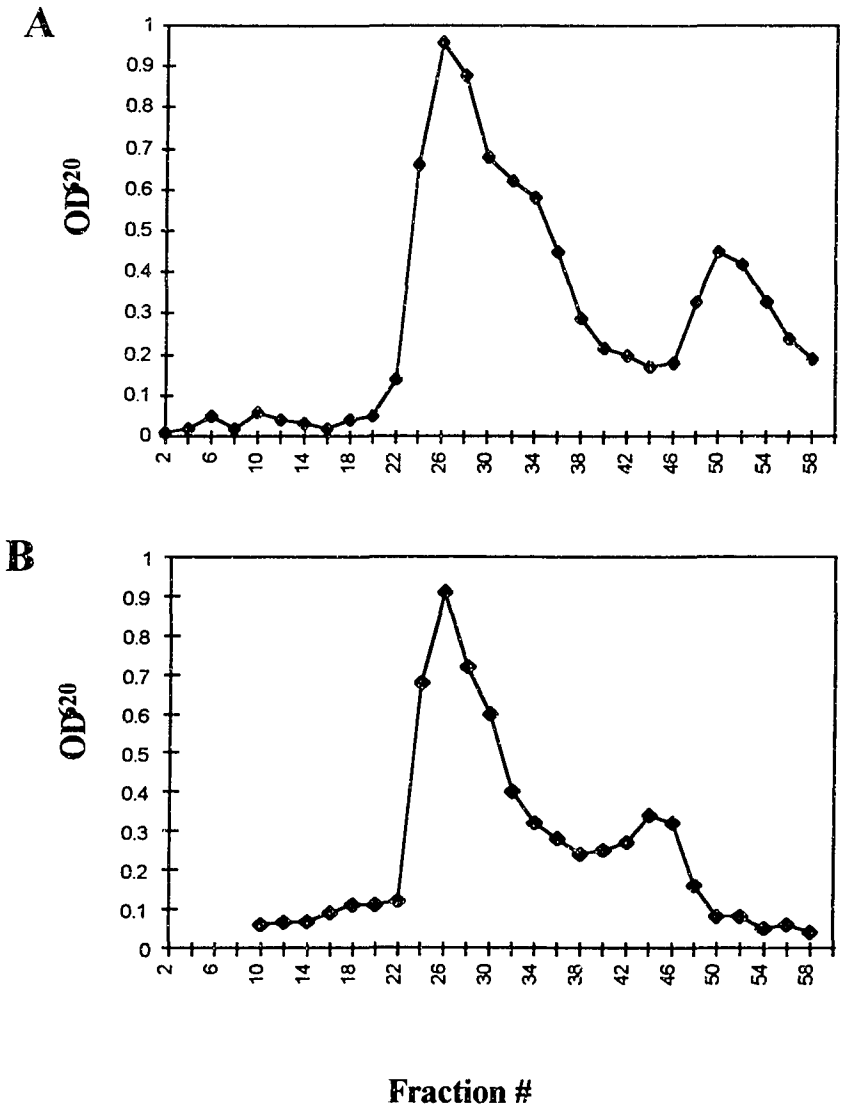


Fig.4.5. Gel filtration of culture supernatant of TAL1145 (A) and mutant NP84 (B) through Bio-Gel A-5m column. TAL1145 (20 mg) and NP84 (0.2 g) lyophilized culture supernatants were loaded on the column. Hexose content of the samples was determined by the procedure given in Materials and Methods section.

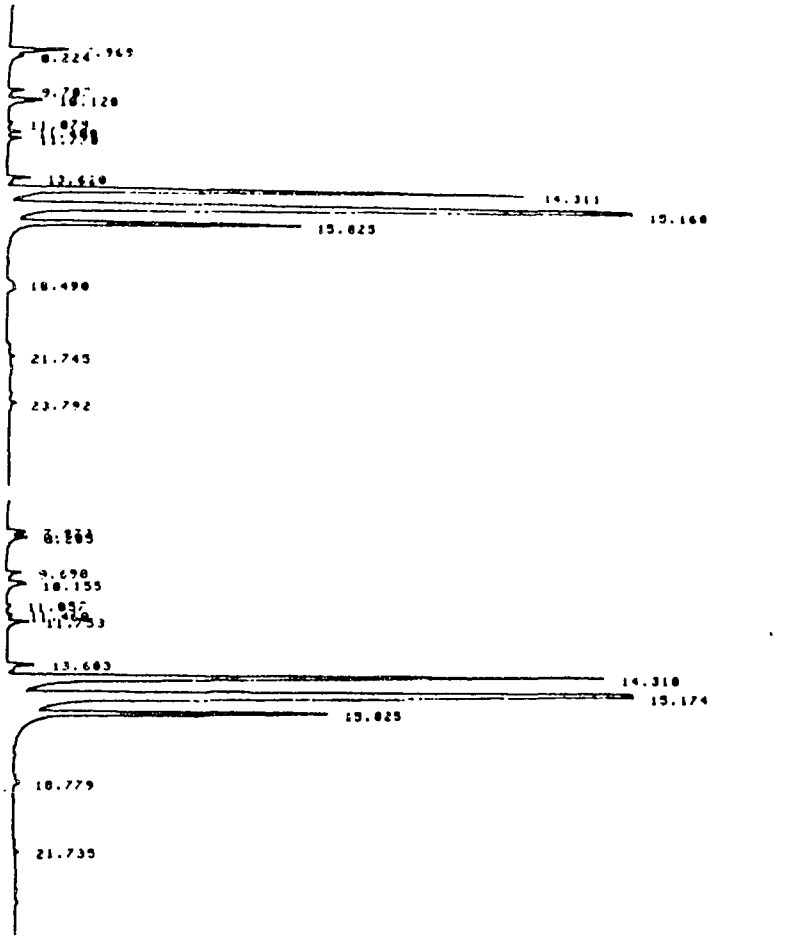


Fig. 4.6 Gas liquid chromatography of alditol acetate derivatives of sugars of partially hydrolyzed EPS of TAL1145 (A) and NP84 (B). Identical peaks are observed in both samples. Prominent galactitol (14.1 min), glucitol (15.1 min), and inositol (15.8 min) peaks are observed. Minor peaks at resolution times of mannitol (13.6 min) and rhamnitol (7.9 min) are also observed.

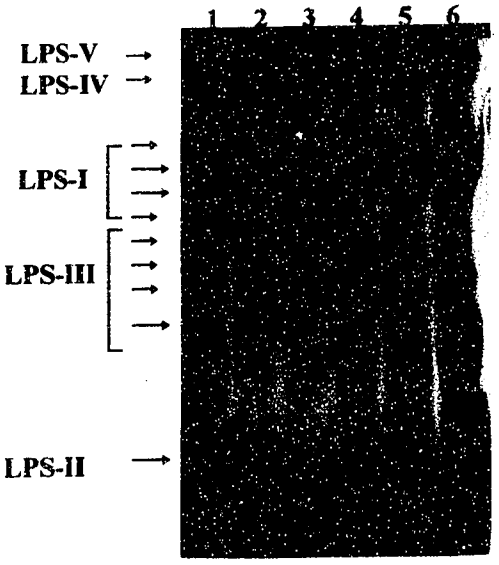


Fig.4.7. Alcian blue-silver stained polyacrylamide gel (18% acrylamide containing, 10% SDS and 0.5% DOC) of crude LPS preparations from *Salmonella typhimurium* (lane 1), TAL1145 (lane 2), NP84 (lane 3), and NP84(pUHR182), NP84(pUHR251) and NP84(pUHR252) (lanes 4, 5 and 6 respectively).

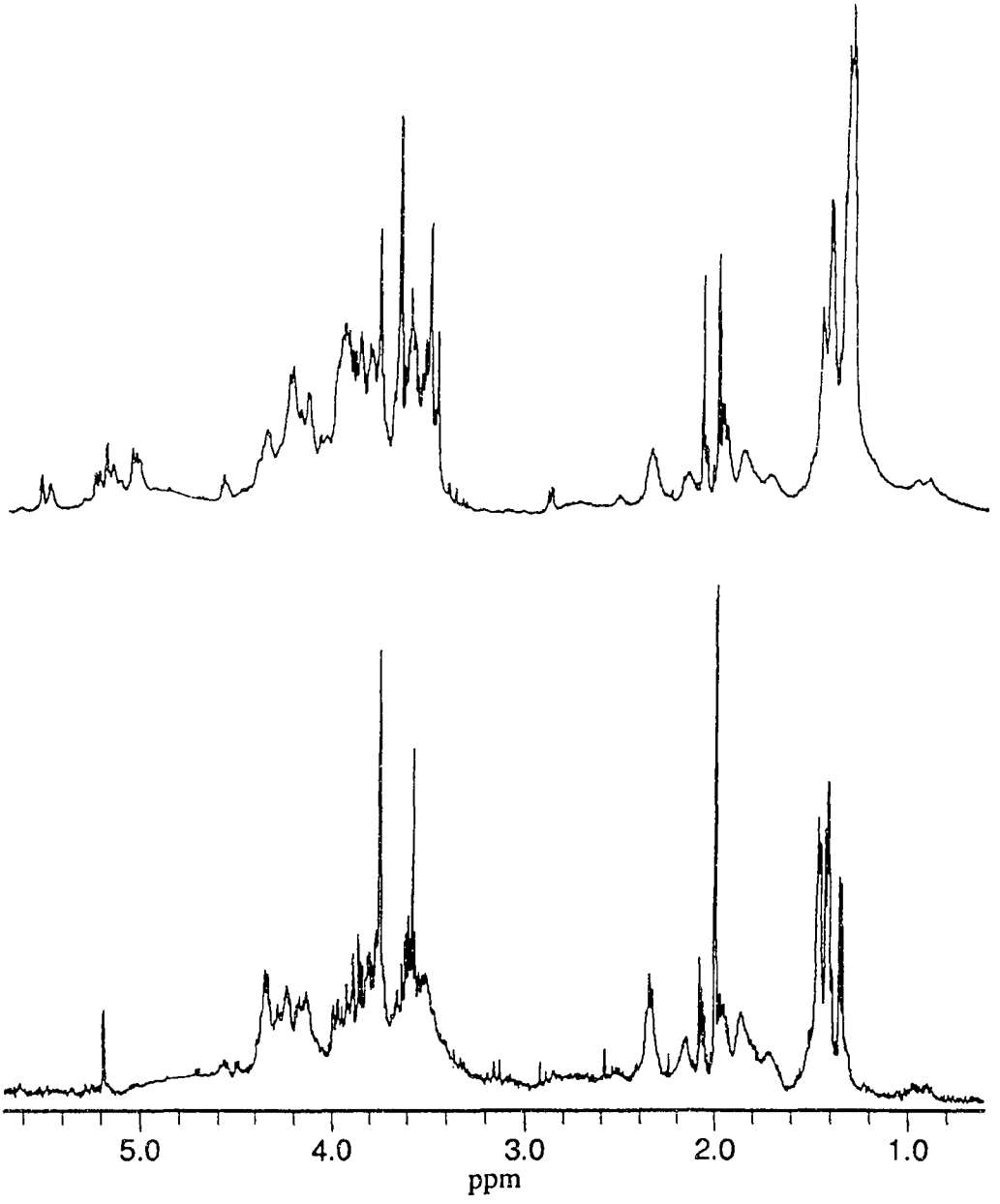


Fig. 4.8 ^{1}H NMR spectra of the crude preparation of LPS of TAL1145 (top) and the mutant NP84 (bottom). Samples were dissolved in D_2O and the spectra were obtained at 500 MHz. Acquisition time was 3.28 sec and 2000 acquisitions were obtained.

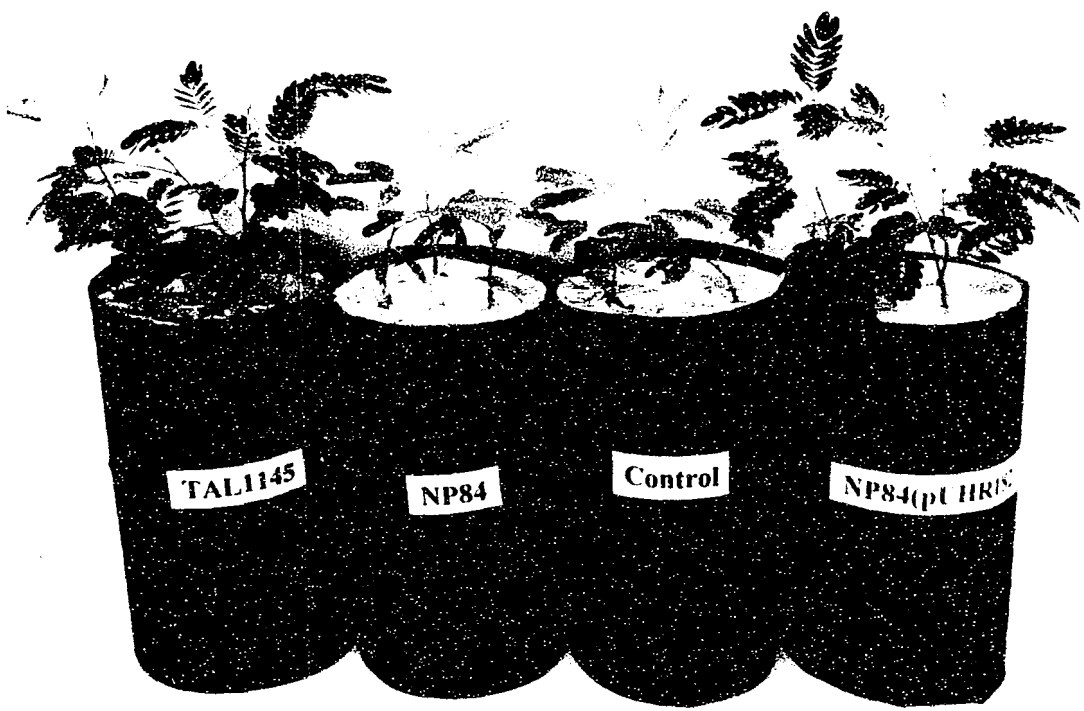


Fig.4.9 Four week old plants of *L. leucocepahala* inoculated as mentioned. Uninoculated control is also included.

4.9 Symbiotic phenotype of NP84 on *L. leucocephala* :

NP84 formed a similar number of nodules on *Leucaena leucocephala* as TAL1145, but they were smaller than those formed by TAL1145 four weeks after inoculation. However, the size and weight of these nodules became very similar to those formed by TAL1145 after eight weeks of inoculation (data not shown). The *L. leucocephala* plants inoculated with the mutant NP84 appeared yellow and stunted in growth after one month of inoculation showing a Fix^- phenotype (Fig. 4.9). Acetylene reduction assay of nodules harvested from these plants did not show detectable nitrogenase activity. The dry weight of these plants was similar to those in the uninoculated control (Table 4.1). However, when the plants nodulated with NP84 were grown for more than five weeks they started to fix nitrogen as judged from the growth of the plants and the color of their leaves. After eight weeks these plants became as green as those nodulated by TAL1145. At this stage the total dry weight of these plants was virtually identical to those nodulated by TAL1145 showing that normal nodule development occurred during the 4-8 weeks period (Table 4.1). Bacteria isolated from the nodules of these eight-week old plants were kanamycin resistant and EPS-defective as judged from colony morphology on YEM agar. The transconjugant NP84(pUHR182) formed effective nodules on *Leucaena* as judged from the acetylene reduction assay and the dry weights of the plants which were similar to those inoculated with TAL1145. This experiment was repeated three times with similar observations.

Nodules formed by NP84 and TAL1145 on *Leucaena* were harvested, fixed and embedded in Spurr resin for microscopic examination. Even the largest nodule formed by NP84 after four weeks showed a much lower number of infected cells than those formed by

Fig.4.10. Light microscopy of 1μ thick sections of five-week-old *L. leucocephala* root nodules, TAL1145 (A), NP84 (B) and NP84(pUHR182) (C). Vascular tissue (vt) and densely stained bacteroid-filled cells (b) and tannins (t) in outer cortex are shown. Bar represents 100μ.

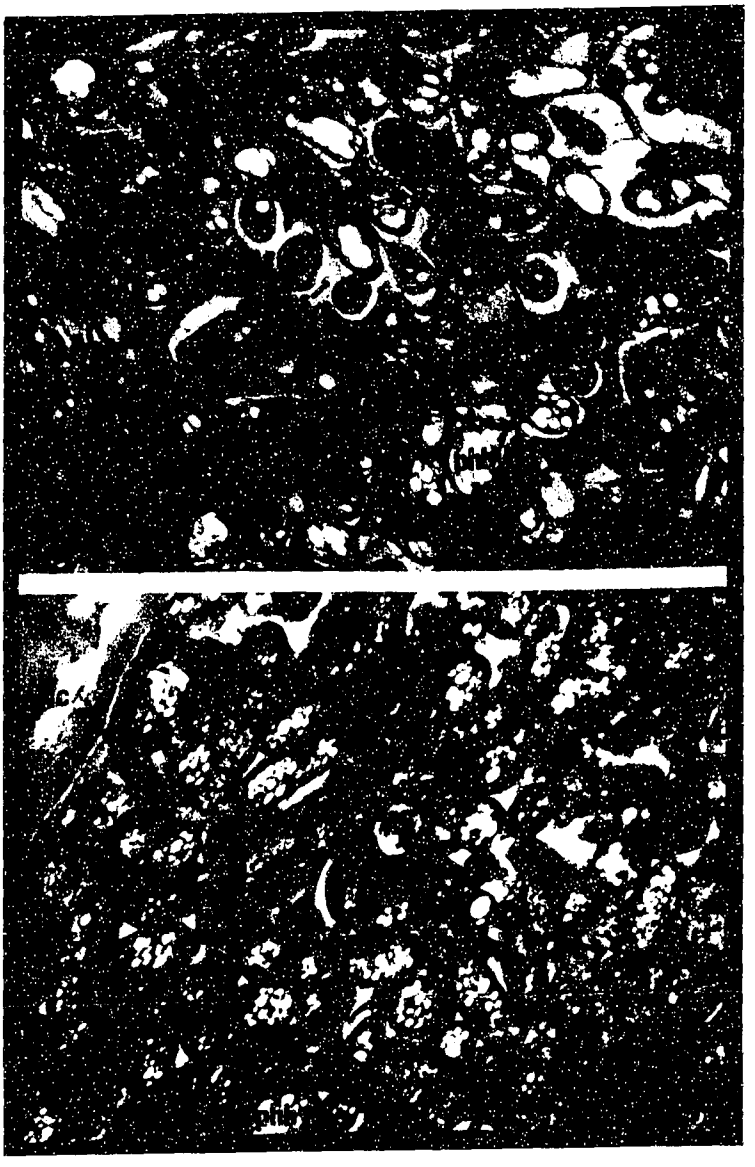


Fig.4.11 Transmission electron microscopy of *L. leucocephala* nodules infected zones TAL1145 (A) and NP84 (B). Bar indicates 4.5μ. Uninfected cell (uic), poly-β-hydroxy butyrate (phb), and peribacteroid space (pbs) are shown.

wild-type strain TAL1145 (Fig. 4.10A and 4.10B). However, normal-looking, bacteroid-filled cells and peripheral vascular bundles were seen in the nodules formed by the mutant. The complemented mutant had a similar nodule phenotype as the wild type (Fig. 4.10C). Electron micrographs of nodules formed by NP84 showed many uninfected cells with fibrillar material which may be an indication of bacterial degeneration (Fig. 4.11B). Bacteroids were more tightly packed than those in nodules formed by TAL1145 (Fig. 4.11A). Bacteroids in nodules formed by NP84 contained more but smaller poly-ß-hydroxy butyrate granules and electron dense cytoplasm compared to TAL1145 bacteroids. The peribacteroid membrane and peribacteroid space enclosing the bacteroids were clearly observed in the core region of the nodules formed by TAL1145 but they were not discernible in the nodules formed by the mutant.

4.9.1 Mutant NP84 is defective in nodulation competitiveness :

Since NP84 forms nodules on *Leucaena* at the same time as TAL1145 experiments were conducted to determine if it can invade *Leucaena* nodules when co-inoculated with the wild-type strain. The competitive nodule occupancy by NP84 and TAL1145 on *Leucaena* in two experiments is shown in Table 4.2. None of the nodules in the plants co-inoculated with both strains showed single occupancy by NP84 even when the inoculum for NP84 was nine times of that for TAL1145. However, in all co-inoculated plants a few nodules were occupied by both TAL1145 and the mutant as indicated by colony morphology of the plated ex-nodule rhizobia. In these nodules where double occupancy was observed, the proportion of the mutant in the ex-nodule rhizobia was estimated to be one per 10^6 colony forming units. Such double occupancy was observed in less than 2% of the nodules when the proportion of

the mutant in the inoculum was 10 or 50%. Eight to 20% of the nodules showed double occupancy when the proportion of the mutant was 90% in the inoculum in these expeeriments. When this experiment was repeated, qualitatively similar results were obtained.

4.10 Symbiotic phenotypes of NP84 on other legumes:

Mutant NP84 formed callus-like pseudonodules on *G. sepium* (Fig. 4.12B) and *P. vulgaris* (Fig. 4.12E) compared to the nitrogen-fixing nodules formed by TAL1145 on these legumes (Fig. 4.12A and 4.12D respectively). The nodules formed by TAL1145 on *G. sepium* were at least three times larger in diameter than those formed on *P. vulgaris*. Similarly, the pseudo-nodules formed by NP84 on *G. sepium* were a little larger than those formed on *P. vulgaris*. Bacteria could not be recovered from the pseudonodules of either host.

No bacteroid zones or bacteroid-filled cells were visible in either host but vascular tissue was observed in the center of some pseudo-nodules. Small meristematic-like cells filled with dense cytoplasm and prominent nuclei were observed in the pseudonodule sections of both hosts (Fig. 4.12F) as compared to darkly stained infected cells observed in the nodules formed by the wild-type. *P. vulgaris* and *G. sepium* inoculated with the transconjugant NP84(pUR182) formed normal, nitrogen-fixing nodules. A section of a *G. sepium* nodule formed by this transconjugant is shown in Fig. 4.12C. Observation of the nodule sections under higher magnification clearly indicates the presence of bacteria in darkly stained cells of the nodules formed by the wild type on three hosts as well as those formed by NP84 on *L. leucocephala* (Fig. 4.13). Small meristematic cells filled with dense cytoplasm can be seen in the pseudonodule of Phaseolus beans formed by NP84.

Table 4.2 : Nodule occupancy by strains TAL1145 and NP84 in paired inoculations on *L. leucocephala*.

Experiment	Strains ratio TAL1145 : NP84	% of nodules with double occupancy (Mean. ± SD)*	% of nodules occupied by TAL1145 only (Mean ± SD)*
1	9 : 1	1.7 ± 2.6	98.3 ± 2.6
	1 : 1	0.7 ± 0.9	99.3 ± 0.9
	1 : 9	19.9 ± 6.0	80.1 ± 6.0
2	9 : 1	0.6 ± 1.4	99.6 ± 1.4
	1 : 1	1.8 ± 1.5	98.2 ± 1.5
	1 : 9	7.2 ± 4.8	92.8 ± 4.8

* Mean values of plants from eight Leonard jars were taken.

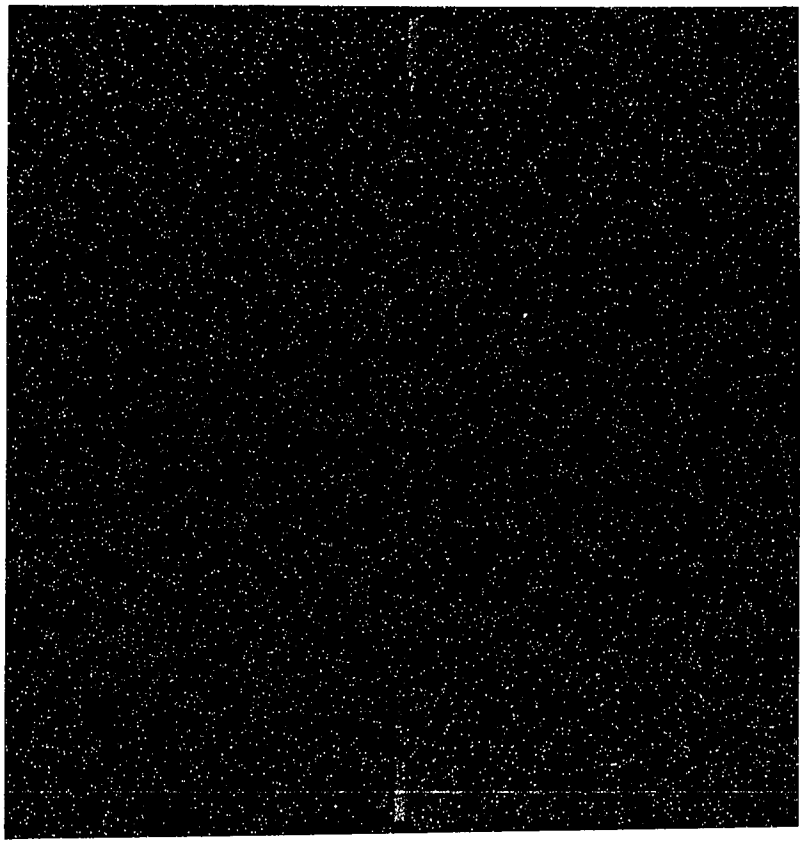


Fig.4.12. Light micrograph of 1μ thick sections of *G. sepium* nodules inoculated with TAL1145 (A), NP84 (B) and NP84(pUHR182) (C) and *P. vulgaris* nodules inoculated with TAL1145 (D) and NP84 (E). Fig. 4.12 shows pseudo-nodules formed by NP84 on beans. Bar from (A) to (E) represents a size of 110μ and (F) indicates 10μ. Bacteroid-filled cells (b), vascular tissue (vt), meristematic like cells (m), vacuoles (v) and plant cell nucleus (n) are shown.

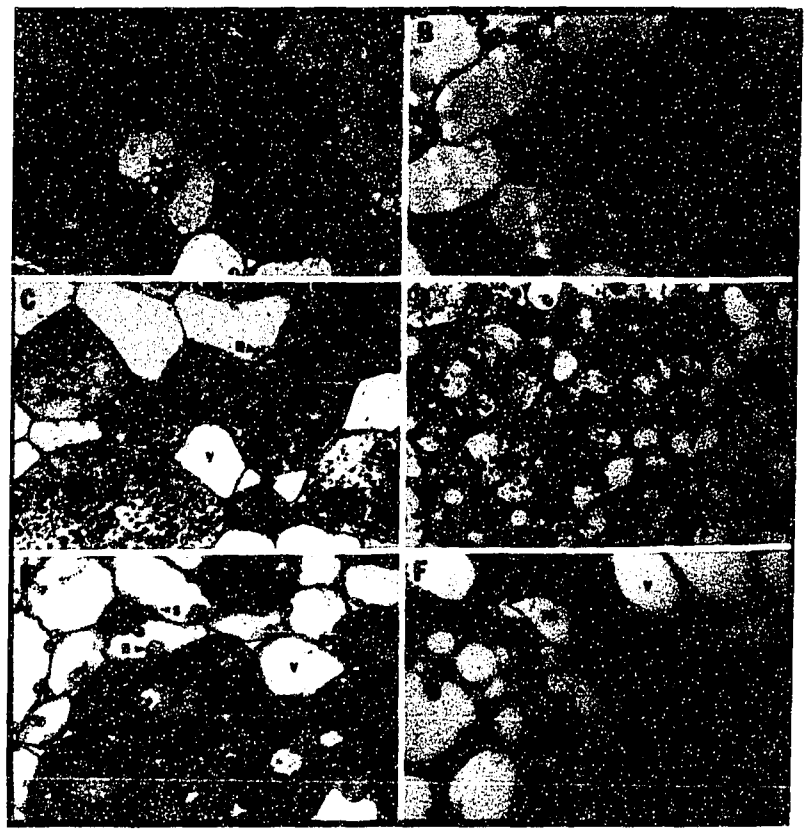


Fig. 4.13 Light microscopic examination of 1 μ thick sections of nodules formed by TAL1145 (A, C, E) and NP84 (B, D, F) on *L. leucocephala* (A and B), *P. vulgaris* (C and D) and *G. sepium* (E and F) under high magnification (800x). Bar indicates 10 μ.

4.11 The 3.8-kb DNA fragment showed homology with DNA from only *Leucaena*-nodulating *Rhizobium* strains:

The 3.8-kb fragment in pUHR252 was used as a probe against genomic DNA from various*Rhizobium* spp. It hybridized with CIAT899 and NGR234, both of which nodulate *Leucaena*. It did not show detectable hybridization with the *R. meliloti* and *R. leguminosarum*, *R. fredii* and *B. japonicum* strains tested (data not shown). When the same probe was used against various plasmid DNA containingdifferent *exo* genes of *R. meliloti* and NGR234, and the *pss* genes of *R. leguminosarum*, ithybridized with a 3.3-kb *Eco*RI fragment and a larger *Eco*RI fragment of approximately 8.0-kb size in plasmid R'3222 that contains *exo* genes of NGR234 (Fig.4.14). This 3.8-kb fragment was also used as a probe against 16 *R. tropici* strains from Mexico (Fig. 4.15). It hybridized stronglywith a 6.6-kb *Eco*RI fragment in the type A strains and a 6.4-kb *Eco*RI fragment in the type B strains of *R. tropici*.

4.12 Gene mutated in NP84 was localized on chromosomal DNA :

Three large, indigenous plasmids of approximately 80 , 150 and 230 Md sizes named as pTAL1145c, pTAL1145b and pTAL1145a respectively were observed in TAL1145 (Fig. 4.16A) by in situ lysis and agarose gel electrophoresis. Southern hybridization of the blot with pUHR182 showed that this cosmid is a part of chromosomal DNA of TAL1145 (Fig. 4.16C and 4.16D). The cosmid pUHR115 that contains *nodDABC* and *nodIJ* was demonstrated to be a part of the largest indigenous plasmid of TAL1145 (George et al. 1994). Transfer of large size DNA fragments from gel to nylon membrane sometimes has difficulties even when DNA is nicked by HCl. Therefore, complete transfer of indigenous plasmids was checked by using hybridization of the same blot with pUHR115 as a control. The plasmid pUHR115

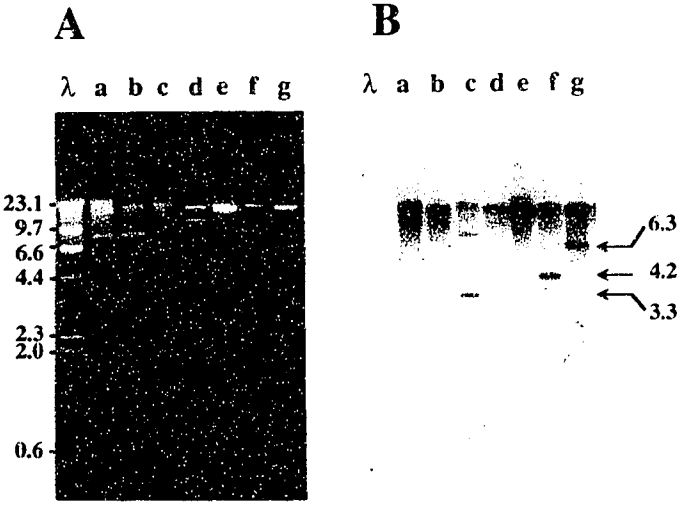


Fig.4.14. (A) Ethidium bromide stained agarose gel showing *Eco*RI restricted plasmids containing *exo, pss* and *psi* genes of different *Rhizobium* species. *R. meliloti* plasmids pD56 and pEX312 (lanes a and b), *R.* sp. strain NGR234 cosmid R'3222 (lane c), *R. leguminosarum* bv phaseoli plasmids pIJ1427 and pIJ1433 containing *pss* and *psi* genes, respectively (lanes d and e), pUHR182 (lane f) and pUHR183 (lane g).

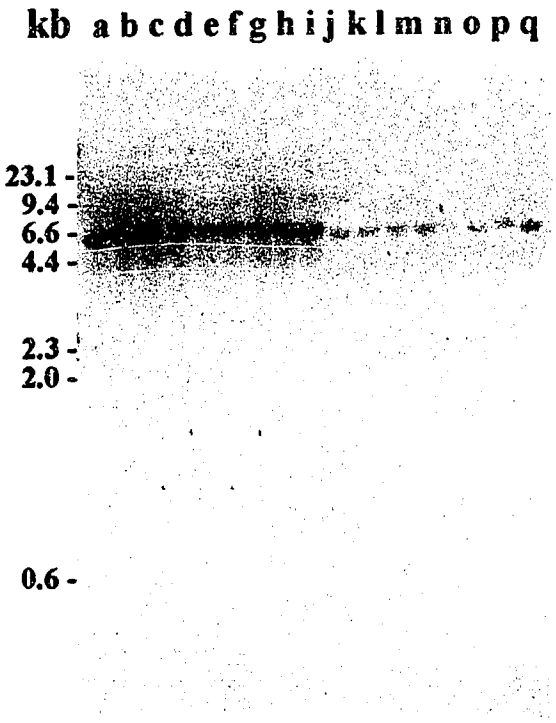


Fig.4.15. Southern analysis of *Eco*RI-digested *R. tropici* strains genomic DNA with ^{32}P-labeled pUHR252 as probe. TAL1145 (lane a), Type A strains are BR828, BR833, BR835, BR836, BR842, BR846, CFN299 and BR10043 (lanes b to i) and Type B strains are BR847, BR850, BR852, BR857, BR858, BR859, BR863, BR864 (lanes j to q).

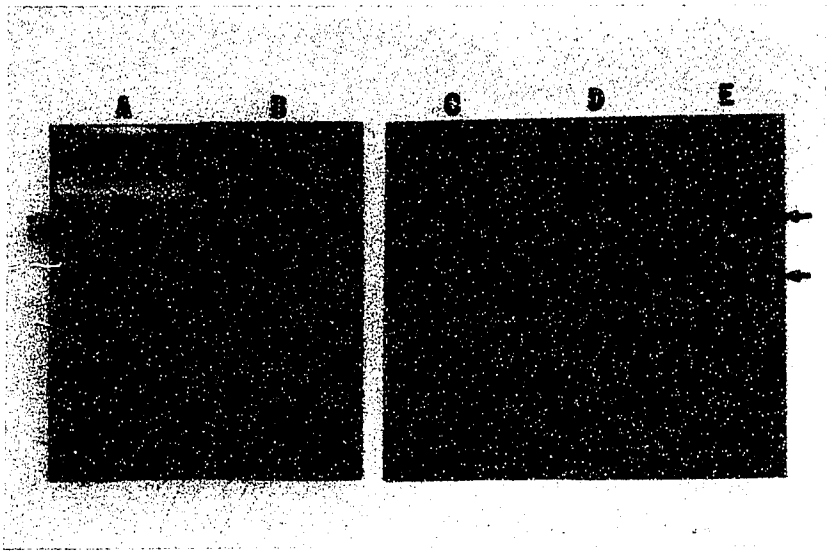


Fig. 4.16 Ethidium bromide stained agarose gels showing large indigenous plasmids of TAL1145 obtained by *in situ* lysis (A) and genomic undigested DNA (B).

Southern hybridization of gels in Figs. A and B using ^{32}P-labelled plasmids pUHR182 (C and D respectively) and pUHR115 (E) as probes.

probe hybridized with pTAL1145a (Fig. 4.16E) confirming that the gene interrupted in mutant NP84 was located on the chromosome of TAL1145.

4.13 Interposon mutagenesis indicates the possible boundary of the gene :

Transposon Tn*5lacZ* insertion in the mutant NP84 was found to be in a 1.2-kb PstI fragment that overlapped with the 6.3-kb *Eco*RI fragment ((Fig. 4.3). Plasmid pBR322 can not replicate in the *Rhizobium* strain TAL1145. Therefore, this plasmid was used for interposon mutagenesis. Several interposon mutants showing two different colony morphologies were observed when 1.2 kb *Pst*I fragment cloned in pBR322 was transferred to TAL1145 and the mutants were selected on tetracycline containing plates. Four mutants were purified two of which showed a colony phenotype like TAL1145 while the other two appeared smaller, opaque and less mucoid like NP84. It suggests a possibility that a *Pst*I site disrupts the gene in the mutant NP84.

4.14 Sequence analysis:

The DNA sequence of a 3.8-kb *Eco*RI fragment was mapped using the Genetic Computer Group (GCG) program. Also, the sequence was compared with gene databases (GenBank/EMBL) using FASTA. The ORFs obtained by mapping were compared with the translation of the known genes that showed a high level of similarity by FASTA. Potential protein sequences were also compared using the Swissport protein data bank from GCG and also by using BLAST.

A hydrophobicity plot was obtained by using the TopPred II software of Claros and von Heijne (1994).

Fig. 4.17 DNA sequence of a 3.8-kb *Eco*RI fragment that complemented the mutant NP84 and the deduced amino acid sequence of the three ORFs. The arrowhead indicates the position of the transposon Tn5*lacZ*. The initiation codons and the amino acid sequence that indicates the prokaryotic membrane lipid-binding domain are underlined. The glyceride-fatty acid lipid binds to the cysteine residue in this domain after cleavage of the signal peptide. The potential ribosome-binding sites are indicated by thick underlines.

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ATAGACGAGTGGCGGGCGGATGATGACGACATCCATGTTCGTCTCCTTGCCGATAGCAAG    60
GAGCGCCTCCTCCGCCTCCCGCTTCGACTGCCCGTAGGGGTCTTCCGGATGCGGCGTATC   120
GGACGCGGTAAACGGCTTGCCGGGCGCGGTCGCCTCACCATTGACCTTGATCGAACTCAA   180
GAAGATGAAGCGCTTTACGCCGGCCTGCGCCGCCTGCCGCGCCAAGTTGACCGTGGCGTC   240
GACATTGACAGCGCGAAAGGCCGCAAGCGGGTCGGCCGCGGTGTCGTTCATGACATGGAC   300
CCGCGCCGCCAGATGCACGACCGTTTCGACGTCGACGAGCGCATTGGTCCAATCCGTCCC   360
GCCATCGATCGTTCCCAGCGCCAGAAATCCCGGCCTCGGGGTACGGCTGACCGGTCGATA   420
GGCCATCTGTCGCCGCGAAAGCTCGGCGCAAAGCGCCTGCCCGACAAAACCCGTCGCGCC   480
CGTCACCAGGATCATGCCTCTGCTACCCCTCGTTCTGCCCGGTTTTCACTCCCGCCATGC   540
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CCTGGCCGCCCTCGGCGCAGCCGTGGTCTTTTCCATCCCGATCCTGATCGTCGCCATCGC   780
 L  A  A  L  G  A  A  V  V  F  S  I  P  I  L  I  V  A  I  A
CGTCCGGTTGACCTCCCGCGGCCCCGTACTCTACTGGTCGGACCGTGTCGGCCGTGAAAA   840
 V  R  L  T  S  R  G  P  V  L  Y  W  S  D  R  V  G  R  E  N
CAGGATTTTTCGCATGCCGAAATTTCGCAGCATGCGGACCGACACCCCGGCGGTTGCCAC   900
 R  I  F  R  M  P  K  F  R  S  M  R  T  D  T  P  A  V  A  T
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 H  L  L  K  D  A  N  A  Y  L  T  P  I  G  S  F  L  R  K  S
CAGCCTGGATGAACTGCCGCAGCTCTGGTGCATCCTGAAGGGCGAGATGAGCATCGTCGG  1020
 S  L  D  E  L  P  Q  L  W  C  I  L  K  G  E  M  S  I  V  G
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                                                                      .
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                                                                      .
GATCCCGCAGAAAGTGGCTTTGGACGAAGACTATCTGCGCCGGCGCTCGTTTCTTTTCGA    1200
 I  P  Q  K  V  A  L  D  E  D  Y  L  R  R  R  S  F  L  F  D
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                                                                      .
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ATCGGCTTGCCGAACATTGGGCTTCGACGAGTGAAAGCCGAATGCTTCCGAGCGGAGGTT   3180
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CACCCAAAAAGAAGACGTTTTTGAGATTGTTCTGCTGCGCATAGGCCTTCAAGGACGCCT   3300
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  F  G  T  S  A  F  E
```

Sequencing of a 3.8-kb *Eco*RI fragment that complemented the mutant NP84 resulted in the identification of three ORFs (Fig. 4.17). The 394 amino acid long ORF which was disrupted in the mutant NP84 may be involved in the LPS synthesis / transport and is designated as *lpsA*. The ORF for *lpsA* codes for a protein of 43-kDa size. The amino acid sequence of LpsA did not show significant homology with the sequences in the data banks. Some similarities with several transmembrane proteins; such as PsaB protein of the plant chloroplast and Lmp1 protein of Epstein-Barr virus, are observed which may be due to the hydrophobic nature of this region and not necessarily due to any specific function. The hydropathy plot of the deduced amino acid sequence (Fig. 4.18A) shows at least 10 hydrophobic domains in the LpsA polypeptide. Analysis of the protein by the TroPred II program based on the algorithms of Claros and von Heijne (1994) shows the topology of the prospective membrane protein to have ten membrane-spanning domains (Fig. 4.18B). A domain observed in LpsA between the third and fourth membrane spanning segments (Fig. 4.17) was similar to the consensus lipid-binding domain observed in a number of prokaryotic lipoproteins. Cleavage of the signal peptide by signal peptidase II, present in cytoplasmic membrane, and post-translational modification takes place in this region in the majority of the lipoproteins in bacteria. The lipoprotein box has been described as Leu-(Ala / Ser)-(Ala / Gly)-Cys with cysteine in the +1 position in the mature protein. The amide bond formation between the fatty acid and a modified cysteine (diglyceride cysteine) has been observed in all lipoproteins (Hayashi and Wu 1990). The presence of a domain similar to the lipid-binding domain of lipoproteins suggests a possibility of LpsA being an outer membrane protein. This domain of LpsA protein

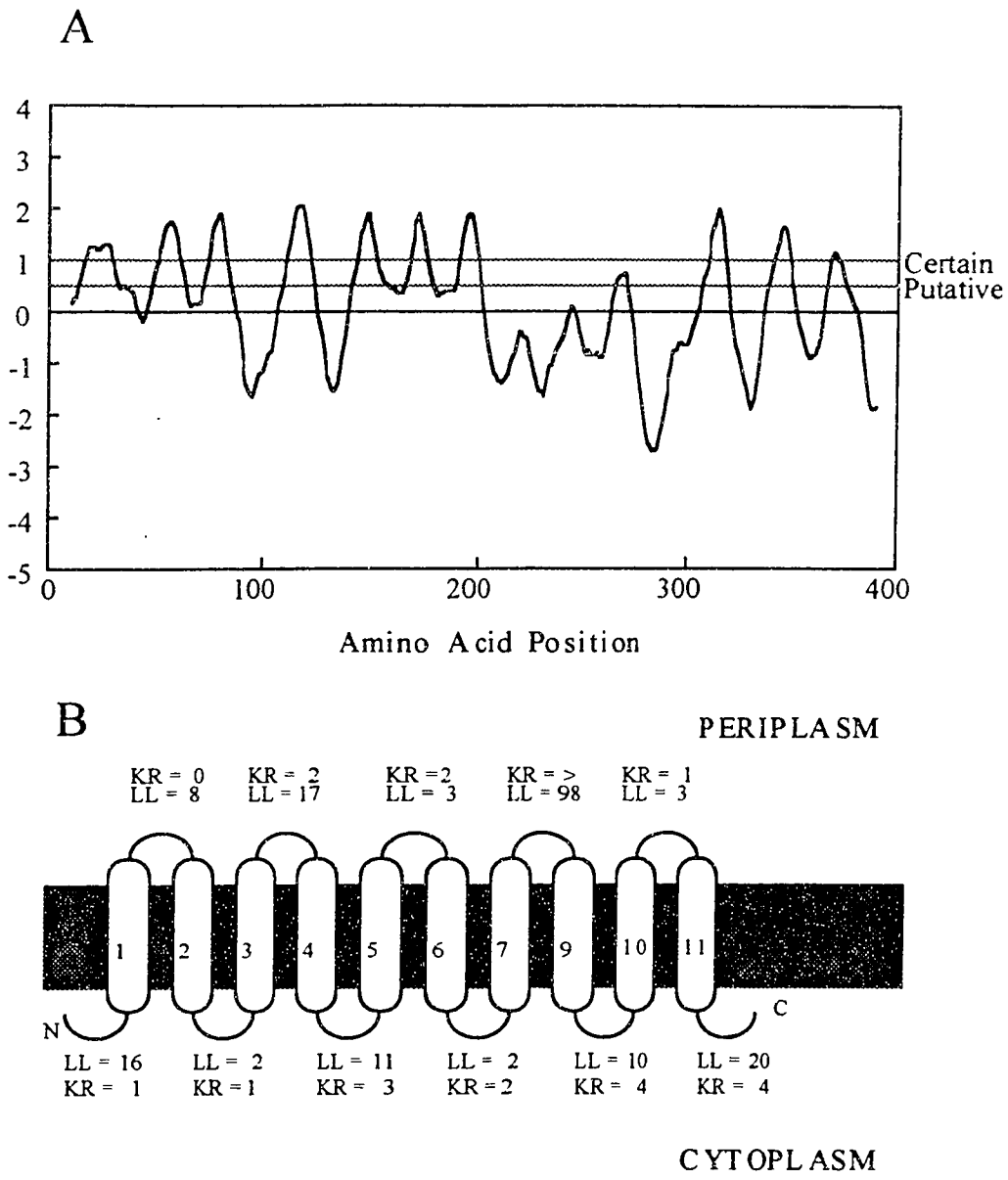


Fig. 4.18A Hydropathy profile of the deduced mino acid sequence of the *lpsA* gene. The plot is drawn using TopPred II based upon algorithm of von Heijne (Claros and Heijne 1994). A 21 amino acid residue window and a critical length of 60 amino acid is used to determine the hydrophobicity.

4.19B Ten prospective transmembrane domains of LpsA protein. Ll indicates the loop length and KR shows the number of Lysine and Arginine residues. The lipid-binding domain similar to consensus lipid-attachment site of prokaryotic is present between third and fourth transmembrane segements with last amino acids of the domain overlapping with the 4th membrane spanning segment.

```
    1                                                            50
 Rlpss2  .......... .......... .......... .......... ..........
   Pss4  VTGLTIDRLL PPRPLEDNRV RSLSITELNN SISTESFRPS RRQQPSLKIQ
ExoYngr  .......... .......... .......... .......... MKSATRSATT
 RmexoY  .......... .......... .......... .......... MKSATRSASS
ExoYTAL  ...MPLLPLV LPGFHSRHAL HIPFAGNKPG KIRDEGDCEQ VRDMRCTPQK


        51                                                   100
 Rlpss2  .......... ...VD..... LVLKRAFDIF SSLSALLVLA PFLLFVALLI
   Pss4  TPVIHSDAPQ APLVD..... LVLKRAFDIV SSLSALLVLA PFLLFVALLI
ExoYngr  AFFIPQETGA IRPIG..... GISKRSFDVL IAILALIALS PLFLLVMGLV
 RmexoY  PFFIPEETGA VRPIG..... GMAKRSFDVL AASVALLLFS PLFLLIMALV
ExoYTAL  ALIGAIETRA PAEPTETMPY TIVKRLFDFL AALGAAVVFS IPILIVAIAV
               ++ +              + ** ** + +++ *  +++    *+++  +

        101                                                  150
 Rlpss2  KLDSPGPVLF KQTRWGKNCK AIKVYKFRSM R......... .....TDLCD
   Pss4  KIDSPGPVLF KQTRWGKNCK AIKVYKFRSM R......... .....TDLCD
ExoYngr  KFSDGGSIFY GHRRIGHNGQ TFKCLKFRTM MENGDRVLQE FFKSNPAAYE
 RmexoY  KFSDGGSVFY GHRRIGHNGQ SFKCLKFRTM MEKGDEVLEE FFRINPDAYE
ExoYTAL  RLTSRGPVLY WSDRVGRENR IFRMPKFRSM RTDTPAVATH LLK.......
          + + *++++    * *      +   ***+* +     *

        151                                                  200
 Rlpss2  VSGVAQTVKN DPRITRIGAI LRRTNVDELP QLLNVLLGHM SVVGPRCHAI
   Pss4  VSGVAQTVMN DPRVTRVGAI LRRTNVDELP QLLNVLLGHM SVVGPRCHAI
ExoYngr  EWRTTRKLQD DPRVTVVGSV LRKLSLDELP QLLNIIRGEM SIVGPR....
 RmexoY  EWRATRKLQN DPRVTVVGAV LRKLSLDELP QLLNIIRGEM SVVGPR....
 ExoYTAL .......DA NAYLTPIGSF LRKSSLDELP QLWCILKGEM SIVGPRPALF
                    *  *+  **+ ++**** **  +  *+* *+****

        201                                                  250
 Rlpss2  GMRAGGMLYE ELVPEYHQRH AMRPGMTGLA QMRGLRGPTD RPAKARARIA
   Pss4  GMRAGGVLYE ELVPEYHQRH AMRPGMTGLA QMRGLRGPTD RPAKARARIA
ExoYngr  PVVEDELELY DSAAEFYLRS ..RPGLTGLW QISGRND... ..VSYATRVA
 RmexoY  PVVEDELELY DSAAVFYLRS ..RPGLTGLW QISGRND... ..VSYATRVA
ExoYTAL  NQHDLIALRT ERGVD..... ALPPGLTGWA QVNGRDE... ..LPIPQKVA
               +   +          *  **+** + *  *+              +*

        251                                 284
 Rlpss2  SDLYYVGNFS IVMDMRIIFG TVVSE.LTRG KGF*
   Pss4  SDLYYVGNFS ILMDMRIIFG TVVSE.LTRG KGF*
ExoYngr  FDTHYVQNWS LLADLVIVFK TIPAVCLSRG SY*.
 RmexoY  FDTQYVQNWS LFADLVIVFK TIPAVCLSRG SY*.
ExoYTAL  LDEDYLRRRS FLFDLKIIVL TVLKVLRSDG VTH*
          *  *    *  +  * *    *+  +  + *
```

Fig.4.19 Alignment of the deduced amino acid sequence of the *exoY* gene of TAL1145 (ExoYTAL) with Pss2 and Pss4 proteins of *R. leguminosarum* bv. *phaseoli* and *R. l.* bv. *viciae*, respectively and ExoY proteins of the *Rhizobium* strain NGR234 (ExoYngr) and *R. meliloti* (RmexoY). Asterisks mark (*) shows conserved amino acid in all proteins and (+) shows amino acid in TAL1145 protein present in some but not all five proteins.

```
      1                                                            50
LpsB  .......... .......... .......... .......... ..........
Mtfc  .......... .......... .......... .......... ..........
Ompx  MPWKTYSRNL MYAVITLMLN VLSEFYLMHL RVIRHKNENK MKVLHVYRTC

      51                                                           100
LpsB  .......... .......... .......... .......... ..EFASTGFS
Mtfc  .......... .......... .......... .......... ..........
Ompx  YPETKGGVEQ VIRFIASGTK PLGIETKILT LSDNQTSSYY CEGTEIISVK


      101                                                          150
LpsB  REAFGRFRES LRKADIVHYH FPWPMMDIVH LAIP...... ....PGKPTV
Mtfc  .......... .......... ....MDMLHL SAR....... ....PDARTV
Ompx  KSIEISSNGF SWKLIRQFKK LSKWADIIHY HYPWPTGDFL SLFGSSNPSI
                                  ++ +                      +++
      151                                                          200
LpsB  VTYHSDIVKQ KFLLKLYGPV MHRFLASVDS IVATSPNYLA TSEVLQRYKD
Mtfc  VTYHSDIVKQ KRLMKLYQPL QERFLASVDC IVASSPNYVA SSQTLKKYQD
Ompx  VTYHSDIIRQ KCLKKLYQPL ESHFLNQANI LVATSPQYAH TSENLLRHKN
      ********++* * * *** *    +**++++  +***+**+* + +*+ * ++++
      201                                                          250
LpsB  KTAVIPLGLD EADYPRASEE DKARWRTRFP KPFFLFVGVL RYYKGVHILL
Mtfc  KTVVIPFGLE QHDVQHDS.Q RVAHWRETVG DNFFLFVGAF RYYKGLHILL
Ompx  KVKIIPLAVD ENTYPIPSND NINKWREKVG EGFFLFVGVL RYYKGLDFLL
      *+ +**++++ + +++  *     + **       *******++ ***** ++**
      251                                                          300
LpsB  EAARQTASDI VIVGEGPMEA SLKAYAQQNN LKNVFFLGAL PDADKTALLE
Mtfc  DAAERSRLPV VIVGGGPLEA EVRREAQQRG LSNVVFTGML NDEDKYILFQ
Ompx  EAAKINQLPV IIAGDGPERV KLESYIAKHN LENVKLVGFI SEEDKVILHL

      +**        +*+* ** ++  +  ++++ + * ** + * +     *  *
      301                                                          350
LpsB  LCTGSN.PFQ PPLGSIRLSL VEAQCSASR* .......... ..........
Mtfc  LCRGVVFPSH LRSEAFGITL LEGARFARPL ISCEIGTGTS FINQDKVSGC
Ompx  LSKAFVFPSH LRSEAFGISL IEAQMYCKAI ISSDIGTGSS YVNINGETGL
      *+ +   *           +*  * +  +

      351                                                          400
LpsB  .......... .......... .......... .......... ..........
Mtfc  VIPPNDSQAL VEAMNELWNN EETSNRYGEN SRRRFEEMFT ADHMIDAYVN
Ompx  VVPPADSQSF SDAMLKIEHD TKLCEKLGIN ARKRFEQEFT AHRYAQSYTK

      401      411
LpsB.......... .
Mtfc  LYTTLLESKS *
Ompx  LYSELFGNVC *
```

Fig.4.20 Alignment of the deduced amino acid sequence of the *lpsB* gene of TAL1145 (LpsB) with OmpX and MtfC (mannosyl transferase C) proteins of *Vibrio cholerae* and *E. coli*, respectively. Asterisks mark (*) shows conserved amino acid in all proteins and (+) shows amino acid in TAL1145 protein present in only one of the other two proteins.

follows the rules formulated to identify the post-translational modifications such as thee presence of a cysteine between position 15 and 35 in the sequence under consideration (starting with the amino acid valine at the 93 position) and the presence of an arginine (can be lysine also) in the first seven position of the mature protein. However, some of the proteins of this class in other bacteria are not membrane lipoproteins.

The *exoY* gene present upstream of the *lpsA* gene showed 36% homology with the deduced amino acid sequence of the *exoY* genes of *R. meliloti* and *Rhizobium* sp. strain NGR234. It also showed 33-39% homology with the Pss 2 and Pss4 proteins of *R. leguminosarum* bv. *phaseoli* and GumD and Xps2A proteins of *Xanthomonas campestris* (Fig. 4.19). All these proteins are sugar transferases suggesting that the ExoY protein of TAL1145 is also a glycosyl transferase. The ExoY of TAL1145 is 256 amino acids long with a predicted molecular weight of 29 kDa.

The *lpsB* gene is present downstream of the *lpsA* gene but in the opposite orientation. The deduced amino acid sequence of LpsB showed 44% homology with Ompx protein of *Vibrio cholerae* and 52% homology with the mannosyl transferase C enzyme of *E. coli* (Fig. 4.20). Both these proteins have been found to be involved in the synthesis of the O-antigen of LPS in the respective bacterial species. The LpsB protein probably has a similar function in the strain TAL1145.

DISCUSSION

In this study a single-transposon insertion mutant NP84 was isolated from a double-insertion mutant NP54. The mutant NP84 showed alterations in both EPS and LPS. Nodule development by this mutant is more severely affected in the determinate

hosts, *P. vulgaris* and *G. sepium,* than in the indeterminate host *L. leucocephala.* A 3.8-kb *Eco*RI DNA fragment cloned from TAL1145 complements this mutant. This 3.8-kb DNA fragment did not show detectable hybridization with DNA of other rhizobia except *L. leucocephala*-nodulating *R. tropici* strains and *R.* sp. strain NGR234.

The plasmid pUHR182 complemented the EPS-defect of the mutant NP54. The plasmid pUHR182::Tn*5lacZ* was isolated from the complemented mutant NP54(pUHR182) by double homologous recombination and was not due to random transposition in the plasmid. This suggestion was confirmed by restriction analysis of pUHR182::Tn*5lacZ* with different enzymes and Southern hybridization which showed the pattern identical to that found in the original mutant NP54 (Fig. 4.3). Southern hybridization of the genomic DNA of two mutants isolated by marker exchange between pUHR182::Tn*5lacZ* and TAL1145 genome with Tn*5* as a probe showed a double insertion. The reason for double insertions with the conservatively transposing Tn*5-lacZ* is not well understood. The initial high frequency of transposition (McCommas and Syvanen, 1988) along with the presence of hot spots (Berg *et al.*, 1988) or rare recombination between the sequence flanking the transposon with partially homologous sequences present elsewhere in the genome are possible explanations. The presence of a 2.8-kb *Eco*RI fragment within pUHR182 which is homologous to 6.3-kb *Eco*RI fragment as indicated by Southern hybridization (Fig. 4.14) supports the last explanation. This method can be used for obtaining single-insertion mutants from double or multiple transposon-insertion mutants. It can also be used to prepare fine restriction maps of the transposon insertions without carrying out extensive Southern hybridizations.

The mutation in NP84 shows a greater effect on LPS than on EPS synthesis. It does not produce the O-antigen of LPS and produces reduced amounts of EPS in medium containing mannitol. The absence of O-antigen in NP84 resulted in an altered banding pattern in the LPS I, III, IV and V regions. Compared to the wild-type strain the mutant NP84 produces a reduced amount of EPS which is chemically indistinguishable from the TAL1145 EPS. Results of the plant experiments with determinate nodule-forming hosts *P. vulgaris* and *G. sepium* suggest that specific oligosaccharide motifs of LPS may be required for normal development of determinate nodules as NP84 formed pseudo-nodules on these hosts. These results are supported by the findings of other workers (Carlson et al. 1987, 1992). Reduction of LPS I or its absence in *R. leguminosarum* bv. *phaseoli* mutants had also been shown to inhibit nodule development of beans (Cava et al. 1989, Noel et al. 1986, Vandenbosch et al. 1985). We observed the presence of central vascular bundles in addition to the normal peripheral conducting tissues in both beans and *Gliricidia*. The presence of central instead of peripheral branched vascular bundles was also reported in bean nodules formed by LPS mutants (Noel et al. 1986).

Bacteroid development and nitrogen fixation is delayed in *Leucaena* nodules formed by NP84. This may be due to some modification of EPS or the absence of O-antigen in the LPS or both. In *R. meliloti* EPS and LPS were shown to perform the same function in nodule development on alfalfa (Williams et al. 1990, Putnoky et al. 1990). It is possible that the EPS produced by NP84 compensated for the absence of some LPS components in *Leucaena* nodules at later stages of development. Leakiness of the Exo phenotype (presence of a few nitrogen-fixing nodules) was observed earlier in *R. meliloti* (Klein et al. 1988). LPS

defects in *R. meliloti* do not affect nodule development in alfalfa that forms indeterminate nodules like *Leucaena* (Clover et al. 1989). On the other hand LPS mutants of *R. leguminosarum* bv. *viciae* formed white Fix$^-$ mutants on indeterminate hosts such as *Vicia hirsuta, Pisum sativum* and *Lens culinaris* (Priefer 1989). These nodules remained small and senesced prematurely. The symbiotic phenotype of NP84 on *Leucaena* in the present study is different from those of *R. meliloti* and *R. leguminosarum* LPS mutants on indeterminate nodule-forming hosts as mentioned above because the *Leucaena* nodules formed by NP84 are Fix$^+$ at four weeks of inoculation and slowly became similar to those formed by the wild-type strain in size, weight and nitrogen fixation. The small Fix$^-$ *V. hirsuta* nodules formed by LPS mutants of *R. leguminosarum* contained infection threads from which bacteria were released into the host cells and became enclosed in peribacteroid membranes (Priefer 1989). Similarly, in the *Leucaena* nodules formed by NP84 bacteria were released from the infection threads and bacteroids formed slowly, but unlike the *V. hirsuta* nodules these nodules did not senesce early. TAL1145 bacteroids had clear peribacteroid membranes and they were surrounded by a distinct peribacteroid space in *Leucaena* nodules while the bacteroids formed by NP84 were more tightly packed and the peribacteroid space was not prominent. It is possible that LPS or EPS or both may be involved in the separation of the peribacteroid membrane from the bacteroids in the nodules formed by the wild-type. However, there is no direct evidence for the presence or absence of polysaccharide components in the peribacteroid space.

Inoculation of *Leucaena* plants with NP84 and TAL1145 separately resulted in appearance of nodules at the same time. However, NP84 was found to be less competitive as

compared to the wild type. When co-inoculated with TAL1145 in various proportions, it was out-competed by the wild type strain for nodule occupancy on *Leucaena*. Bhagwat et al. (1991) suggested that the reduction of EPS synthesis by a *Bradyrhizobium* mutant was responsible for its defect in competitive nodule occupancy. The competition defects of NP84 may be due to the EPS or the LPS defect or both. Reduced competitiveness of *B. japonicum exoB* mutants, that show alterations in both EPS and LPS, for nodulation of *Glycine max* has also been reported by Parniske et al. (1993).

The wild-type DNA fragment homologous to the Tn*5lacZ*-inserted fragment in NP84 has been cloned. A 3.8-kb fragment cloned in plasmid pUHR 252 complemented NP84 for both EPS and LPS defects and allowed the transconjugant of NP84 to nodulate *Gliricidia* and *P. vulgaris*. The *exoB* mutants of *R. meliloti* have been found to be defective in both EPS and LPS synthesis (Leigh and Lee 1988). Therefore, the cloned 3.8-kb fragment was checked for the presence of *exoB* gene of TAL1145. The 3.8-kb fragment in plasmid pUHR252 did not hybridize with plasmid pD56 containing *exoB* gene of *R. meliloti* suggesting that this DNA fragment did not contain a gene structurally similar to the *exoB* gene and therefore NP84 is not an *exoB* mutant. Although NP84 is similar to exo-320 and exo-343 mutants of *R. leguminosarum* bv. *phaseoli* that were defective in both LPS and EPS synthesis, the mutated gene in NP84 may not be the same as any *exo* genes in *R. leguminosarum*, since the 3.8-kb fragment in pUHR252 did not show significant homology with the genomic DNA of any *R. leguminosarum* strains tested. The gene(s) present in the 3.8-kb cloned DNA of TAL1145 may be more conserved in *Leucaena*-nodulating rhizobia because this fragment also showed significant homology with all *R. tropici* strains tested and with the NGR234 genes present on

plasmid R'3222. Previously, the *exoB* region of NGR234 was also thought to be involved specifically in nodulation of *Leucaena* (Zhan et al. 1990). However, unlike NP84, the *exoB* mutants of NGR234 are only defective in EPS but not in LPS synthesis (Gray et al. 1991). Two *Eco*RI fragments of NGR234 that hybridized with the 3.8-kb cloned fragment of TAL1145 do not include the four *exo* regions described by Zhan et al. (1990).

Sequence analysis of the 3.8-kb *Eco*RI fragment showed the presence of three ORFs which encode for proteins involved in the synthesis of LPS and EPS. The *lpsA* which was disrupted in the mutant NP84 did not show significant homology with the genes in the data banks. The LpsA protein is suggested to be a transmembrane protein but further work is needed to confirm its localization in the cell and its function in LPS and EPS synthesis. The presence of lipid binding and a post-translation modification domain in LpsA is similar to the consensus sequence of various lipoproteins; however, some important differences have been observed. A leucine residue observed at the -3 position in the consensus sequence is replaced by a small neutral residue (Ala) in the prospective mature polypeptide and the hydrophobicity of LpsA is also higher than the outer membrane proteins of bacteria. Also, this protein is larger than most known lipoproteins present in the outer membrane and the signal peptide present before the presumptive cleavage site is also unusually large (92 amino acids) as compared to other outer membrane lipoproteins. The presence of small neutral residues in the -1 and -3 positions in the mature protein like that observed in LpsA has been suggested to define the cleavage site of nonlipoprotein precursors (von Heijne 1989). On the basis of these results it is suggested that LpsA may be a lipoprotein present in the cytoplasmic membrane, outer

membrane or in the adhesion zone or Bayer's junction between the inner and outer membrane in *Rhizobium* sp. strain TAL1145. Some known lipoproteins have been found to be located in the inner membrane of the bacterial cell envelope such as Lipp-28 of *E. coli* (Ichihara et al. 1981).

The LpsA protein is probably involved in the transport of lipopolysaccharide and exopolysaccharide in strain TAL1145 as a mutation in the *lpsA* gene in NP84 resulted in changes in the O-polysaccharide of LPS and also in the quantity of EPS secreted. Transfer of lipopolysaccharide through an adhesion zone and its assembly in the outer membrane has been shown by Muhlradt et al. (1973).

The *lpsA* gene present in this 3.8-kb *Eco*RI fragment is probably a part of the larger cluster of genes involved in LPS and EPS synthesis in TAL1145. The presence of two genes flanking *lpsA* that showed significant homology to the genes involved in EPS and LPS synthesis in other bacterial species confirmed this hypothesis. The *exoY* gene present upstream of *lpsA* has 33-39% homology to different sugar transferases. The GumD protein of *X. campestris* is a glucosyl transferase while the ExoY of *R. meliloti* has been shown to be a galactosyl transferase. Therefore, the ExoY of TAL1145 is also suggested to be glycosyl transferase which may be involved in the synthesis of EPS or LPS or both. This protein may be membrane associated like the ExoY of *R. meliloti* and Pss2 protein of *R. l.* bv. *phaseoli* which are both membrane associated (Borthakur et al. 1988, Muller et al. 1993). Gluckmann et al. (1993) have reported the presence of several glycosyl transferases in *R. meliloti,* which are involved in EPS synthesis, that are cytoplasmic. High homology of the deduced amino acid sequence of the gene *lpsB* with

OmpX of *V. cholerae* and mannosyl transferase C of *E. coli* suggests that the LpsB protein is also involved in the synthesis of the O-polysaccharide in the LPS of TAL1145. The function of protein products of *exoY* and *lpsB* genes in LPS and / or EPS synthesis can be confirmed by isolating mutants defective in these genes.

In conclusion, a 3.8-kb DNA fragment of TAL1145 containing genes involved in both EPS and LPS synthesis has been identified in this study. This DNA fragment is essential for the infection of the determinate nodules and is required for normal nodule development and competitiveness in the indeterminate-nodule-forming host, *Leucaena.*

CHAPTER V

ANALYSIS OF A CLUSTER OF GENES INVOLVED IN EPS SYNTHESIS IN *RHIZOBIUM* SP. STRAIN TAL1145

5.1 Introduction :

Bacteria belonging to the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* produce various kinds of polysaccharides that have important roles in the establishment of normal, nitrogen-fixing nodules on their legume hosts. Acidic exopolysaccharide (EPS) of different *Rhizobium* spp. is required for the invasion of nodules and possibly for later stages of nodule development on some legume hosts. The EPS produced by some fast-growing *Rhizobium* spp. such as *R. meliloti, R. loti* and some strains of *R. fredii* produce acidic exopolysaccharide (EPS) that binds the dye Calcofluor. Acidic EPS of *R. meliloti* contains a succinyl group and is, therefore, also known as succinoglycan. In transposon-insertion mutants that do not produce Calcofluor-binding, acidic EPS have been described in different *Rhizobium* spp. (Finan et al. 1985, Hotter and Scott 1991, Leigh et al. 1985, Long et al. 1988a). These mutants formed empty nodules on indeterminate-nodule-forming hosts such as *M. sativa* and *L. leucocephala.* Mutants of *R. meliloti* that produced non-succinylated exopolysaccharide but failed to produce the low-molecular weight form of polysaccharide formed haloless colonies on Calcofluor-containing medium and were also unable to invade the nodules of *M. sativa.*

The *exo* genes in *R. meliloti* and *Rhizobium* sp. strain NGR234 have been found to be clustered together. Long et al. (1988a) identified a cluster of *exo* genes of *R. meliloti* located on the megaplasmid pRmeSU47b that contains 12 complementation groups. In

addition, *exoC* and *exoD* were found to be located on the chromosome. The *exoC* is necessary for phosphoglucomutase activity and mutation in this gene results in pleiotropic effect on synthesis of different polysaccharides while *exoD* was suggested to modulate the quantity of succinoglycan and its distribution between high- and low-molecular-weight fractions (Finan et al. 1986, Gray et al. 1990). The *exp* gene cluster responsible for the synthesis of an alternative exopolysaccharide, EPS II, is also located on the second megaplasmid in *R. meliloti* strain Rm1021 while the regulatory gene, *expR,* was found to be present on the chromosome (Glazebrook and Walker 1989). Chen et al. (1988) found that genetic loci involved in EPS synthesis (*exo* region) in NGR234 are also linked. Zhan et al. (1990) identified four complementation groups in the NGR234 *exo* gene cluster.

Gene fusions with promoterless *lacZ, phoA* and *gus* have been used by various workers to study the expression of genes involved in EPS synthesis in the nodules. The *exoR,* a negative regulatory gene, was suggested to play a role in the inhibition of EPS I synthesis in nodules since the mutant *exoR95* of *R. meliloti* induced Fix$^-$ nodules on alfalfa (Doherty et al.1987 and Reuber et al. 1991). A high level of expression of alkaline phosphatase was observed in *exoF-, exoA-* and *exoP::phoA* gene fusions in *exoR* mutants only in the early symbiotic or invasion zone of the alfalfa nodules. There was almost no detectable expression in the late symbiotic or bacteroid zone suggesting that EPS is essential for invasion of nodules but needs to be attenuated at later stages of symbiosis (Reuber et al. 1991). In *R. meliloti,* transcription of *exo* genes was also observed within the nodules using gene fusions with *lacZ* (Keller et al. 1988). However, expression of *pssA::gus* gene transcriptional fusions of *R. l.* bv. *phaseoli* was shown to be undetectable

in the bean nodules (Latchford et al. 1991). The *pssA* gene is involved in EPS synthesis in this species.

An exogenous supply of EPS restored the nitrogen-fixing ability to *exo* mutants of *R. meliloti* and *Rhizobium* sp. strain NGR234 (Battisti et al. 1992, Djordjevic et al. 1987). Some strains of *R. meliloti* produce a second exopolysaccharide, EPS II, that overcomes symbiotic defects of *exo* mutants on some but not all legume hosts (Glazebrook and Walker 1989, Zhan et al. 1989). In some EPS-defective mutants the presence of specific *lps* genes in some of the *R. meliloti* strains (*lpsX, lpsY, lpzZ* etc.) results in the functional replacement of EPS during symbiosis by producing alternative LPS or K polysaccharides. This underlines the importance of EPS in symbiotic interactions with legumes (Putnoky et al. 1990, Reuhs et al. 1995, Williams et al. 1990a and 1990b). On the basis of these findings, Glazebrook et al. (1990) suggested that exopolysaccharides may have more than one function in nodulation such as avoidance of plant defense response, determining the host range, forming part of the infection matrix and acting as a signal molecule for the host or serving as a carrier for signal molecules.

RESULTS :

5.2 Isolation of three EPS-defective mutants and their complementation by the genomic library of TAL1145 :

Three spontaneous EPS-defective mutant colonies were isolated by screening approximately ten thousand colonies of *Rhizobium* sp. strain TAL1145 on 10 YEM agar plates. These colonies appeared smaller, dry and opaque as compared to the mucoid and

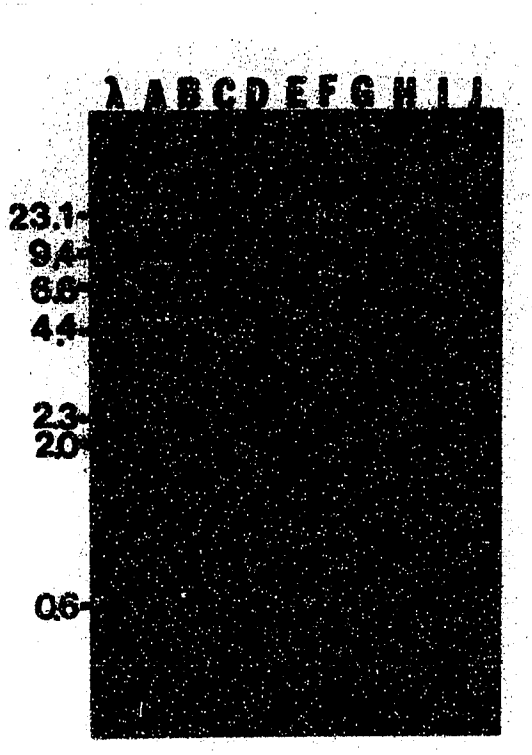


Fig. 5.1 Ethidium bromide stained gel showing five overlapping cosmid clones containing the cluster of genes involved in EPS synthesis in TAL1145. The plasmids pUHR221, pUHR222, pUHR223, pUHR224 and pUHR225 are digested with *Eco*RI (lanes A, B, C, D and E) and *Hin*dIII (lanes F, G, H, I, J).

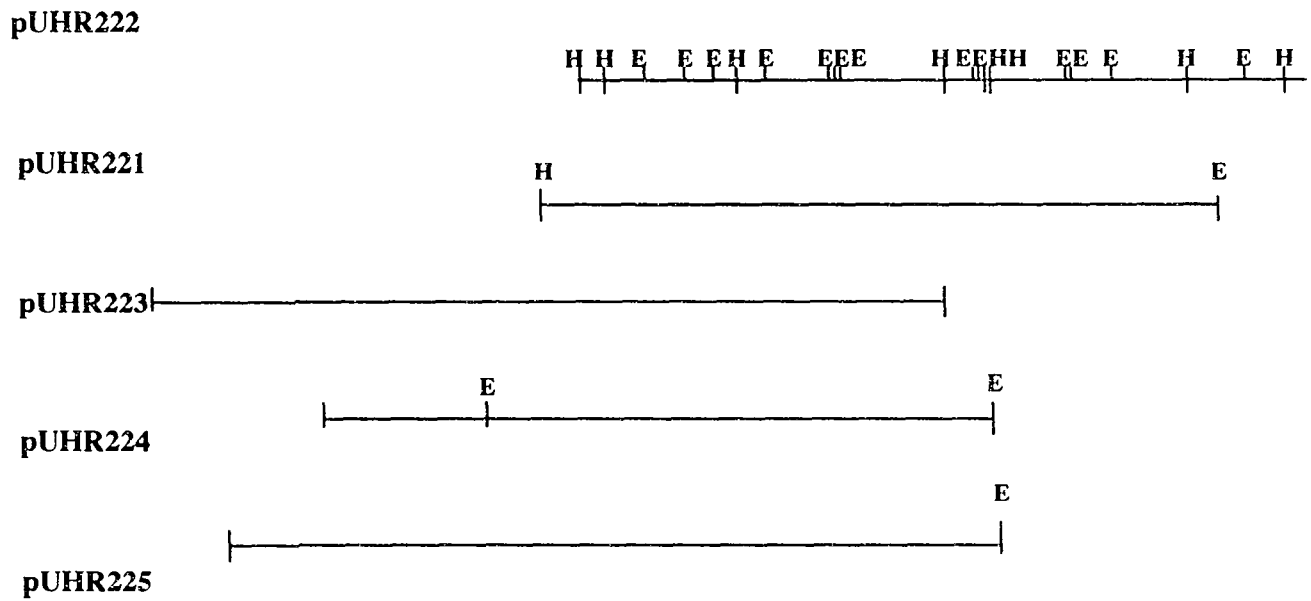


Fig. 5.2 Overlapping cosmid clones containing a cluster of genes involved in EPS synthesis in TAL1145

translucent colonies of the wild type. The colonies were streaked on YEM agar containing0.02% Calcofluor , incubated for 7 days at 28°C and examined under UV light. All three EPS-defective mutants showed bright yellowish green fluorescence as compared to the bluish fluorescence shown by TAL1145. They also appeared pale yellow on YEM agar. Using a cosmid clone library of TAL1145 (George et al. 1994) the mutants were complemented and a number of transconjugants showing wild-type colony morphology were selected. Plasmids were isolated from these transconjugants and used to transform *E. coli*. The plasmids were reisolated from *E. coli* and used for restriction analysis. Five overlapping cosmids, pUHR221, pUHR222, pUHR223, pUHR224 and pUHR225 were obtained by this method that complemented the spontaneous EPS-defective mutants, and contained genes for EPS synthesis (Fig. 5.1). A restriction map of these cosmids is shown in Fig.5.2. Two cosmids, pUHR221 and pUHR222, almost completely overlap each other except for the presence of an artificial 4.6- kb *Eco*RI fragment generated by Sau3AI digestion during the preparation of the genomic library in pUHR221 which is replaced by the real 6.6- kb fragment. Also an additional 1.6- kb *Hin*dIII fragment was observed in pUHR221 which hybridized with a 0.6- kb fragment in pUHR222.

5.3 Isolation of EPS-defective mutants by site-directed mutagenesis :

Plasmids pUHR221 and pUHR222 were used to obtain transposon-insertion mutants of the strain TAL1145. Fifty seven EPS-defective mutants were isolated by site-directed mutagenesis as described in Material and Methods. All mutants formed more opaque and drier colonies than the wild type. The mutants were screened on YEM agar

Table 5.1 Characterization of EPS-defective mutants of TAL1145

Mutant class	Mutants	Colony morphology	Calcofluor fluorescence	Insertion on *Eco*RI fragment	Symbiotic phenotype
I	NP98, NP99, NP118, NP119, NP120, NP130, NP131, NP136	Slightly wet	F^B	2.2 kb	Nod^+, Fix-
II	NP85, NP86, NP87,NP88, NP89, NP90, NP91, NP100, NP101, NP102, NP103, NP105, NP106 NP107, NP108, NP109, NP110, NP111, NP121, NP125, NP126, NP127, NP129, NP132, NP133, NP134, NP135, NP137, NP138, NP139, NP141 to NP143, NP145, NP146, NP147 and NP148	Dry	-	4.4 kb	Nod^+, Fix^+
III*	NP92, NP93, NP94, NP95, NP96, NP113, NP114, NP115, NP116, NP117, NP128, NP140	Slightly wet	-	1.8 / 2.2 kb	Nod^+, Fix-

F^B indicated dim bluish and F^Y shows delayed yellowish-green Calcofluore-fluorescence. F- shows Calcofluor-dark phenotype.
Fix^- : Non-nitrogen fixing on Leucaena, Gliricidia and beans

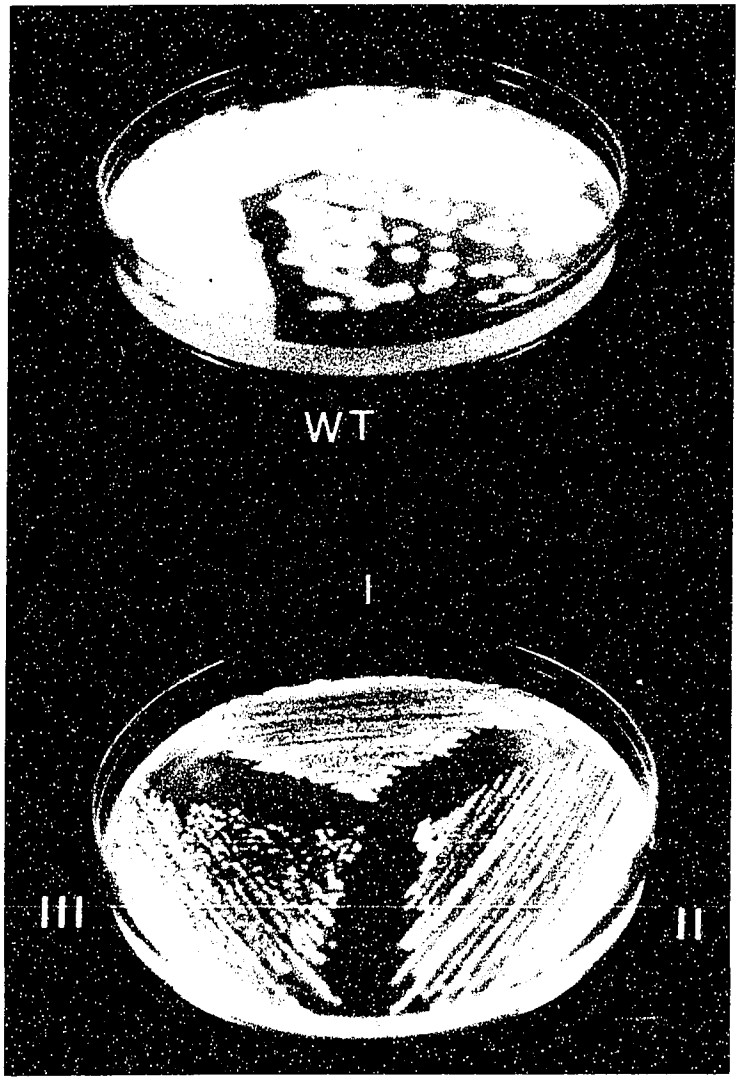


Fig. 5.3 Colony morphology of seven-day old culture of wild-type strain TAL1145 (WT) and representative strains of three classes of mutants, NP98 (I), NP91 (II) and NP95 (III) on yeast extract mannitol agar medium.

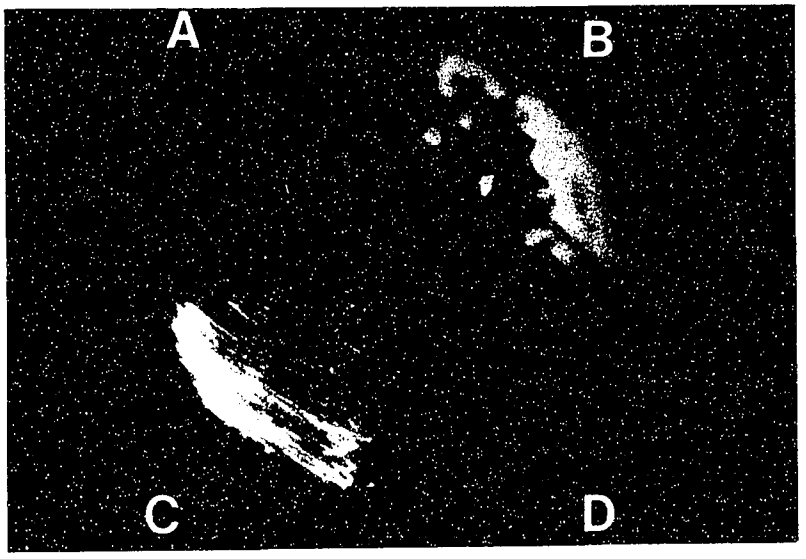


Fig. 5.4 Fluorescence shown by wild-type strain TAL1145 (B), and EPS-defective mutants, NP98 of class I (A), NP95 of class III (C) and NP91 of class II (D) grown in Calcofluor-containing yeast extract mannitol medium and examined under UV.

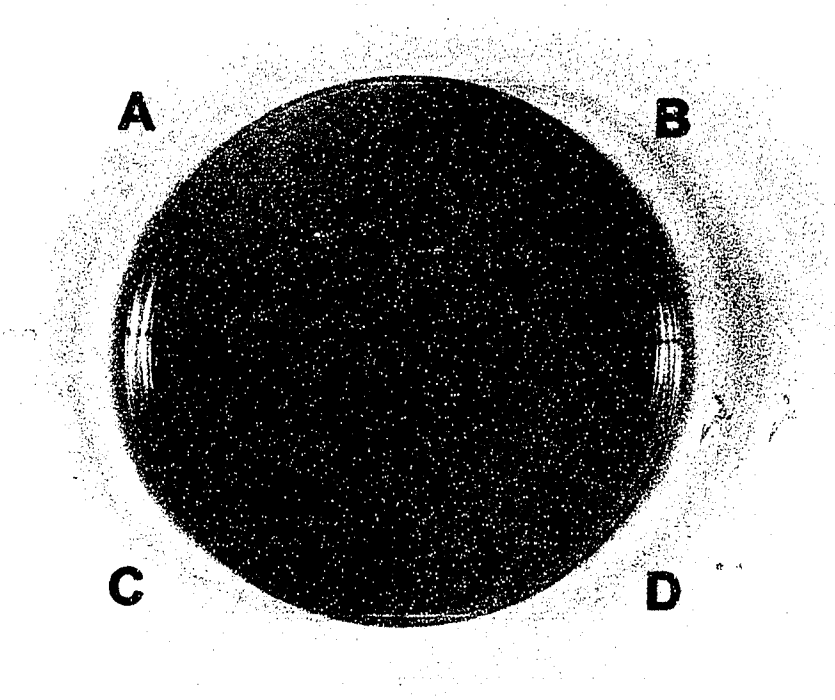


Fig. 5.5 β-glucuronidase activity shown by the mutants NP91 and NP97 (B and C respectively) as compared to no activity shown by wild-type strain TAL1145 (A) and mutant NP86 (D) on X-gluc-containing YEM medium.

containing 0.02% Calcofluor dye. On the basis of colony morphology and phenotype on Calcofluor-containing medium, these mutants were grouped into three classes (Table 5.1). All mutants formed more opaque and drier colonies than the wild type. The mutants were screened on YEM agar containing 0.02% Calcofluor dye. On the basis of colony morphology and phenotype on Calcofluor-containing medium, these mutants were grouped into three classes (Table 5.1).

Class I and Class III mutants produced significantly reduced quantities of EPS and appeared partially mucoid while class II mutants were completely dry (Fig.5.3). Also, when examined under UV, class I and class III mutants showed bluish but dim fluorescence on Calcofluor-containing YEM agar while a yellowish green fluorescence was observed on prolonged incubation of class III mutants. Fluorescence was undetectable in class II mutants (Fig. 5.4).

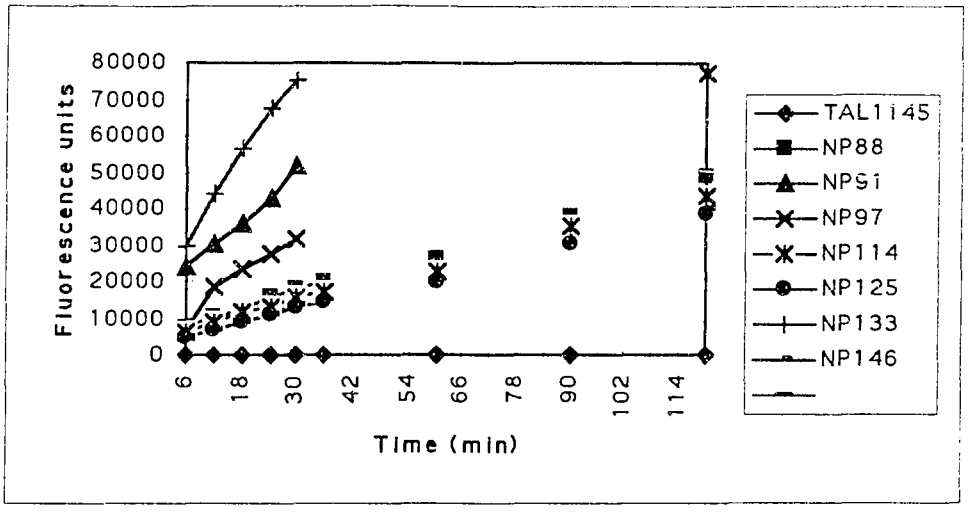
5.4 Expression of GUS-gene fusions :

Insertion of transposon Tn3Ho*gus* in the correct orientation of the target gene results in transcriptional fusions with the *uidA* or *gus* gene. All mutants obtained by site-directed mutagenesis using Tn3Ho*gus* in this study were screened for β-glucuronidase (GUS) activity. On the basis of GUS expression, a plausible direction of transcription of genes involved in EPS synthesis was determined (Fig.5.23).

5.4.1 Assay of β-glucuronidase activity in the mutants:

Twenty mutants expressed GUS activity on YEM containing 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) as the substrate. Mutants, such as NP85, NP89,

a



b

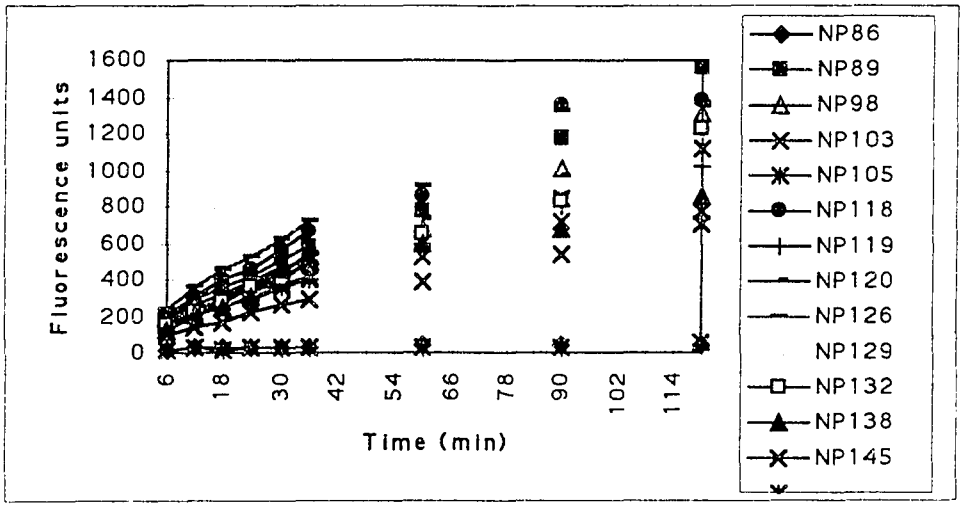


Fig. 5.6 Quantitation of GUS activity by fluorometry using MUG as a substrate

NP103, NP105,NP118, NP119, NP120, NP138 and NP145, showed low levels of gene expression on plates while very high GUS activity was observed in the mutants NP88, NP91, NP97, NP125, NP133 and NP146. GUS activity of two mutants, NP91 and NP97,is shown in Fig. 5.5 as compared to no activity shown by the wild-type strain. The mutant NP86 which was one of the 35 mutants that do not show any GUS activity was

Table 5.2. β-glucuronidase activity in Tn3Ho*gus*-insertion strains

Classes of mutants	Strains	GUS activity (nM 4-MU / ml / h)
Class I	NP98	548
	NP118	660
	NP119	416
	NP120	511
Class II	NP86	13
	NP88	2262
	NP89	488
	NP91	6412
	NP103	412
	NP105	436
	NP125	1595
	NP126	730
	NP129	475
	NP132	464
	NP133	10,123
	NP138	524
	NP145	299
	NP146	1627
Class III	NP97	4160
	NP114	2074
Wild-type	TAL1145	10

used as a negative control here as well as in further studies.

GUS activity of nineteen mutants, that turned blue on X-gluc-containing YEM agar, was also determined by the fluorometric assay method. Neither the negative control mutant NP86 nor the wild type strain TAL1145 showed any enzyme activity (Fig. 5.6a and 5.6b). Quantitation of high and low GUS activity in the mutants is shown in Figs. 5.6a and 5.6b, respectively.

β-glucuronidase enzyme activity of Tn3Ho*gus*-insertion mutants was determined to study the level of promoter activity of the *exo* genes (Table 5.2). Absence of background enzyme activity was confirmed in TAL1145. Also, the control mutant NP86 showed less than 15 units of GUS activity. NP145 showed 23-fold while NP133 showed approximately 800-fold enzyme activity above the background level activity in NP86. Enzyme activity in the mutants shown in Fig.5.6a was 2- to 34-fold higher than those of the mutants in Fig.5.6b.

5.5 Quantification of EPS in some selected mutants:

After removing the cells by centrifugation, the EPS present in the supernatant was precipitated by 2 volumes of ethanol. Dry weights of EPS obtained from the wild-type and some selected mutants are shown in Table 5.3. None of the five class II mutants showed a detectable level of EPS production. The mutants belonging to class I and class III produced less than 25% of the EPS produced by the wild-type. The quantity of EPS produced by the same mutant showed slight differences when the experiment was repeated. However, none of the mutants produced more than 900 μg of ethanol-precipitated EPS per milligram of total cell protein at any time.

Table 5.3 EPS produced by EPS-defective mutants in culture supernatants

Class	Strains	Dry weight of ethanol precipitated EPS (μg / mg protein)	EPS %
wild type	TAL1145	3595	100
	NP98	455	13
I	NP118	550	15
	NP120	355	10
	NP86	0	0
	NP88	0	0
II	NP91	0	0
	NP125	0	0
	NP146	0	0
	NP92	710	20
III	NP95	790	22
	NP97	680	19

5.6 Size fractionation of EPS of selected mutants by gel filtration:

Two major fractions of a high- and a low-molecular-weight size EPS were collected when TAL1145 culture supernatant was passed through a Biogel A-5m column. Results of column chromatography of one class I, four class II and one class III mutants are shown in Fig. 5.7. The class II mutants that did not fluoresce on Calcofluor-containing medium and also had not shown detectable levels of ethanol-precipitated EPS lacked the high molecular weight EPS fraction peak.

Two major fractions of a high- and a low-molecular-weight size EPS were collected when TAL1145 culture supernatant was passed through a Biogel A-5m column.

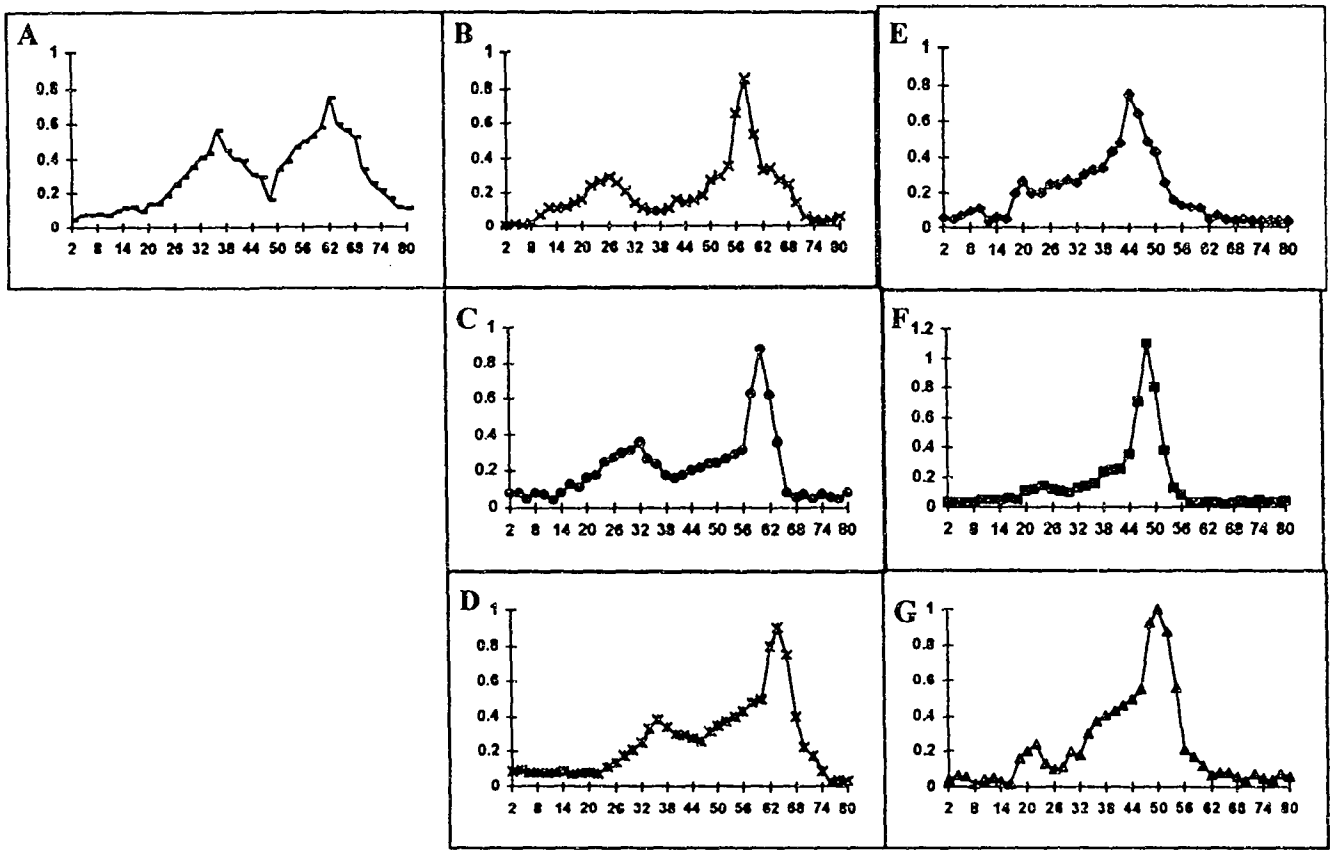


Fig. 5.7 Size fractionation of EPS by column chromatography. (A) TAL1145, (B) NP125, (C) NP98, (D) NP95, (E) NP86, (F) NP88, (G) NP91

Results of column chromatography of one class I, four class II and one class III mutants are shown in Fig. 5.7. The class II mutants that did not fluoresce on Calcofluor-containing medium and also had not shown detectable levels of ethanol-precipitated EPS lacked the high molecular weight EPS fraction peak. However, class I mutant NP98 and class III mutant NP95 were found to produce reduced levels of high-molecular-weight EPS. All six mutants produced almost 1/10 of the levels of low-molecular-weight EPS as compared to the wild-type.

5.7 SDS-DOC-PAGE analysis of LPS of the selected mutants :

The LPS profiles of seven selected mutants belonging to all three classes were also studied by conducting an SDS-DOC-PAGE analysis of crude extract followed by alcian blue-silver staining (Fig. 5.8). Alcian blue is a cationic dye that is effective in staining polyanionic polysaccharides. Two major banding regions are observed in SDS-DOC-PAGE analysis of TAL1145 which is common in various *Rhizobium* spp. Four bands were observed in high molecular weight LPS I while a single band was observed in high mobility LPS II. In addition, some faint bands were observed between LPS I and LPS II on overloading the gel which are probably equivalent to the non-LPS,cell-associated, 3-deoxy-D-manno-2-octulonic acid-rich acidic polysaccharide (KPS) reported in some *Rhizobium* spp. (Reuhs et al. 1993, 1995). The absence of these bands in SDS-PAGE confirmed that they are not components of LPS. The SDS-polyacrylamide gels were stained with Bio-Rad silver stain which specifically stains LPS . None of the mutants showed significant differences in LPS I and LPS II banding regions suggesting that LPS synthesis is not affected in these EPS-defective mutants.

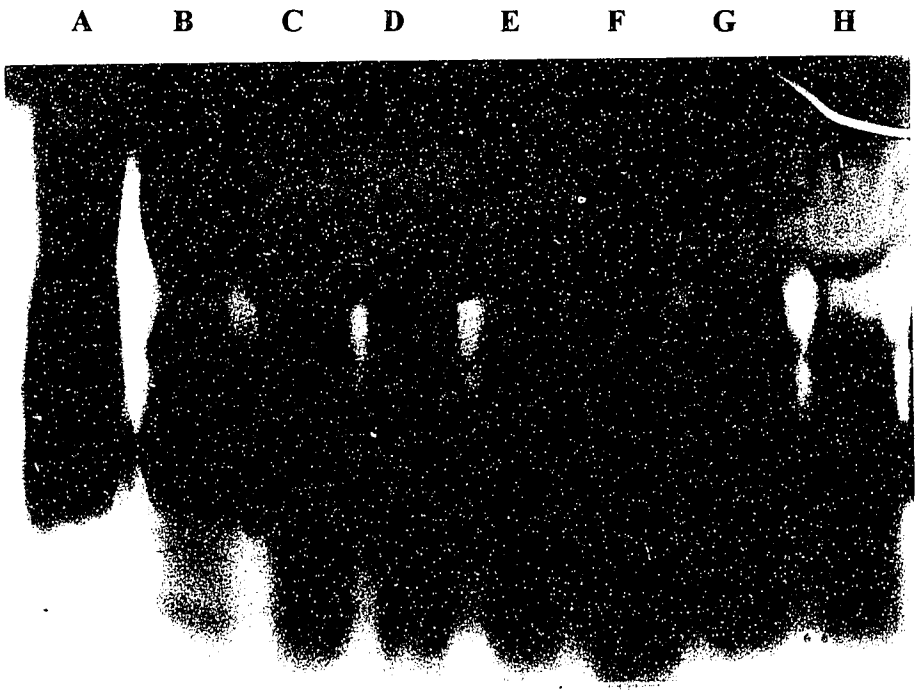


Fig. 5.8 Alcian blue-silver stained SDS-DOC-PAGE gel of crude preparation of LPS from TAL1145 (lane B), and class II mutants NP86 (lane C), NP88 (lane D), NP91 (lane E) and NP125 (lane F), class I mutant NP98 (lane G) and class III mutant NP95 (lane H). *Salmonella typhimurium* (lane A) was used as a control. Gel was overloaded to resolve the band between the brownish yellow LPS I banding region at the top and the LPS II banding region at the bottom of the gel.

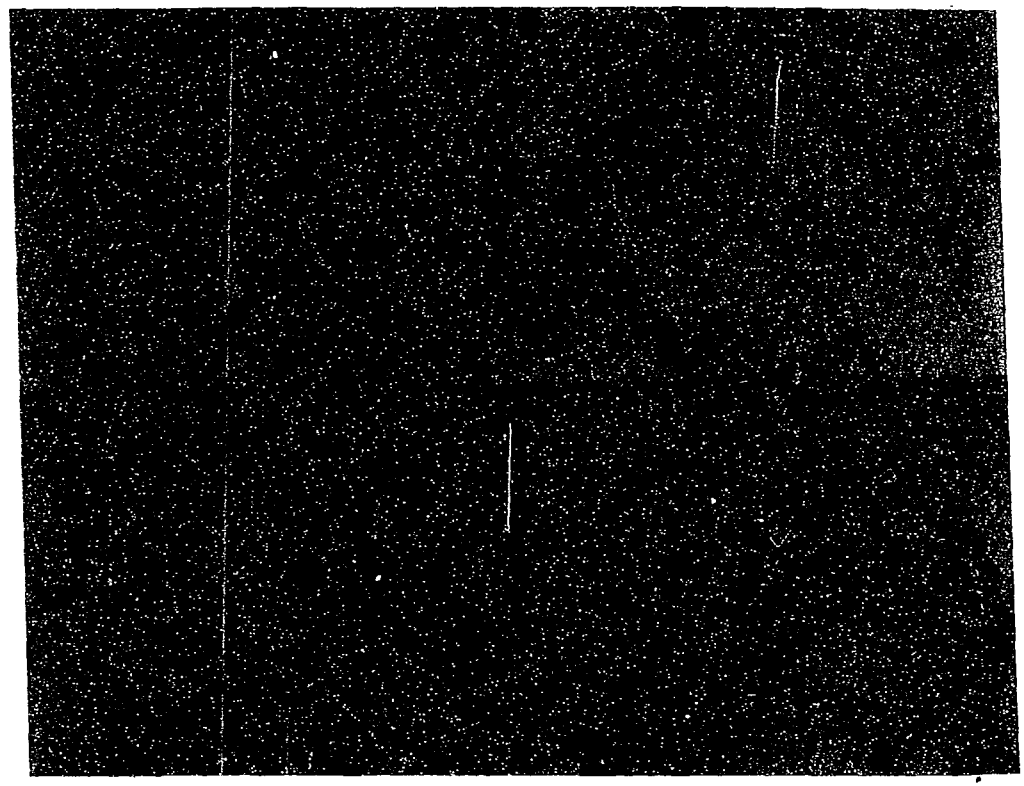


Fig. 5.9 Nodule phenotype of *L. leucocephala* plants inoculated with TAL1145 (A), NP98 (B), NP91 (C) and NP95 (D). Arrows indicate pseudonodules. Bar equals 5.0 cm.

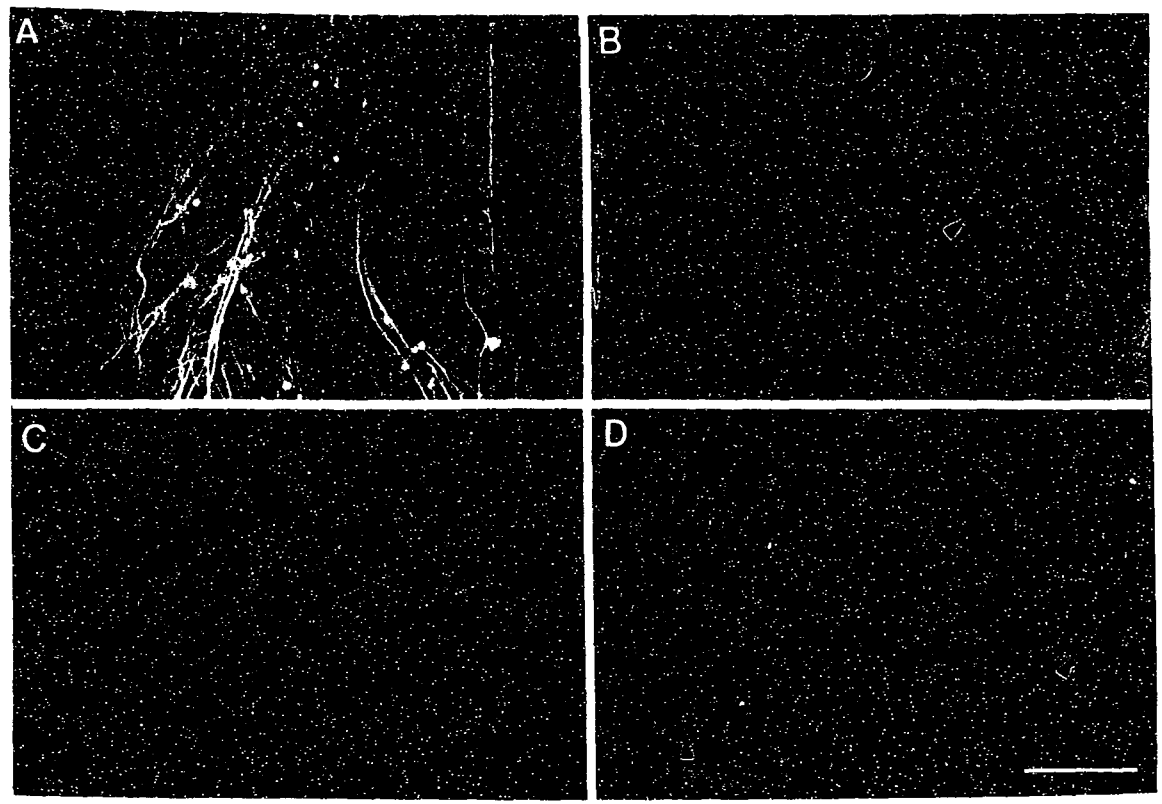


Fig. 5.10 Nodule phenotype of *P. vulgaris* plants inoculated with TAL1145 (A), NP98 (B), NP91 (C) and NP95 (D). Arrows indicate pseudonodules. Bar equals 5.0 cm.

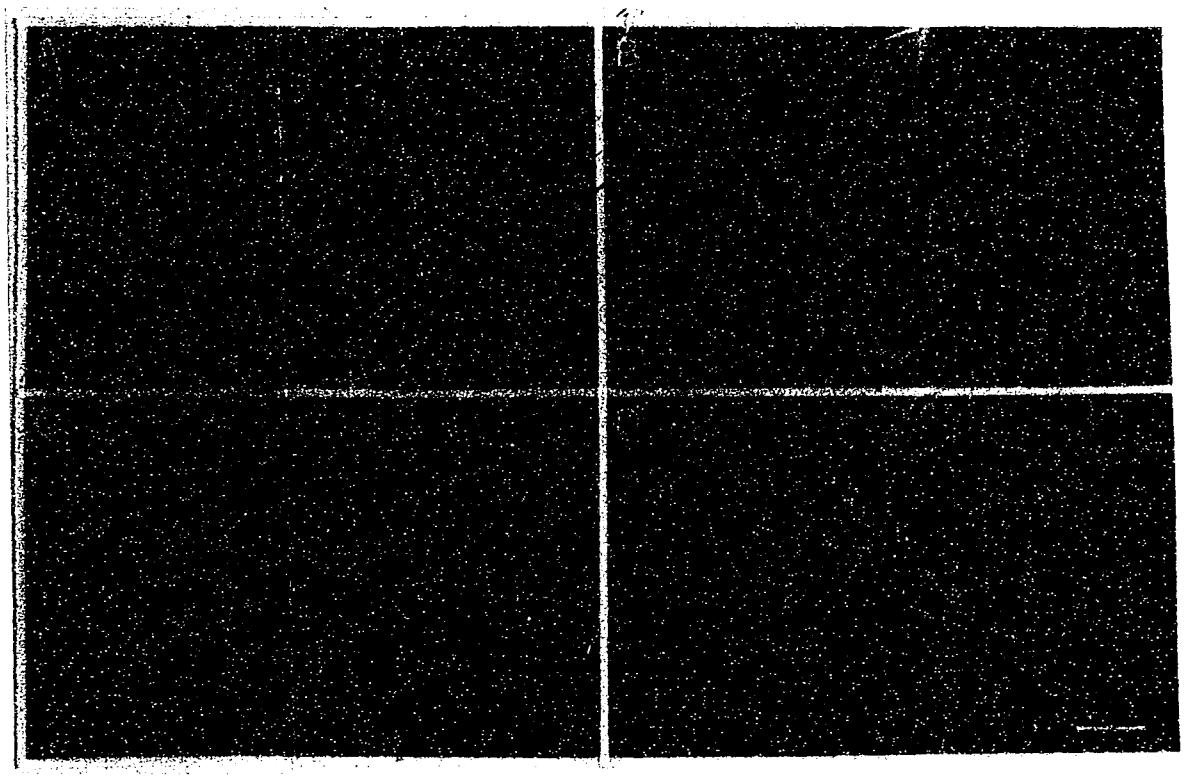


Fig. 5.11 Nodule phenotype of *G. sepium* plants inoculated with TAL1145 (A), NP98 (B), NP91 (C) and NP95 (D). Bar equals 2.5 cm.

5.8 Symbiotic behavior of mutants on three legume hosts:

Symbiotic phenotypes of all mutants on three legume species, *L. leucocephala, Phaseolus vulgaris* and *Gliricidia sepium* were studied by inoculating plants grown under a controlled environment. The phenotypes of the nodules on three legume species are shown in Figs. 5.9, 5.10 and 5.11 and results are also summarized in Fig. 5.23. The class II mutants that did not produce high-molecular-weight EPS and appeared dark on Calcofluor-containing medium showed normal nodulation and nitrogen fixation on all three legume species. The nodule phenotype of NP91 belonging to this category is shown in Figs. 5.9, 5.10 and 5.11.

Mutants belonging to class I (NP98) and class III (NP95) which produced reduced amounts of high- and low-molecular weight EPS fractions formed tumor-like structures on all three legumes (Fig. 5.9, 5.10 and 5.11). These pseudonodules were unable to fix nitrogen and the plants became yellow within 4 weeks of inoculation. These results suggest that a defect in EPS synthesis in TAL1145 affects nodulation of both indeterminate- and determinate-nodule-forming hosts. Complementation of both class I and class III mutants with pUHR221 restored normal symbiotic phenotype in both *Leucaena* and beans. The nodule phenotypes of mutants NP97 and NP98 as compared to their complemented derivatives are shown in Figs. 5.12 and 5.13.

5.9 Expression of *exo* genes of TAL1145 in nodules by using GUS-fusions:

Selected mutants of each class that showed GUS activity under free-living conditions were also analyzed for GUS expression in the nodules formed on all three

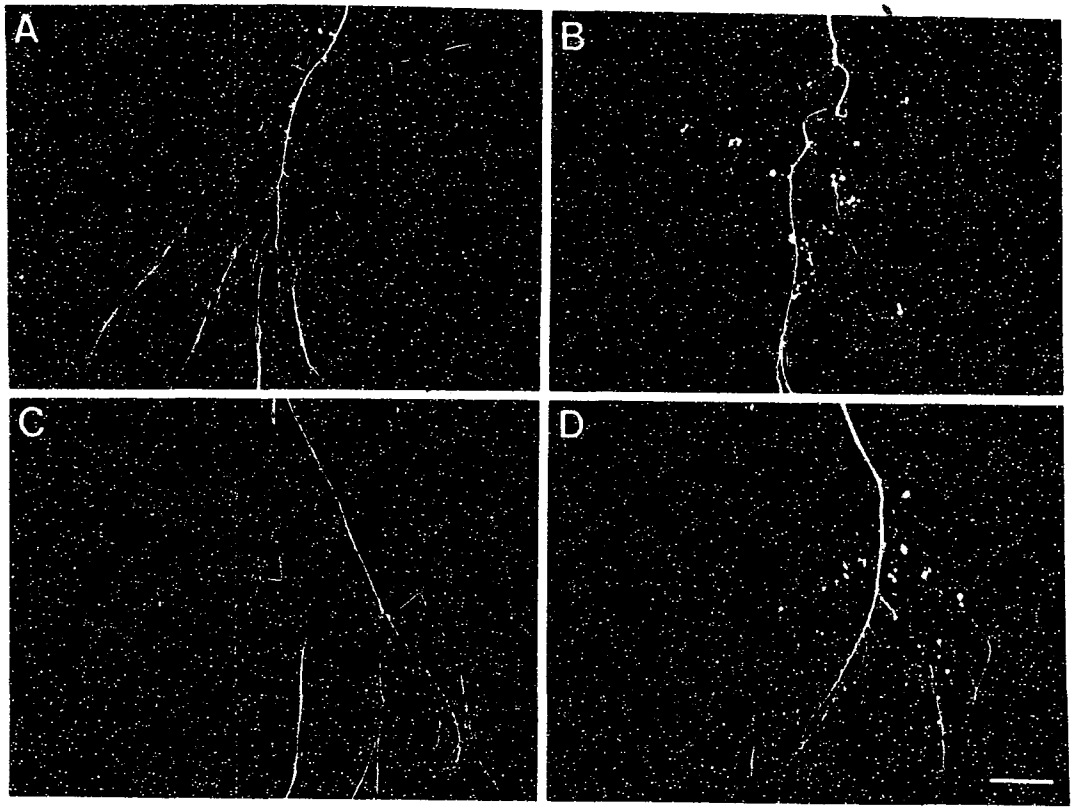


Fig. 5.12 Nodule phenotype of *L. leucocephala* plants inoculated with class I mutant NP98 (A) and class III mutant NP97 (C) as compared to plants inoculated with the complemented mutants NP98(pUHR221) and NP97(pUHR221) (B and D, respectively).
Arrows indicate pseudonodules. Bar equals 2.5 cm

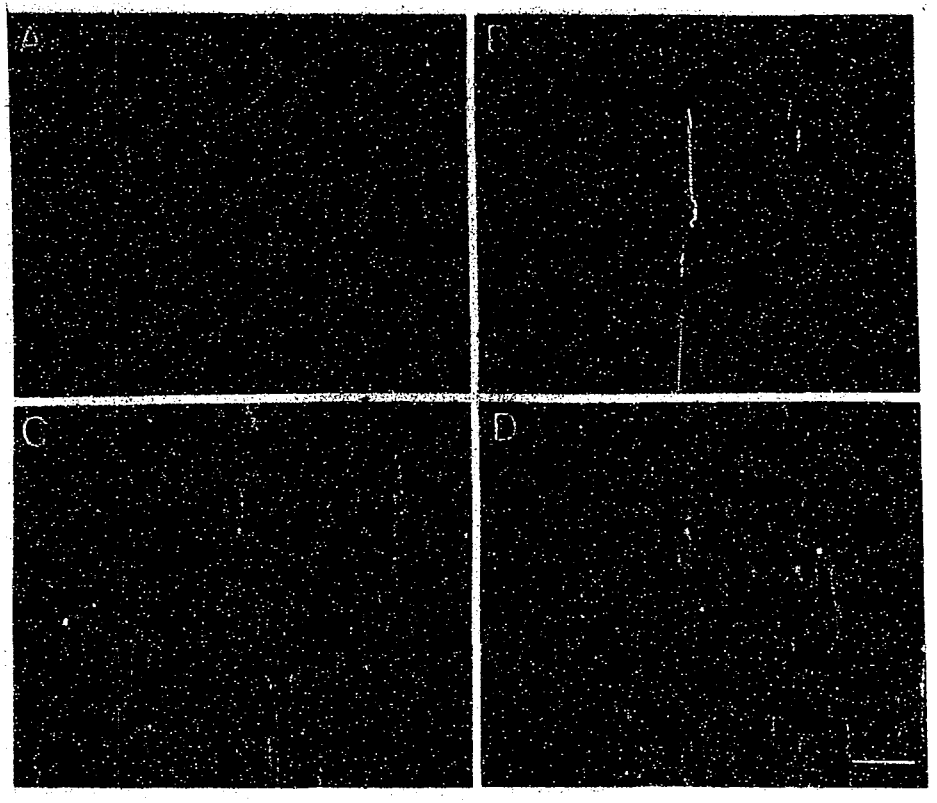


Fig. 5.13 Nodule phenotype of *P. vulgaris* plants inoculated with class I mutant NP98 (A) and class III mutant NP97 (C) as compared to plants inoculated with the complemented mutants NP98(pUHR221) and NP97(pUHR221) (B and D respectively). Arrows indicate the pseudonodules. Bar equals 2.5 cm.

hosts. Thick sections (15-20μ) of nodules of all three legumes were reacted with X-gluc and examined under the light microscope for color development due to GUS activity. Class II mutants that formed normal nodules on all three hosts expressed GUS activity in the nodules indicating that the gene(s) involved in EPS synthesis that has (have) been interrupted in these mutants is (are) expressed in the nodules. These mutants showed high GUS activity in the infected cells of nodules formed on all three legume hosts (Figs. 5.14C, 5.17B and 5.18A). Rhizobia in the infected cells that appeared blue are more clearly visible in the infected cells of nodules formed by NP91 and NP88 in thin sections under higher magnification (Figs. 5.15 and 5.16, respectively). In contrast, bacteria appeared colorless in nodules formed by NP86 that did not show GUS expression (Fig. 5.16A).

Infected cells and GUS activity were indistinguishable in the pseudonodule formed by NP95 on *L. leucocephala* (Fig. 5.14 D). Only a few scattered infected cellswere observed in the small pseudonodules formed by NP97 on *Leucaena* (Fig. 5.15C). Pseudonodules formed by NP119, a class I mutant, showed some infected cells but GUS activity was not discernible in the infected cells of *L. leucocephala* (Fig. 5.14 C). Thick sections of pseudonodules formed by class I and class III mutants on *P. vulgaris* and *G. sepium* showed neither infected cells nor GUS activity (Figs. 5.17 and 5.18).

5.10 Electron microscopic examination of nodules formed by three classes of mutants on *L. leucocephala*:

Infected cells of nodules formed by three mutants and the wild-type were examined by transmission electron microscopy (Fig. 5.19). Bacteroids of TAL1145 were oval in shape and were filled with poly-β-hydroxy butyrate (PHB). A clear peribacteroid membrane and a prominent peribacteroid space surrounded the bacteroids. The bacteroids were tightly packed in the infected cells (Fig. 5.19A). The bacteroids in *Leucaena* nodules formed by NP86 appeared normal but were not as densely packed as the wild-type. Also, the peribacteroid space was not clearly seen around the bacteroids (Fig. 5.19C).

Mutants NP95 and NP97 showed two types of bacteroids which were more dispersed than the wild-type. Only a few bacteroids appeared normal in these cells. The majority of the bacteroids were irregular, lacked PHB as well as a peribacteroid space and appeared to be degenerating (Figs. 5.19 A and D). Many undifferentiated bacteria are observed in nodules formed by NP97.

5.11 Localization of Tn3Hog*us*-insertion in the mutants:

The position of Tn3Hog*us* in the mutants was determined by Southern hybridization using several different probes. Southern analysis using pUHR221 as the probe showed that all class II mutants that formed dry colonies contained Tn3Hog*us*-insertion in a 4.4-kb *Eco*RI fragment. In these mutants the 4.4-kb *Eco*RI fragment was replaced by an *Eco*RI fragment of sizes 5-kb to 8.2-kb depending on the site of transposon insertion. Similarly, class I and class III mutants were located on 2.2-kb and 1.8-kb *Eco*RI fragments respectively except one class III mutant, NP97, which had a transposon insertion in the 2.2-kb *Eco*RI fragment. The results of a few mutant hybridization studies is shown in Fig. 5.20. The positions of the transposon insertions within these fragments

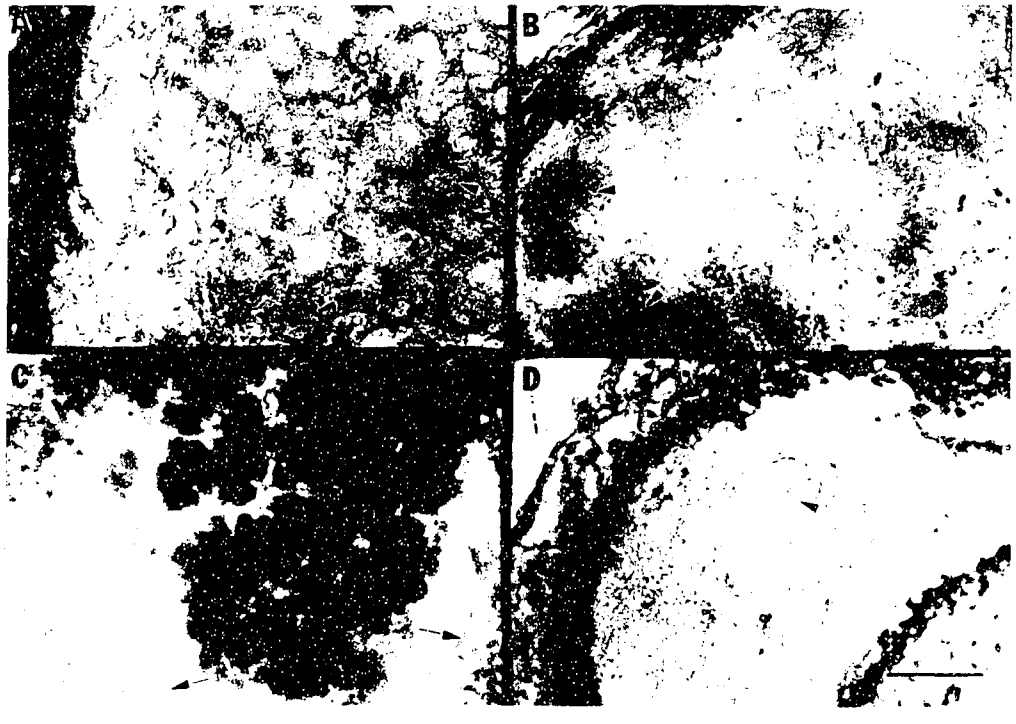


Fig. 5.14 Light microscopic examination of thick sections(15-20 μ) of nodule of *L. leucocephala* formed by the mutants NP86 (A) and NP88 (C) of class II, NP119 (B) of class I, and NP95 (D) of class III after reaction with X-gluc for expression of GUS activity.

Fig. 5.15 Light microscopic examination of thick sections (4 μ) of nodule of *L. leucocephala* formed by the mutants NP86 (A) and NP91 (B) of class II, NP97 (C) of class III after reaction with X-gluc for expression of GUS activity. Bar equals 80 μ (magnification 200x).

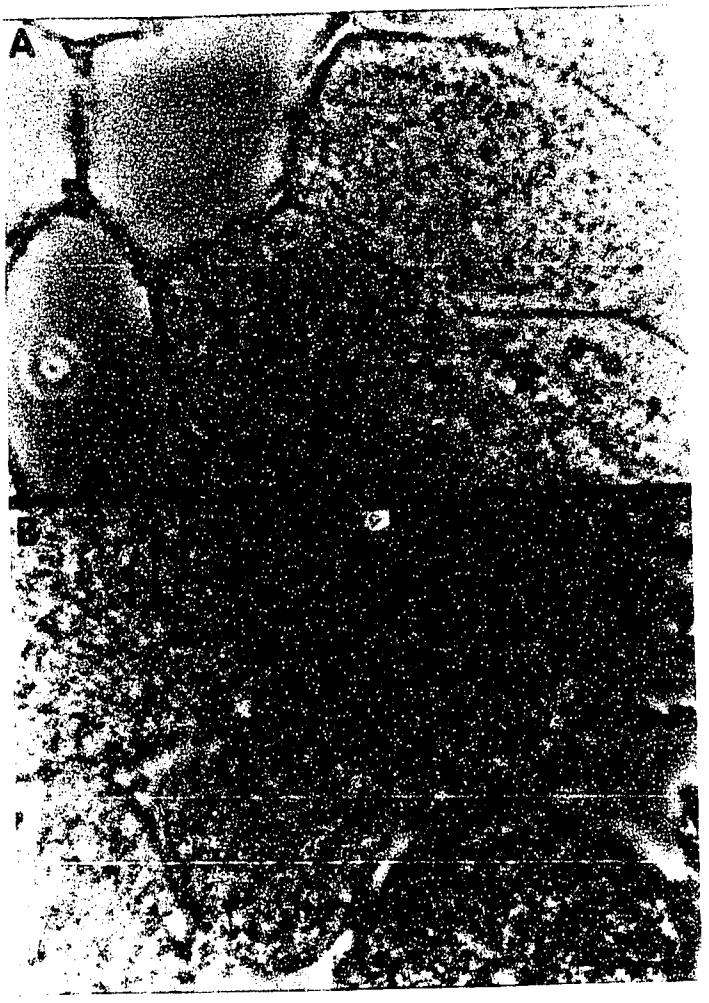


Fig. 5.16 Light microscopic examination of thick sections (4 μ) of nodule of *L. leucocephala* formed by the mutants NP86 (A) and NP88 (B) after reaction with X-gluc for expression of GUS activity under higher magnification (800x). Bar equals 20 μ.

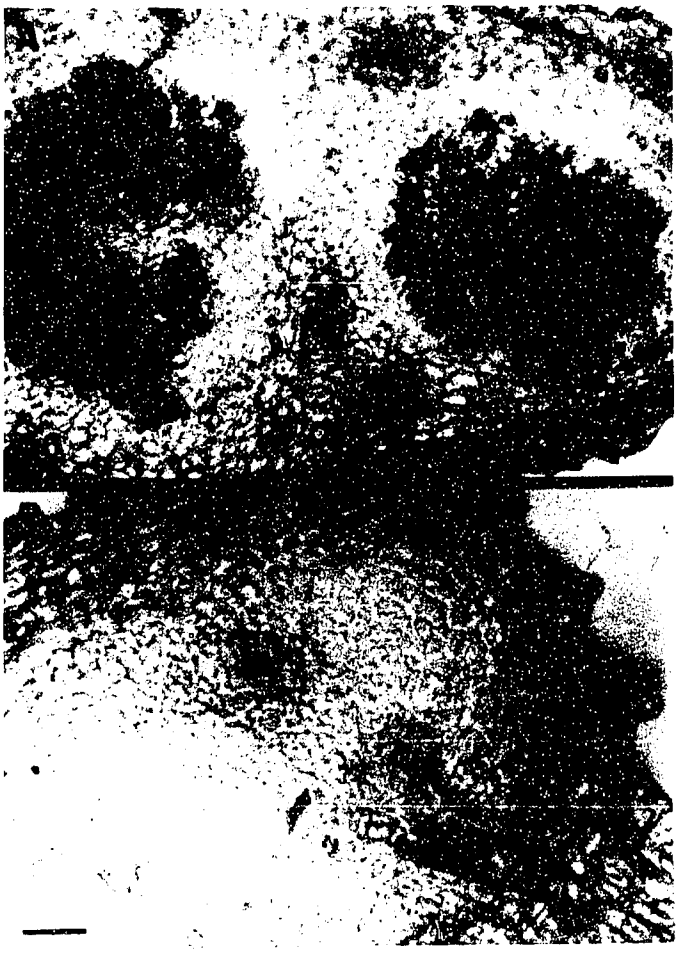


Fig. 5.17 Light microscopic examination of thick sections (4 μ) of nodules of *P. vulgaris* formed by the mutants NP88 (A) and NP86 (B) after reaction with X-gluc for expression of GUS activity. Bar equals 110 μ.

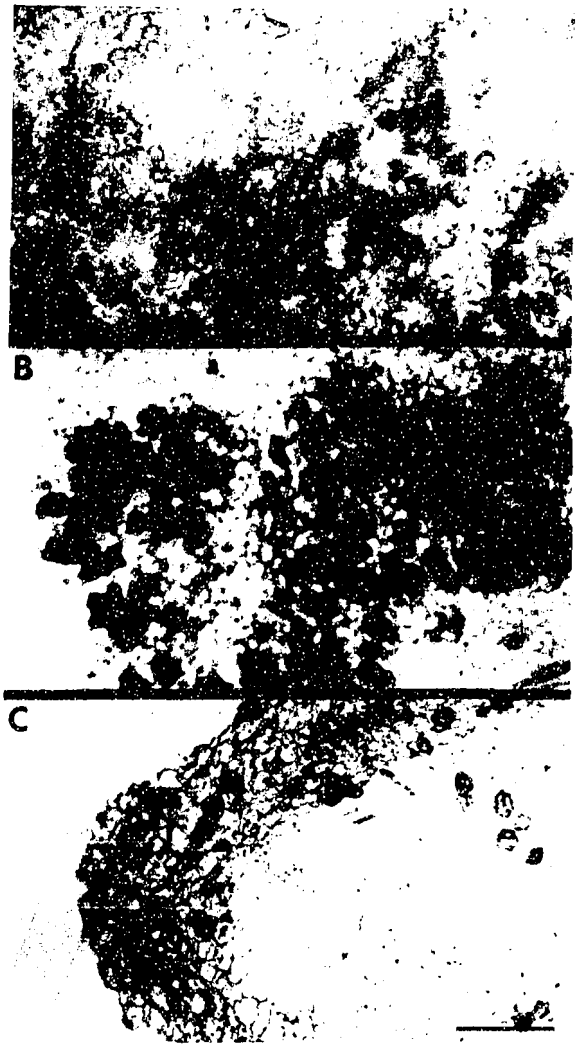


Fig. 5.18 Light microscopic examination of thick sections (4μ) of nodules of *G. sepium* NP86 (A) and NP91 (B) of class II, NP97 of class III after reaction with X-gluc for expression of GUS activity. Bar equals 80μ (magnification 200x).

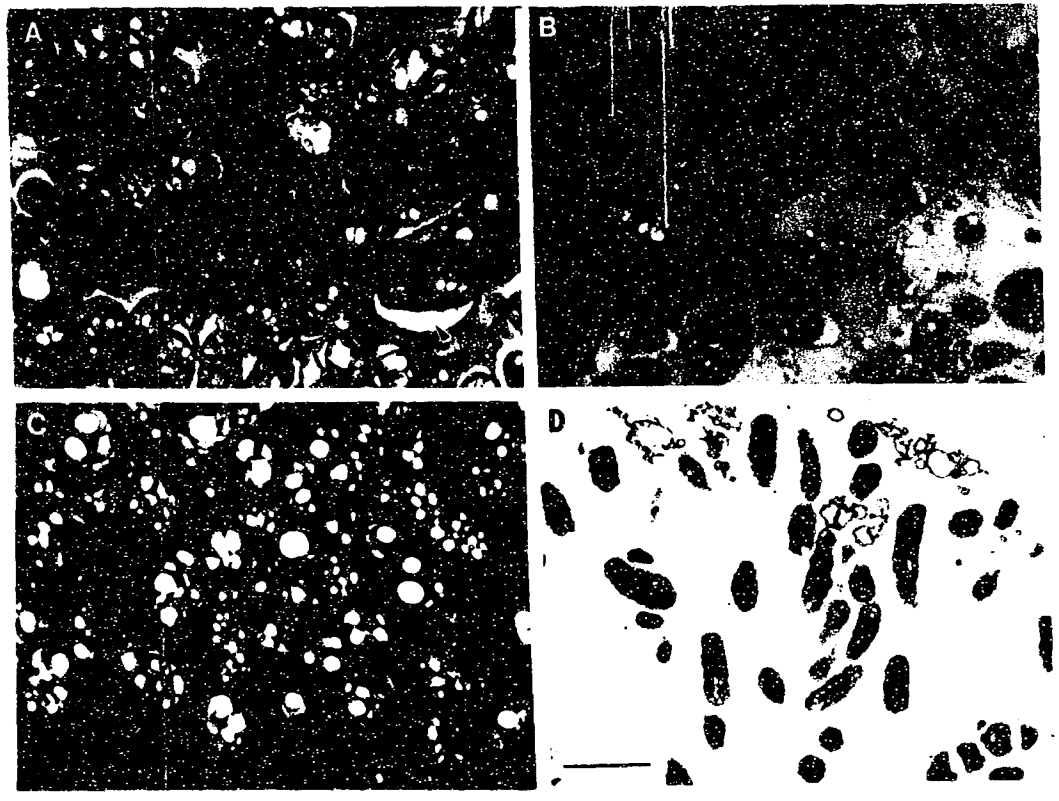


Fig. 5.19 Transmission electron microscopic examination of infected zones of nodules of *L. leucocephala.* (A) Wild-type strain TAL1145, (C) NP86 mutant of class II, (B) NP95 and (D) NP97 of class III. Peribacteroid space is indicated by arrow in A. Bar equals 4.5μ

were determined by using the neomycin phosphotransferase gene as a probe for the same blots. The exact position of the transposon insertion in the mutants was determined on the basis of the size of the shifted band. The results were confirmed by additional hybridization using subcloned 2.2- kb, 4.4- kb and 1.8- kb *Eco*RI fragments of pUHR221 as probes. Fig. 5.21 shows some mutants' genomic DNA restricted with *Eco*RI and their hybridization with 2.2- kb cloned DNA as an example. The map of Tn3Hog*us* and the method of determining the exact site of transposon-insertion is shown diagrammatically in Fig. 5.22. *Eco*RI fragments of sizes 2.2- kb and 1.8- kb were found to be adjacent to each other but mutant phenotypes due to the insertion of a transposon in these fragments were observed to be different.

5.12 Map of EPS-defective mutants :

Fig. 5.23 shows the restriction map of pUHR221 indicating the location of transposon-insertions in different mutants and the cultural and symbiotic characteristics of these mutants. It was mentioned earlier that the symbiotic phenotypes of these mutants on *L. leucocephala, G. sepium* and *P. vulgaris* were similar. Possible boundaries of the genes are also shown as predicted on the basis of the complementation experiments, transposon-insertion sites and GUS activity. On the basis of GUS gene expression, the direction of transcription of *exo* genes was also determined. At least three transcripts have been identified in this cluster of *exo* genes. A polycistronic operon or multiple monocistronic operons may be present in the 4.4-kb *Eco*RI fragment which defines the class II mutants.

5.13 Complementation of mutants:

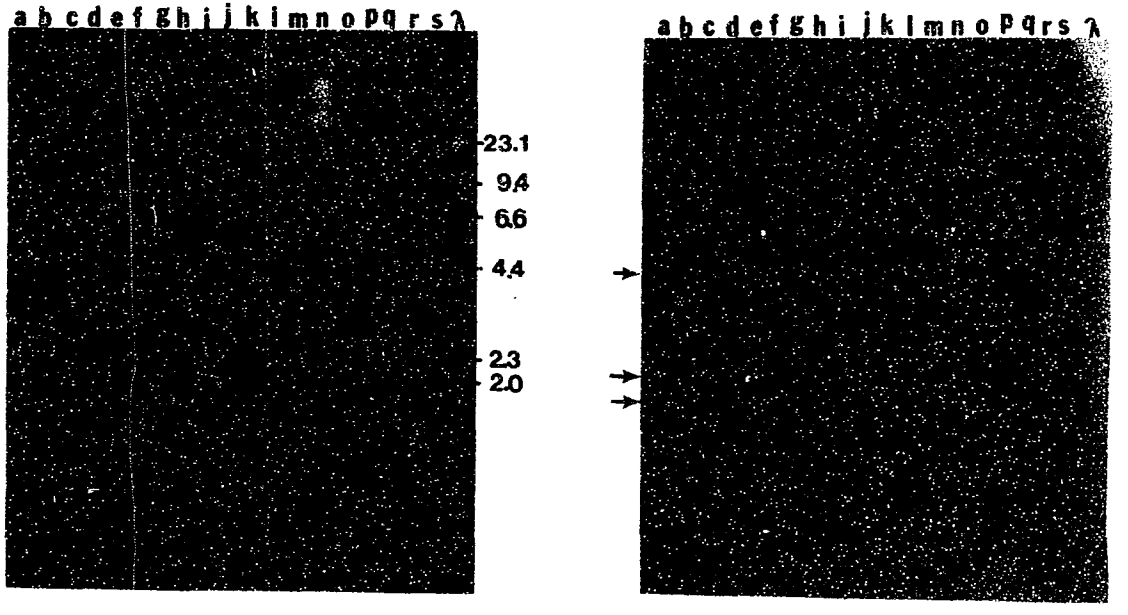


Fig.5.20 (A) Etidium bromide stained gel picture of the genomic DNA digested with *Eco*RI of some mutant strains (b to s) and wild-type (a).
(B) Southern hybridization of the gel blot in (A) using pUHR221 as a probe.

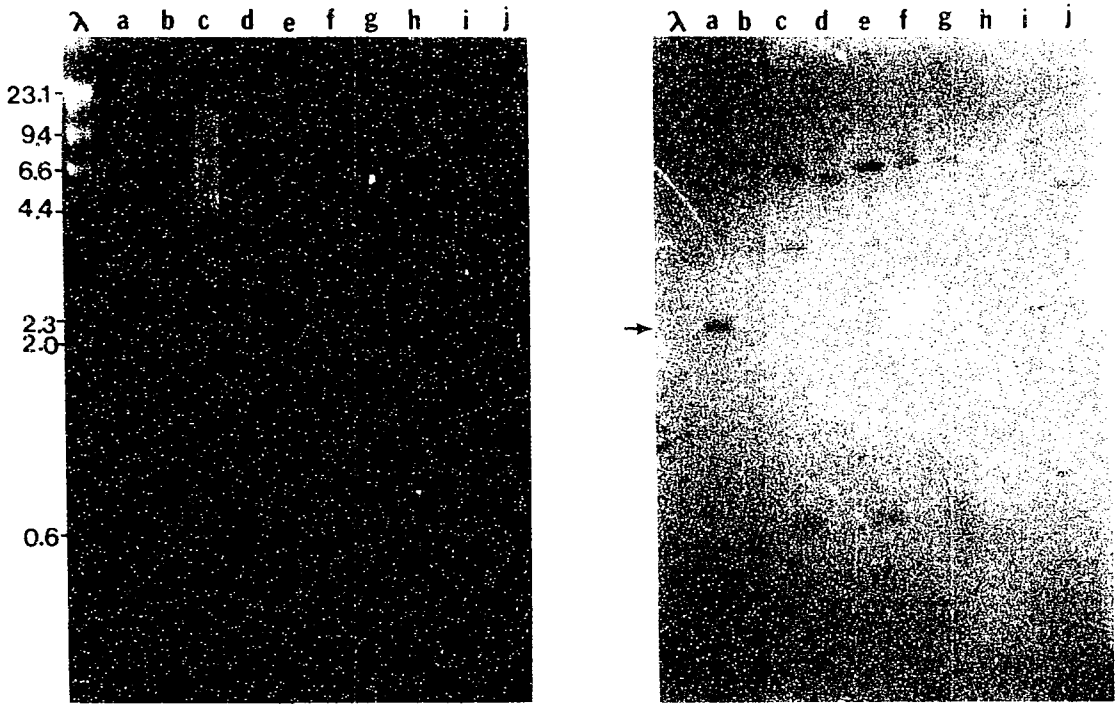


Fig. 5.21 (A) Ethidium bromide stained agarose gel showing *Eco*RI digested genomic DNA of wild-type (a), and mutants (b to j) (B) Southern hybridization of the gel in (A) with 2.2-kb *Eco*RI fragment probe

Complementation of selected mutants with different plasmids is shown in Table 5.4. The mutants were complemented by the plasmids pUHR221 and pUHR222. Southern hybridization showed insertion of transposon in the mutants NP92 and NP95 in the 1.8- kb *Eco*RI fragment of pUHR221. However, plasmid pUHR236 containing 1.8-kb *Eco*RI fragments from pUHR221 failed to complement these mutants. Furthermore, plasmid pUHR230 containing 2.2- kb *Eco*RI did not complement the mutants NP97, NP98, NP118, NP120, all of which have the insertion of Tn3Hog*us* in the 2.2-kb *Eco*RI fragment of the chromosome. This fragment is the same as that present in pUHR221. These results indicate that part of the operon extend across these individual fragments. Similar results were obtained with plasmids pUHR256, pUHR257 and pUHR258 that have 7.6- kb, 7.0- kb and 4.6- kb *Hin*dIII fragments of pUHR221, respectively, cloned in the wide-host range plasmid vector pRK404.

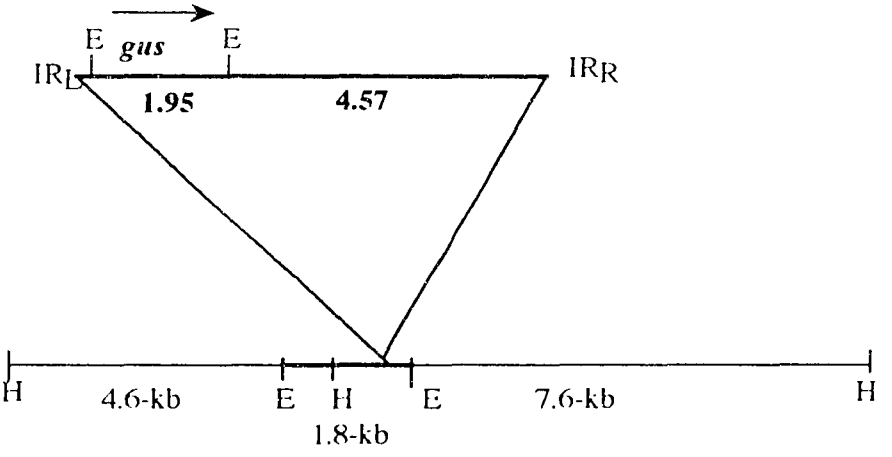
Plasmids pUHR221::Tn3Hog*us*-92 did not complement class I mutants and plasmid pUHR221::Tn3Hog*us*-98 failed to restore the wild type phenotype to class III mutants. Thus, genes on these two *Eco*RI fragments are placed in the same complementation groups. PlasmidpUHR221::Tn3Hog*us*-87 complemented both class I and class III mutants indicating that mutants located within the 4.4-kb *Eco*RI fragment constitute another complementation group in this cluster of *exo* genes.

The cosmid R'3222 containing the *exo* region of *Rhizobium* sp. strain NGR234 and cosmid pEX312 containing the cluster of *exo* genes of *R. meliloti* that are located in pRmeSU47b megaplasmid failed to complement these mutants.

5.14 Sequence analysis :

Table 5.4 Complementation of EPS-defective phenotype of TAL1145 mutants

Mutants	Complementation by plasmids												
	221	222	230	236	237	256	257	258	87Tn	92Tn	98Tn	pEX312	R'3222
NP86	+				+		−		−			−	−
NP87	+	+					−		−				−
NP88	+	+					−		−			−	−
NP89	+								−				
NP91	+	+			+		−		−		+	−	−
NP125	+				−				±	−		−	
NP146	+											−	
NP92	+	+		−	−	−	−	−		−			−
NP93	+	+		−				−		−	−		
NP95	+	+		−	−	−		−		−	−		−
NP97	+	+	−	−	−						−		−
NP98	+	+	−		−	−		−	+		−		−
NP118	+	+	−		−	−		−			−		−
NP119	+	+	−			−		−			−		−
NP120	+	+	−			−		−			−		−



***Eco*RI fragments : 1.5-kb and 5.0-kb**
(hybridization with 1.8-kb *Eco*RI fragment

***Hin*dIII fragments : 4.3-kb and 10.9-kb**
(hybridization with 7.6-kb *Hin*dIII fragment

Fig. 5.22. Map of transposon Tn3H*gus* and method of its localization in a mutant

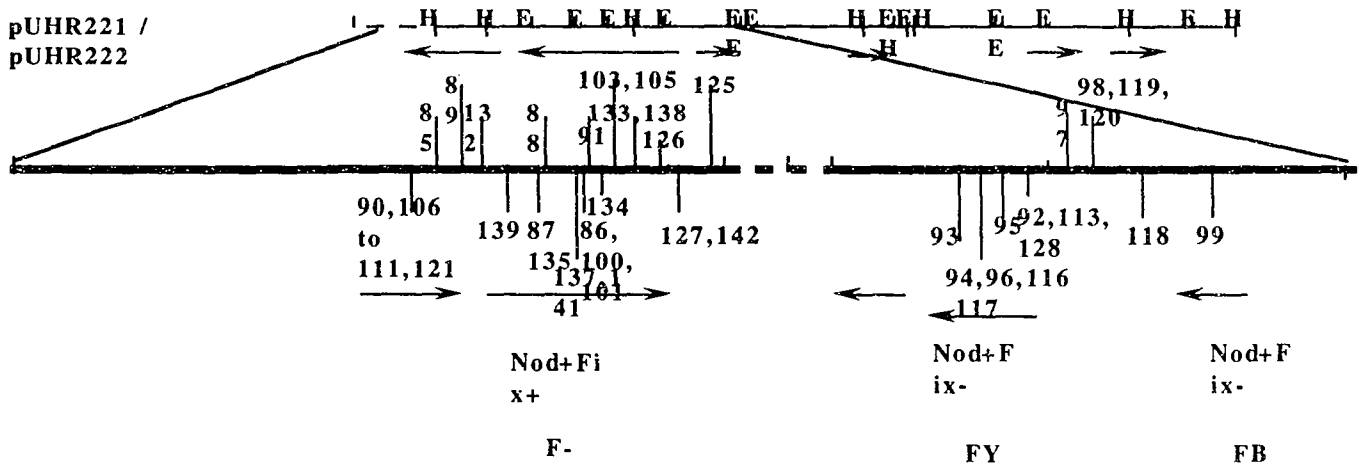


Fig. 5.23. Map of cluster of EPS-defective mutants

Arrowheads indicate direction of *gus*, mutants above the line show GUS activity while below the line are GUS negative. F-- Cacofluor negative, FY-yellowish fluorescence, FB -dim bluish fluorescence, Nod+-nodulation, Fix+ nitrogen fixation, Fix-- No nitrogen fixation

Fig. 5.24 Nucleotide sequence of 4.7-kb DNA fragment fron cosmid pUHR221. The deduced amino acid sequence of four *exo* genes of TAL1145 are also shown. The initiation codons of the proteins are underlined. The putative ribosome-binding sites are shown by thick underlines. Arrows show the direction of the transcripts.

```
GAGGGATTGCGGGTGGATCATTATCCATTGCTGTTGAAAGATATTCATGCCTTTCGCCAA   60
   P  I  A  P  P  D  N  D  M  A  T  S  L  Y  E  H  R  E  G

CGCAGCGCGCCCGGGTATCTTCACACCAACAACACGCGTTTGGCCGCAAGGGATGCAGTC  120
V  C  R  A  R  T  D  E  C  W  C  C  A  N  P  R  L  P  H  L

GCAAGGAGGACCAAACACATCCTCTGCCGCCAGCCGCAATGCGGGCCTTCGAAACGGGAT  180
R  L  S  S  W  V  C  G  R  G  G  A  A  I  R  A  K  S  V  P

TTTCTCGGAACAGTGGTCGGCCGATCACCATGCAGGAGAGATGCAGAAAGCTCCAATCTT  240
N  E  R  F  L  P  R  G  I  V  M  C  S  L  H  L  F  S  W  D

TCAGCATGACGCTGGCGATATCAGGAACTTCGATAAGCGACGGCGTTTGGGAGAGACGGG  300
K  L  M  V  S  A  I  D  P  V  E  I  L  S  P  T  Q  S  L  R

TGGCATTCACTGATTTTGCCAGTTCCGCCATGCTGAAATGGTAGGAGAATTCCTCGCCGC  360
T  A  N  V  S  K  A  L  E  A  M  S  F  H  Y  S  F  E  E  G

CATGGATCGAGTCCCAATAACAATCAGCATCGTACCGCGTCAGGGCCTCGAAGGCGTTGC  420 ExoW
G  H  I  S  D  W  Y  C  D  A  D  Y  R  T  L  A  E  F  A  N

TGAGATGTTGCGGCGCCCATTCGTCGTCGGAATCCAGGAAGGCGACAAAACGGCTTGAAG  480
L  H  Q  P  A  W  E  D  D  S  D  L  F  A  V  F  R  S  S  A

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 P  S  E  D  D  V  I  L  I  D  F  D  Q  F  V
                          <----------

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CAATAAGCTTGCATCATCGCCCTATTTTGATCACGATTGATCGTGCGATCTGCGGCAATG 1080

CAAAAAAAATCCTCGGCCGGTGGAAAAAAATTTGGTGCTGCCGCAGCGTGTAATTCCACG 1140

CTATCGCGTTAACATTATACCACTCTTAAAATCGTTTTAAACCAACCACTTCCATAGGTA 1200
```

```
                                                                    .
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                                                                    .
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                                                                    .
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                                                                    .
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                                                                   M
                                                                   .
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                                                                    .
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                                                                    .
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  F  R  V  G  V  P  C  L  S  A  I  S  G  Y  L  L  F  R  H  G
                                                                    .
GCGCGGCCTCGCTGGACTACGGCAAGACCGTCCGCCGCAAGACAAGGACCGTTCTCCTGC    1680  ExoH
  A  A  S  L  D  Y  G  K  T  V  R  R  K  T  R  T  V  L  L  P
                                                                    .
CATTTCTGCTATGGAACAGCGCCTTTTTTCTTTTTGTGCTGATCCTGCAGGCCAGCGGTA    1740
  F  L  L  W  N  S  A  F  F  L  F  V  L  I  L  Q  A  S  G  I
                                                                    .
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                                                                    .
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                                                                    .
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  C  I  L  L  S  P  L  L  A  M  L  I  R  H  Y  P  L  P  T  L
                                                                    .
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  V  F  L  L  M  L  A  A  L  P  V  P  L  G  I  V  L  R  N  S
                                                                    .
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  I  L  F  S  F  S  F  G  I  Y  L  S  L  Y  R  I  D  L  T  I
                                                                    .
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                                                                    .
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                                                                    .
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  M  V  L  A  G  I  P  G  F  W  A  L  S  A  I  L  I  K  S  K
                                                                    .
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  V  G  Q  Q  L  A  E  T  G  G  L  S  F  W  I  F  C  A  H  H
                                                                    .
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  P  L  L  L  S  L  W  I  L  W  N  R  T  G  V  D  L  Y  P  V
                                                                    .
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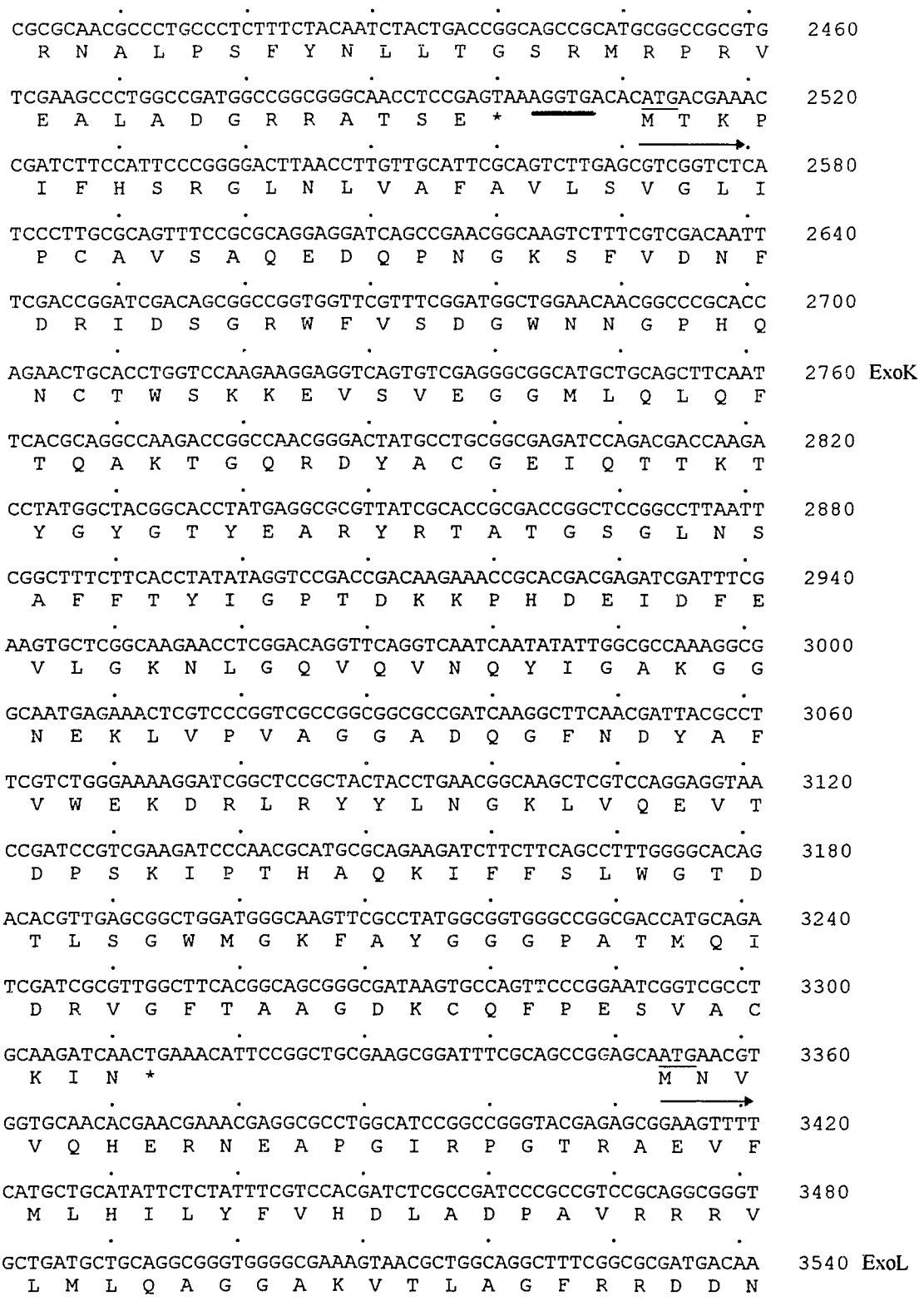
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E A L A D G R R A T S E * M T K P
CGATCTTCCATTCCCGGGGACTTAACCTTGTTGCATTCGCAGTCTTGAGCGTCGGTCTCA 2580
I F H S R G L N L V A F A V L S V G L I
TCCCTTGCGCAGTTTCCGCGCAGGAGGATCAGCCGAACGGCAAGTCTTTCGTCGACAATT 2640
P C A V S A Q E D Q P N G K S F V D N F
TCGACCGGATCGACAGCGGCCGGTGGTTCGTTTCGGATGGCTGGAACAACGGCCCGCACC 2700
D R I D S G R W F V S D G W N N G P H Q
AGAACTGCACCTGGTCCAAGAAGGAGGTCAGTGTCGAGGGCGGCATGCTGCAGCTTCAAT 2760 ExoK
N C T W S K K E V S V E G G M L Q L Q F
TCACGCAGGCCAAGACCGGCCAACGGGACTATGCCTGCGGCGAGATCCAGACGACCAAGA 2820
T Q A K T G Q R D Y A C G E I Q T T K T
CCTATGGCTACGGCACCTATGAGGCGCGTTATCGCACCGCGACCGGCTCCGGCCTTAATT 2880
Y G Y G T Y E A R Y R T A T G S G L N S
CGGCTTTCTTCACCTATATAGGTCCGACCGACAAGAAACCGCACGACGAGATCGATTTCG 2940
A F F T Y I G P T D K K P H D E I D F E
AAGTGCTCGGCAAGAACCTCGGACAGGTTCAGGTCAATCAATATATTGGCGCCAAAGGCG 3000
V L G K N L G Q V Q V N Q Y I G A K G G
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N E K L V P V A G G A D Q G F N D Y A F
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V W E K D R L R Y Y L N G K L V Q E V T
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D P S K I P T H A Q K I F F S L W G T D
ACACGTTGAGCGGCTGGATGGGCAAGTTCGCCTATGGCGGTGGGCCGGCGACCATGCAGA 3240
T L S G W M G K F A Y G G G P A T M Q I
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D R V G F T A A G D K C Q F P E S V A C
GCAAGATCAACTGAAACATTCCGGCTGCGAAGCGGATTTCGCAGCCGGAGCAATGAACGT 3360
K I N * M N V
GGTGCAACACGAACGAAACGAGGCGCCTGGCATCCGGCCGGGTACGAGAGCGGAAGTTTT 3420
V Q H E R N E A P G I R P G T R A E V F
CATGCTGCATATTCTCTATTTCGTCCACGATCTCGCCGATCCCGCCGTCCGCAGGCGGGT 3480
M L H I L Y F V H D L A D P A V R R R V
GCTGATGCTGCAGGCGGGTGGGGCGAAAGTAACGCTGGCAGGCTTTCGGCGCGATGACAA 3540 ExoL
L M L Q A G G A K V T L A G F R R D D N

```
                                                                    .
CGCGTTGGTTGCCGTGCACGGTGTCGAACCGATCGAACTCGGGCGGACGCGTGATGCGCA   3600
  A  L  V  A  V  H  G  V  E  P  I  E  L  G  R  T  R  D  A  Q
                                                                    .
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  F  A  Q  R  I  A  V  V  G  K  S  A  L  Q  L  R  G  L  L  R
                                                                    .
TTCGATTGAAAAGCCAGACGTGATCATCGGCCGCAATCTTGAAATGCTGGCCGTGGCCAA   3720
  S  I  E  K  P  D  V  I  I  G  R  N  L  E  M  L  A  V  A  N
                                                                    .
TCGCGCCAAGTCGATCTTCGGCGGCGATATGCCCGTTGTCTACGAGTGCCTCGATATTCA   3780
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                                                                    .
CCGGCTGCTGCTGCGCAAGGACGTCTTTGGCGGCGCCCTTCGTGGCATCGAGCGCCATTT   3840
  R  L  L  L  R  K  D  V  F  G  G  A  L  R  G  I  E  R  H  F
                                                                    .
CGGCGCGGATGCGGCGCTGCTGCTGACCAGTTCGCCGGCCTTTGTCGAGCATTATTTCCG   3900
  G  A  D  A  A  L  L  L  T  S  S  P  A  F  V  E  H  Y  F  R
                                                                    .
CTCTCGCTCGGGCCTCGACCTGCCGATCCTCCTTCTCGAGAACAAAGTCCTGGCGATCGA   3960
  S  R  S  G  L  D  L  P  I  L  L  L  E  N  K  V  L  A  I  E
                                                                    .
GGGTATCGGGGCTGAAATTACCGTTGCGCCGCGATTGCCAGCCGCCAACGAGCCCTGGAA   4020
  G  I  G  A  E  I  T  V  A  P  R  L  P  A  A  N  E  P  W  K
                                                                    .
GATCGGNTGGTTCGGTGCGCTTCGGTGTCGCAAATCACTGGAAATGCTTGCTGAATTTTC   4080
  I  G  W  F  G  A  L  R  C  R  K  S  L  E  M  L  A  E  F  S
                                                                    .
ACGTCAGATGGAAGGCCGCTTCGAGATCGTCTTGCGTGGCCGGCCGGCCTATTCCGAGTT   4140
  R  Q  M  E  G  R  F  E  I  V  L  R  G  R  P  A  Y  S  E  F
                                                                    .
CAGTGATTTCGATGGCTTCGTGCGCGATGCGCCCTTCATGCGCTTCGGTGGCCCATACAA   4200
  S  D  F  D  G  F  V  R  D  A  P  F  M  R  F  G  G  P  Y  K
                                                                    .
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  N  P  E  G  S  R  G  D  L  M  A  R  V  Q  I  L  L  G  Y  R
                                                                    .
ACTTCTTCGAAGAAGGACTGAATTCCAGTTGGCTATTGCCGAACCGTCTCTATGAAGGCG   4320
  L  L  R  R  R  T  E  F  Q  L  A  I  A  E  P  S  L  *
                                                                    .
GCCTGCATGGCGCCGTGCCGATCGCCTTCAATGGCACTGAAACCGCACGGTTCCTGGCAA   4380
                                                                    .
ACCGAAAAATTGGGCTGACGCTCGAAAAGGCGGATGCTGCGCACCTTGTCGCCCTCCTGG   4440
                                                                    .
GCGACATAAATAAGGAGCGCTACCTCGCCGCTTTCAACGCCTTGGCCGCGCAGGACCGCA   4500
                                                                    .
GGCAATGGATGACCGACCGCGCCGAATGCCAAGGATTGGTGCAGCGGCTGGCCTCCCTTA   4560
                                                                    .
CCCGCGCCAGTGGCCAAACCGCCCGAATTAAAAGCCCTCCCACAAATGCATCGCAATAGA   4620
                                                                    .
GGTTGGATTGNAATGAAGACCGGAATTCCTATGATATGNGTTTGGTTAATCGGTATCCCC   4680
                                                                    .
TGCCTCAATGAGGAGGAAGGATNTCGAGCCGTTGATGGCAAGTT   4724
```

The nucleotide sequence of a 4.7-kb DNA from the cosmid pUHR221 that includes the 1.8-kb and 2.2-kb *Eco*RI fragments was determined (Fig. 5.24). The analysis of this region revealed the presence of one incomplete and three complete ORFs. The complete ORFs showed the same orientation while the fourth ORF was in the opposite direction. A comparison of the deduced amino acid sequence of the four ORFs with the sequences in the EMBL/ GenBank showed significant homology with *exoW, exoH, exoK* and *exoL* genes of *R. meliloti* strains Rm2011 and Rm1021. Therefore, the same nomenclature was used for these four genes of TAL1145.

The sequence comparison of ExoW protein of TAL1145 showed a 52% homology to the deduced amino acid sequence of the *exoW* gene of *R. meliloti*. The ExoW in *R. meliloti* is known to be a glucosyl transferase that adds the seventh sugar to the growing EPS subunit. Therefore, ExoW of TAL1145 is also suggested to be a glycosyl transferase. Also, the ExoW of *R. meliloti* is membrane associated with the carboxy terminus of the protein attached to the membrane. The carboxy terminal region of the *exoW* gene is not included in the sequence of the 4.7-kb DNA fragment. Therefore, it is not possible to determine the location of the ExoW protein in the cell at this stage.

The deduced amino acid sequence of the *exoH* gene of TAL1145 showed a 51% homology to the ExoH protein of *R. meliloti.* This comparison suggests that ExoH of TAL1145 may also be responsible for the transfer of non-carbohydrate substituents to the EPS. The size of the ExoH protein (354 amino acids long) is predicted to be 39-kDa. The *exoH* mutants of TAL1145 showed a dim bluish fluorescence initially and a yellowish green fluorescence at a later stage. In *R. meliloti* strain Rm1021 *exoH* mutants have a

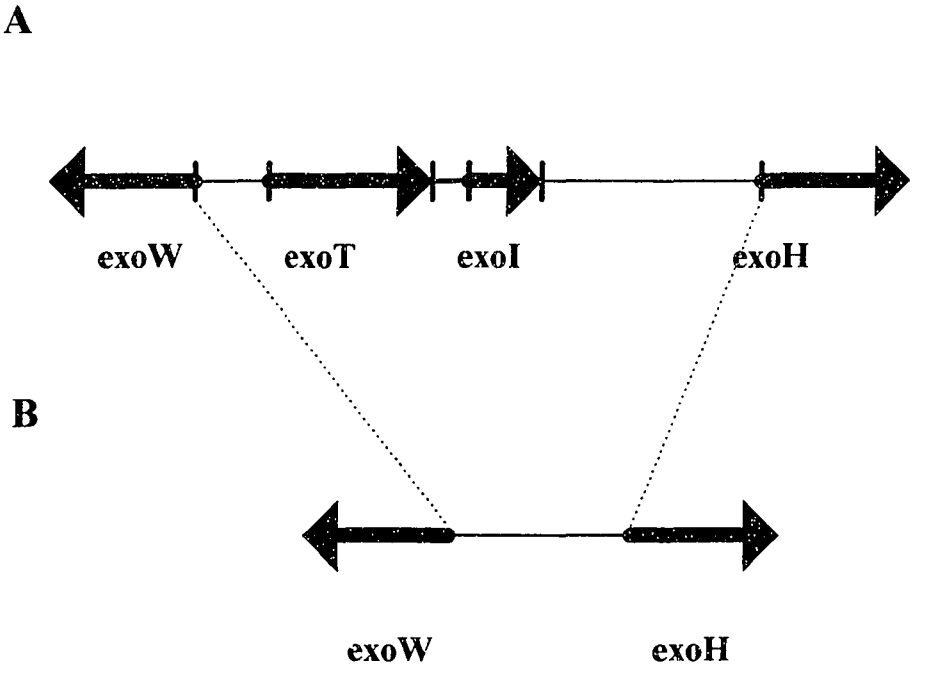

Fig. 5.25 The arrangement of *exoW* and *exoH* genes in *R. meliloti* (A) and in *Rhizobium* sp. strain TAL1145.

```
1                                                          50
   Bgln  ..MPYLKRVL LLLVTGLFMS LFAVTSTASA Q....TGGSF FDPFNGYNSG
   Bgls  ..MPYLKRVL LLLVTGLFMS LFAVTATASA Q....TGGSF FDPFNGYNSG
   Bgla  .....MKRVL LILVTGLFMS LCGITSSVSA Q....TGGSF FEPFNSYNSG
ExokTAL  MTKPIFHSRG LNLVAFAVLS VGLIPCAVSA QEDQPNGKSF VDNFDRIDSG
   Exok  MTIDRYR.RF ARLAFIATLP LAGLATAAAA QEG.ANGKSF KDDFDTLDTR
         ++ +    +  + *+  + ++    +  +++* *+   +*+**  + *+  +++

         51                                                   100
   Bgln  FWQKADGYSN GNMFNCTWRA NNVSM.TSLG EMRLALTSPS YNKFDCGENR
   Bgls  FWQKADGYSN GNMFNCTWRA NNVSM.TSLG EMRLALTSPA YNKFDCGENR
   Bgla  LWQKADGYSN GDMFNCTWRA NNVSM.TSLG EMRLALTSPS YNKFDCGENR
ExokTAL  RWFVSDGWNN GPHQNCTWSK KEVSVEGGML QLQFTQAKTG QRDYACGEIQ
   Exok  VWFVSDGWNN GGHQNCTWSK KQVKTVDGIL ELTFEEKKVK ERNFACGEIQ
          *++++++** * ++++++* + *+   + +  + +   +    +  +****+

         101                                                  150
   Bgln  SVQTYGYGLY EVRMKPAKNT GIVSSFFTYT GPTDGTPWDE IDIEFLGKDT
   Bgls  SVQTYGYGLY EVRMKPAKNT GIVSSFFTYT GPTDGTPWDE IDIEFLGKDT
   Bgla  SVQTYGYGLY EVRMKPAKNT GIVSSFFTYT GPTEGTPWDE IDIEFLGKDT
ExokTAL  TTKTYGYGTY EARYRTATGS GLNSAFFTYI GPTDKKPHDE IDFEVLGKNL
   Exok  TRKRFGYGTY EARIKAADGS GLNSAFFTYI GPADKKPHDE IDFEVLGKNT
         + ++++++* *+*   * ++ *++*+++++ **++++*+** **+*+++*+

         151                                                  200
   Bgln  TKVQFNYYTN GAGNHEKIVD LGFDAANAYH TYAFDWQPNS IKWYVDGQLK
   Bgls  TKVQFNYYTN GAGNHEKIVD LGFDAANAYH TYAFDWQPNS IKWYVDGQLK
   Bgla  TKVQFNYYTN GAGNHEKFAD LGFDAANAYH TYAFDWQPNS IKWYVDGQLK
Exoktal  GQVQVNQYIG AKGGNEKLVP VAGGADQGFN DYAFVWEKDR LRYYLNGKLV
   Exok  AKVQINQYVS AKGGNEFLAD VPGGANQGFN DYAFVWEKNR IRYYVNGELV
           ** *+*   ++*++*+++  + ++* ++++ +******++ +  +** +* *+

         201                                                  250
   Bgln  HTATN..QIP TTPGKIMMNL WNGTGVDEWL GSYN.GVNPL YAHYDWVRYT
   Bgls  HTATN..QIP TTPGKIMMNL WNGTGVDEWL GSYN.GVNPL YAHYDWVRYT
   Bgla  HTATT..QIP AAPGKIMMNL WNGTGVDDWL GSYN.GVNPI YAHYDWMRYR
ExokTAl  QEVTDPSKIP THAQKIFFSL WGTDTLSGWM GKFAYGGGPA TMQIDRVGFT
   Exok  HEVTDPAKIP VNAQKIFFSL WGTDTLTDWM GTFSY.KEPT KLQVDRVAFT
          ++*++ +**   ++***+** *+++++  *+ * + +*  *    + *++ ++

         251                  273
   Bgln  KK*.......  .......  ...
   Bgls  KK*.......  .......  ...
   Bgla  KK*.......  .......  ...
ExokTAL  AAGDKCQFPE SVACKIN*..  ...
   Exok  AAGDECQFAE SVACQLERAQ SE*
         ++++ +++ + ++++
```

Fig. 5.26 Tha alignment of deduced amino acid sequence of *exoK* gene of TAL1145 (ExokTAL) with ExoK protein of *R. meliloti* strain Rm1021 and β-glucanases of *Bacillus amyloliquefaciens* (Bgla), and *B. subtilis* (Bgln and Bgls), respectively. The asterisks indicate conserved amino acid in all five proteins while (+) shows that amino acid is present in some (including ExoK of TAL1145) but not all five proteins.

haloless phenotype (Long et al. 1988). The *exoH* gene is present upstream of the *exoW* gene in the strain TAL1145 but is oriented in the opposite direction. A gap of 794 nucleotides was observed between the *exoW* and *exoH* genes. Two genes, *exoT* and *exoI*, are present in the region between *exoW* and *exoH* in the *R. meliloti* strain Rm2011 (Fig. 5.25) The ExoT is a 494 amino acids long transmembrane protein. The presence of a prokaryotic signal peptide in the N-terminal end of the 191 amino acid long ExoI protein indicates that it may be a periplasmic protein (Becker et al. 1993c). Analysis of the hydrophobicity of deduced amino acid sequence of *exoH* gene showed 11 hydrophobic or membrane spanning domains. Therefore, ExoH is suggested to be a transmembrane protein. The *exoK* gene product of TAL1145 is 268 amino acids long with a predicted molecular size of 29 kDa . The deduced amino acid sequence of the *exoK* gene of TAL1145 showed 66% homology to the ExoK of *R. meliloti.* In addition, it also showed significant homology to a number of endo-β-1,3-1,4-glucanases of several *Bacillus* species (Fig. 5.26). These extracellular enzymes have been shown to cleave β-1,4 linkages adjacent to β-1,3 linkages. ExoK in *R. meliloti* is suggested to be involved in converting high-molecular-weight EPS to a low-molecular-weight fraction. In the *R.meliloti*-alfalfa symbiosis, a LMW component has been found to be important in the invasion of the nodules. Further investigation is needed to confirm similar role for ExoK in TAL1145.

The deduced amino acid sequence of *exoL* gene in TAL1145 showed 63% homology to the ExoL protein of *R. meliloti.* The predicted translation product of *exoL* consists of 321 amino acids. The predicted size of this protein is 36-kDa. Mutation in *exoL* in *R. meliloti* resulted in a calcofluor-dark phenotype. ExoL has been found to be a

glucosyl transferase which transfers the second glucose in the EPS subunit of *R. meliloti*. ExoL of TAL1145 also appears to be a cytoplasmic protein like ExoL of *R. meliloti* and is suggested to be a glucosyl transferase.

DISCUSSION

In different *Rhizobium* and *Bradyrhizobium* spp. the genes involved in nodulation, and nitrogen fixation are clustered together on the symbiosis plasmid or on the chromosome. Long et al. (1988a) and Glazebrook and Walker (1989) reported the presence of clusters of *exo* and *exp* genes, that are involved in succinoglycan and galactoglycan synthesis, respectively, on the second largest megaplasmid of *R. meliloti*. Chen et al. (1988) and Zhan et al. (1990) found that genes for EPS-synthesis are also clustered in the genome of *Rhizobium* sp. strain NGR234. In the present study, a cluster of genes involved in EPS synthesis in *Rhizobium* sp. strain TAL1145 has been identified. Mutation in these genes affected the synthesis of Calcofluor-binding acidic exopolysaccharide.

TAL1145 forms nitrogen-fixing nodules on indeterminate-nodule-forming tree legumes such as *Leucaena* as well as determinate-nodule-forming tree legumes such as *Gliricidia*. Several previous studies with *R. leguminosarum* and *R. loti* EPS-defective mutants showed that the mutants had different symbiotic phenotypes on indeterminate- and determinate-nodulating hosts. It is shown here that the EPS-defective mutants of TAL1145 have the same phenotypes on both indeterminate- and determinate-nodulating hosts. The majority of the mutants isolated and characterized in this study did not produce any detectable level of EPS, formed non-Calcofluor fluorescent, dry colonies and

were grouped as class II mutants. Interestingly, these mutants formed normal nitrogen-fixing nodules on both indeterminate- and determinate-nodulating hosts. This is in contrast to the previous reports with *R. leguminosarum, R. loti, R. meliloti* and *Rhizobium* sp. strain NGR234 EPS-defective mutants that were defective for nodulation on indeterminate hosts such as *Pisum sativum, Vicia hirsuta, M. sativa, Trifolium* spp. and *L. leucocephala* (Borthakur et al. 1988, Chen et al. 1986, Diebold and Noel 1989, Gray et al. 1991, Hotter and Scott 1991, Leigh et al. 1985, Long et al. 1988a, 1988b, Lopez-Lara et al. 1993).

Complementation of three spontaneous EPS-defective mutants with the cosmid clone library of TAL1145 resulted in the isolation of five overlapping cosmid clones. Fifty seven mutants were isolated by site-directed mutagenesis using cosmids pUHR221 and pUHR222. On the basis of the colony morphology and phenotype on Calcofluor-containing media these mutants were classified into three major categories: class I, class II and class III. Succinoglycan in *R. meliloti* and acidic EPS of *R. loti* had been shown earlier to bind the dye Calcofluor and fluoresce under UV light (Hotter and Scott 1991, Leigh et al. 1985, Long et al. 1988a). The physical map of the overlapping cosmids showed that the genes for EPS synthesis were present in the overlapping region of the five complementing cosmids (Fig. 5.23). Transposon-insertion in the class I and class II mutants were located on 2.2-kb and 4.4 -kb *Eco*RI fragments. All insertions in the class III mutants were localized on a 1.8-kb *Eco*RI fragment, except NP97, which had insertions on the 2.2-kb *Eco*RI fragment.

At least two complementation groups have been identified in this cluster of genes on the basis of complementation studies. Genetic analysis showed that the class I and class III mutants were present in one complementation group while the complementation group containing class II mutants was 2.4-kb from the complementation group containing class III mutants. This 2.4-kb region may not contain any *exo* genes. Leigh et al. (1985) identified six complementation groups in the *exo* cluster of *R. meliloti*. Chen et al. (1988) suggested the presence of five genetic loci that were linked and were involved in the synthesis of acidic EPS in *R.* sp. strain NGR234. Later, Zhan et al. (1990) found that there were only four genes in this region three of which are functionally related to the *exo* genes of Rm1021.

Four *exo* genes of TAL1145 present in a cluster have been identified within a 4.8-kb DNA fragment. The genes showed very high homology to the respective *exo* genes of *R. meliloti* suggesting that they perform a similar function in EPS synthesis of TAL1145. The gene arrangement was also found to be similar. For example, *exoH, exoK,* and *exoL* are present in one direction in the same order as *R. meliloti* and *exoW* is in the opposite orientation of that of *exoH*. However, two more genes, *exoT* and *exoI* are present between *exoW* and *exoH* in *R. meliloti* which are not observed in TAL1145. The function of ExoI is not known but ExoT is suggested to be involved in EPS subunit polymerization and export (Leigh and Walker 1993, Reuber and Walker 1993).

The ExoL and ExoW proteins in TAL1145 are also suggested to be glucosyl transferases involved in EPS synthesis as they are in *R. meliloti.* Probably none of the mutants in the 2.2-kb and 1.8-kb *Eco*RI fragments are located in *exoL* or *exoW* genes in

TAL1145. Therefore, a dark phenotype was not observed in the mutants with the *Tn3Hogus* insertion in this region.

Mutation in the *exoH* gene also reduced fluorescence on Calcofluor-containing medium in TAL1145 like that of *R. meliloti.* However, delayed yellowish-green fluorescence observed in TAL1145 mutants was not reported in *R. meliloti* (Leigh et al. 1987, Long et al. 1988). The *exoH* mutants of TAL1145 also secreted some amounts of high-molecular-weight EPS and formed empty nodules on hosts like the *R. meliloti exoH* mutants. On the basis of the high homology observed between the deduced amino acid sequence of *exoH* genes and similar phenotypes exhibited by the *exoH* in two *Rhizobium* spp., the ExoH in TAL1145 is suggested to be a succinyl transferase.

Mutation in the *exoK* gene of TAL1145 showed reduced levels of the HMW EPS secretion as well as a dim fluorescence on Calcofluor-containing medium like the *exoK* mutants of *R. meliloti.* A high homology between the deduced amino acid sequence of *exoK* of TAL1145 and ExoK protein of *R. meliloti* suggests that ExoK of TAL1145 may also be responsible for the cleavage of HMW EPS to an LMW EPS fraction. The *exoK* mutants formed empty nodules on both determinate- as well as indeterminate-nodule-forming hosts. These results were different from that of the *exoK* mutants of *R. meliloti* which did not show invasion defects on *M. sativa* nodules. A different function for ExoK in EPS synthesis in TAL1145 and a significant role during symbiosis with different hosts is suggested. The class I or *exoK* mutants in TAL1145 produced reduced levels of LMW as well as HMW EPS fractions. An insufficient level of a LMW EPS without the proper structural configuration in the *exoK* mutants may be responsible for formation of

pseudonodules on all three hosts by these mutants. Battisti et al. (1992) observed that the exogenous application of a fraction containing charged tetramers of succinoglycan subunits only promoted invasion of alfalfa by *exoH* mutants. Leigh et al. (1987) suggested that degradation of exopolysaccharide by bacterial or plant enzymes may be required for the production of appropriate oligosaccharides which then act as a signal to plants during nodulation. ExoK may play such a role in TAL1145 during symbiosis.

Class II mutants which showed a Calcofluor dark phenotype did not produce detectable levels of ethanol-precipitable EPS when grown in *Rhizobium* minimal medium while class I and class III produced 25% or less of the amount of EPS as compared to the wild type. In *R. meliloti* strain Rm1021, Leigh and Lee (1988) and Long et al. (1988a) showed that *exoP, exoM, exoA, exoL, exoF, exoQ* and *exoB* mutants that showed dark phenotype on Calcofluor-containing medium produced negligible amounts of anthrone-reactive material in the culture supernatant while Calcofluor-dim *exoG* and *exoJ* mutants produced less than 10% of the carbohydrate in the culture supernatant as compared to the wild type.

Size fractionation of the culture supernatant of the class II mutants showed the presence of only low-molecular weight anthrone-reactive material while the high-molecular-weight fraction was missing even when 10 times more lyophilized supernatant was loaded on a Bio-Gel A5m column. The *exo mutant of R.* sp. strain GRH2 (*Acacia* sp.) was also found to be impaired in the synthesis of HMW EPS (Lopez-Lara et al. 1993). Leigh and Lee (1988) observed the presence of only the low-molecular-weight fractions on Bio-Gel A5m column chromatography of culture supernatants of Calcofluor-dark

exoA, *exoB* and *exoF* mutants as well as Calcofluor-dim *exoD* mutant of *R. meliloti* when grown in salt-glutamate-mannitol medium. However, they observed reduced amounts of high-molecular-weight succinoglycan in *exoD* mutants when the cultures were incubated in M9-glutamate-salt-medium. NP98, a representative of class I mutants and NP95, a class III mutant produced reduced quantities of high- and low-molecular-weight EPS fractions as compared to the wild-type. The difference between class I and class III mutants is that the class III mutants showed a yellowish fluorescence after 10 days of incubation while Calcofluor-fluorescence of class I mutants did not change on prolonged incubation. The difference in the chemical structure of EPS of class I and class III mutants is not established in this study. None of the mutants showed differences in the LPS profile when compared with the wild-type.

Gene-fusion is an ideal method to determine the direction of transcription and study the expression of the genes of interest. Tn3Ho*gus* was used to generate fusions with the β-glucuronidase gene in the *exo* mutants. Trascription fusions between *exo* genes and the promoterless *gus* gene present in Tn3Ho*gus* resulted in the expression of GUS activity in 20 mutants. On the basis of GUS activity, and the direction of transposon-insertion as determined by Southern hybridization (Table 5.2 and Fig. 5.21), two transcripts in the opposite direction were identified within a 4.0-kb region containing the adjacent 2.2- and 1.8-kb *Eco*RI fragments. Long et al. (1988b) identified several symbiotic loci in *R. meliloti* on the basis of Tn*phoA* mutagenesis. *TnphoA* creates translational fusions resulting in the synthesis of hybrid proteins composed of an amino region of the target protein and the carboxy-terminal region of alkaline phosphatase.

Active translational-fusions only result if the target genes encode for a secretory or membrane-spanning protein. Long et al. (1988b) used these mutations to study membrane and periplasmic proteins specifically. They were able to find the location of the target protein in the membrane or the periplasm by studying the alkaline phosphatase activity in subcellular fractions of *R. meliloti* mutants. It was not possible to determine the subcellular localization of genes in the present study using *gus*-fusions because the transposon Tn3Hog*us* creates transcription fusions.

The presence of strong or weak promoters in different *exo* genes could be determined on the basis of variation in GUS activity in different mutants. Among class II mutants the *exo* genes interrupted in mutants NP88, NP91; NP125 and NP133, NP138 are suggested to have strong promoters while genes interrupted in NP85, NP89, NP132, NP147; NP103, NP105 and in NP126 may have weak promoters. Mutant NP129 showed low GUS activity and mutants NP114 and NP146 showed high enzyme activity but the exact site of transposon insertion in these mutants has not be determined at this stage. All four mutants, NP98, NP118, NP119 and NP120 that showed low GUS activity have the insertion of the transposon within a 1-kb region and appear to interrupt the same gene. The presence of strong promoters was indicated by GUS activity for the genes that were interrupted in the mutants NP97 and NP114.

Class II mutants either span a long polycistronic transcript or multiple monocistronic transcription units. On the basis of sites of transposon insertion and the level of GUS activity it is suggested that at least two transcripts are synthesized within the 4.4-kb DNA region. Mutations in this region result in the complete absence of EPS

production as indicated by the lack of Calcofluor fluorescence and undetectable levels of ethanol-precipitable EPS in culture supernatants in class II mutants.

All class II mutants that showed high GUS activity in vitro also showed high levels of enzyme activity in the nodules. Early as well as late symbiotic regions showed the enzyme activity suggesting that active transcription of these *exo* genes occurs in the nodules. Keller et al. (1988) showed transcription activity of some unidentified genes involved in EPS synthesis in *R. meliloti* by creating *lacZ* fusions. Only two mutants, which differed in EPS quantity from that of the wild-type, showed significant β-galactosidase activity. The remaining EPS-defective mutants were defective in nodule invasion. Thus, they studied the activity in the bacteroids by creating a merodiploids. In the present study, it is suggested that in the complete absence of EPS synthesis the symbiotic function is carried out by some other rhizobial cell surface component(s). Putnoky et al. (1990) observed that EPS and LPS of *R. meliloti* strain RM41 can have the same function during plant-bacterium interaction. These two regions were found to functionally substitute for each other in symbiosis. A single mutation in either region in the RM41 background induced normal nitrogen-fixing nodules on alfalfa while *exo-lps* double mutants showed Fix- phenotype. Williams et al. (1990) found that the symbiotic defect of EPS mutants in *R. meliloti* strain Rm1021 can be suppressed by transfer of the *lpsZ* gene of RM41. Strain Rm1021 lacks this gene. Recently, Reuhs et al. (1995) have suggested that modification of polymerization of a cell-associated polysaccharide, named KPS, by *lpsZ* in RM41 may be significant in symbiosis. A component similar to KPS described by Reuhs et al. (1993b) in *R. meliloti* strain AK631, a derivative of RM41, and in *R. fredii* strain

USDA205 may also be produced by TAL1145. It is suggested that in class II mutants KPS, LPS or a low-molecular-weight specific oligosaccharide fraction of EPS substitutes for the functions of EPS during symbiosis.

Both class I and class III mutants formed Fix- nodules on all three legume hosts. Lack of normal EPS in these mutants might have prevented normal bacteroid development in these mutants resulting in the Fix- phenotype. A preliminary observation of nodule sections formed by NP97 and NP98 complemented with cosmid pUHR221 showed the presence of significant GUS activity in the early symbiotic or invasion zone only, further indicating the function of these *exo* gene products during the invasion of the nodules. This observation shows that expression of these *exo* genes is not needed at later stages of symbiosis. Reuber et al. (1991) also showed that high alkaline phosphatase activity in the early symbiotic region and relatively little or no activity in the late symbiotic region of alfalfa nodules formed by *exoF*::Tn*phoA* fusion strains (merodiploids) of *R. meliloti*. All these mutants showed reduced levels of production of both high-molecular weight and low-molecular-weight EPS. Leigh et al. (1987) observed that *exoH* mutants that failed to succinylate their EPS formed empty nodules in alfalfa. These mutants also showed invasion-deficiency like class I and class III (*exoH* and *exoK, respectively*) mutants of TAL1145 while *exoK* mutants of *R. meliloti* showed normal nodule formation on *M. sativa*. The *exoK* gene in TAL1145 is also located downstram of *exoH* gene like *R. meliloti* ruling out the possibility of the polar effect on *exoH* gene expression. Structural changes in EPS in the *exoK* and *exoH* mutants of TAL1145 have not been determined in this study which can indicate if changes in non-carbohydrate substituents or the

composition of the low molecular weight EPS fraction or both were responsible for the symbiotic defect of these mutants.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

Legume root nodules are the products of nitrogen-fixing symbiosis involving bacteria of three genera, *Rhizobium, Bradyrhizobium* and *Azorhizobium* collectively known as rhizobia. Although there is considerable variation in nodule morphology as well as details in symbiotic interactions among different species, four common features have been observed in all effective nodules : meristematic activity in the root cortical cells; invasion of root cortical cells by rhizobia; development of a central bacteroid zone with reduced access to oxygen where nitrogen fixation occurs; and peripheral vascular tissue development (Brewin 1991). Different rhizobial polysaccharides have been known to play important functions throughout the symbiotic process. Modified oligosaccharides of N-acetyl glucosamine or Nod factors' signals from rhizobia stimulate plant root meristem and hence nodule formation. These signals are major determinants of host-specificity. The later steps of nodule development involve manifold signalling mechanisms that probably include EPSs and LPS, respectively, in the indeterminate- and determinate nodules.

The present study describes the identification of a cluster of genes involved in EPS synthesis in *Rhizobium* sp. strain TAL1145. In addition, a gene involved in LPS synthesis in this strain is also identified and characterized. This gene is not included in the gene cluster involved in EPS synthesis but it does affect EPS synthesis in TAL1145. Several mutants were obtained in these genes to study the changes in EPS and LPS and to investigate their effect on symbiosis with three legumes. Mutations in these genes showed some similarities to the symbiotic phenotypes observed in LPS- and EPS-defective

mutants of other *Rhizobium* spp. in their respective hosts. However, this study identifies some novel symbiotic phenotypes not observed in EPS- and LPS-defective mutants of other *Rhizobium* spp.

The mutant NP84 severely affected LPS I (a fraction that shows slower mobility in polyacrylamide gel electrophoresis) synthesis and also caused some changes in EPS. This mutant formed empty nodules on both *Phaseolus vulgaris* and *Gliricidia sepium.* Both *P. vulgaris* and *G. sepium* form spherical, determinate type nodules. In earlier studies, intact LPS has been shown to be essential for effective nodulation of several determinate-nodule-forming legumes (Cava et al. 1989, Carlson et al. 1987, Maier and Brill 1978, Noel et al. 1986, Puvanesarajah et al. 1987, Stacy et al. 1991, Vandenbosch et al. 1985). Results of Southern hybridization and complementation experiments indicated that a 3.8-kb *Eco*RI fragment contains the gene mutated in NP84. High homology as indicated by Southern hybridization was not observed with strains of other *Rhizobium* spp. that were included in this study. The gene appears to be conserved among typical *Leucaena*-nodulating rhizobia.

Sequence analysis of 3.8-kb *Eco*RI fragment indicated presence of three ORFs. The deduced amino acid sequence of the *exoY* of TAL1145 showed significant homology (33-39%) to various exopolysaccharide synthases. Alignment of the various protein products (Figs. 4.20 and 4.21) showed presence of some conserved motifs that are suggested to be the enzymes' active sites (Ivashira et al. 1994). Also, the *lpsB* gene product of TAL1145 showed very high (44 and 52%, respectively) homology to OmpX protein of *Vibrio cholerae* and mannosyl transferase C of *E. coli.* Both of these proteins

have been found to be involved in the O-polysaccharide synthesis in respective bacterial species. Therefore, LpsB is suggested to be involved in the O-antigen synthesis in TAL1145 LPS. The product of *lpsA* gene that is disrupted in NP84 did not show significant homology to the proteins in the data bank. The hydrophobicity analysis of the deduced amino acid sequence of *lpsA* showed that it may be a transmembrane protein. The role of LpsA in synthesis / transport of LPS is suggested

Although NP84 formed nodules on *L. leucocephala*, an indeterminate host, at the same time as the wild-type strain TAL1145, the nodule development by this mutant was delayed. Nodules showed much less infection in the mutant in four-week old plants and plants appeared yellow and stunted as compared to those inoculated with the wild-type. However, after eight weeks, the plants inoculated with NP84 completely recovered and showed normal nitrogen fixation ability. Normal or only slightly impaired nodule development was observed on alfalfa and peas inoculated with *R. meliloti* and *R. leguminosarum* mutants, respectively, that showed only mild alterations in LPS (Brewin et al. 1993, Clover et al. 1989). However, it has been generally observed that mutants of different *Rhizobium* spp. that lack the O-antigen of the lipopolysaccharide affect symbiosis with indeterminate-nodule-forming hosts at the later stages of symbiosis such that some invasion of the root cortical cells occurs (deMaagd et al. 1989, Noel 1992, Priefer 1989). Mutants of *R. leguminosarum* with a moderately affected LPS also formed abnormal nodules with relatively lower number of infected host cells on peas. Leghaemoglobin production was induced in these mutants and the endosymbiotic bacteria enclosed by peribacteroid membrane also stimulated the synthesis of nitrogenase (Brewin et al. 1993).

The mutant NP84, in the present study probably resembles the mutants of other *Rhizobium* spp. that lack O antigen. However, none of the above mentioned studies have shown complete restoration of symbiosis on longer incubation of plants infected with LPS mutants. Thus, it is suggested that like EPS, the presence of normal LPS structure, as observed in free-living TAL1145, may not be essential during symbiosis with indeterminate-nodulating hosts past the invasion step.

Brewin et al. (1993) observed that severe LPS-defective mutants of *R. leguminosarum* formed empty nodules on peas, an indeterminate-nodule-forming host. Small, white nodules that were devoid of bacteria were induced by LPS-defective mutants of *Rhizobium* sp. strain GRH2 on the tree legume, *Acacia cyanophylla.* Contrary to the general observation, LPS mutants of this strain were able to nodulate the determinate-nodulating host, *Phaseolus,* but not the indeterminate-nodulating host, *Lotus* (Lopez-Lara et al. 1995). These authors suggested that in this tree-legume-nodulating *Rhizobium* strain, a symbiotic defect shown by LPS-defective mutants is not nodule ontogeny-dependent.

The mutant NP84 was also found to be less competitive than TAL1145. An LPS-defective mutant of *Bradyrhizobium japonicum* which also produced less EPS but formed a determinate type of nodules was found to be less competitive like NP84 (Bhagwat et al. 1991). Whether this lack of competitiveness of NP84 as compared to the wild-type is due to a defect in LPS, EPS or both is not known at this stage.

None of the tested EPS-defective mutants of TAL1145 obtained by Tn3Hogus insetion and that are present in a cluster could be complemented by pEX312 and R'3222

plasmids that contain the *exo* gene cluster of *R. meliloti* strain Rm1021 and *Rhizobium* sp. strain NGR234. Yellowish-green Calcofluor fluorescence observed upon prolonged incubation of class III EPS-defective mutants of TAL1145 has not been reported in any other *Rhizobium* sp. These observations suggest that the EPS structure as well as function of the genes involved in EPS synthesis during symbiosis may be different in TAL1145 from the other *Rhizobium* spp.

Sequence analysis of a 4.7-kb DNA fragment that includes the 1.8-kb and 2.2-kb *Eco*RI fragments showed the presence of four *exo* genes of TAL1145. The gene products showed high homology with the ExoW, ExoH, ExoKand ExoL proteins of *R. meliloti* strains Rm1021 and Rm2011 (Becker et al. 1993a, 1993b and 1993c, Reuber and Walker 1993). Therefore, the ExoW and ExoL proteins of TAL1145 are suggested to be glucosyltransferases, while ExoH may be a succinyl transferase. The deduced amino acid sequence of the *exoK* gene of TAL1145 showed high homology with the ExoK protein of *R. meliloti* and to the β-glucanases of several *Bacillus* spp. The product of the *exoK* gene of TAL1145 probably binds β-1,3-1,4-glucans like various glucanases with which it shows homology. However, the function of this enzyme is not known. The presence of a signal peptide suggests that it may be a periplasmic or extracellular protein. This protein may be involved in modification of EPS in addition to playing a role in degradation of HMW EPS to LMW EPS as suggested in *R. meliloti.*

Class II mutants that formed dry, Calcofluor-dark colonies formed normal, nitrogen-fixing nodules on all three hosts. Extensive evidence indicates that acidic EPS is essential for successful symbiosis with the indeterminate-nodulating hosts but not for

determinate-nodulating hosts (Borthakur et al. 1986, Chakravorty et al. 1982, Chen et al. 1985, Diebold and Noel 1989, Hotter and Scott 1991, Kim et al. 1989, Ko and Gadya 1990, Leigh et al. 1985, Long et al. 1988, Lopez-Lara et al. 1993). However, this is the first report to show that function of some *exo* genes that are essential for EPS synthesis in TAL1145 is replaceable during symbiosis with both determinate- as well as indeterminate-nodulating hosts.

Root hair curling is usually induced by EPS-defective mutants on indeterminate hosts in most cases. However, the infection thread was either completely absent or abnormal infection thread developed which aborted in the peripheral or outer cortical regions of the roots resulting in the formation of empty nodules (Chen et al. 1985, Finan et al. 1985, Leigh et al. 1987, Muller et al. 1988, Niehaus et al. 1993, Yang et al. 1992). Stacey et al. (1991) suggested that EPS could form an important component of the infection-thread matrix of broader infection threads that are observed in indeterminate nodules as compared to the determinate nodules. A block in the nodule development pathway by the EPS-defective mutants of different *Rhizobium* spp. may occur due to: the delay in root hair curling, formation of infection threads but their lack of penetration and/or aborted infection threads indicating the role of EPS in protection against the plant defense mechanisms. Lack of a discrete, persistent meristem, and the induction of only a few nodulins in the nodules formed by EPS defective mutants suggest that EPS also acts as a signal molecule for normal nodule development (Leigh et al. 1987, Norris et al. 1988, Yang et al. 1992).

It is suggested here that in class II mutants of TAL1145 the presence of high molecular weight EPS is not necessary for normal symbiosis with either indeterminate- or determinate-nodule forming hosts. The modified, specific LPS or KPS (polysaccharide resembling K or capsular antigen of *E. coli*) or a low molecular weight EPS fraction may substitute for functions performed by normal EPS in the wild-type strain in the initial stage of nodule development in these mutants. Battisti et al. (1992) reported that the invasion defects of *exo* mutants of *R. meliloti* were corrected when a specific, charged (therefore highly modified with pyruvate and succinate substituents) low-molecular-weight EPS fraction from the wild type strain Rm1021 was applied exogenously to alfalfa roots. The invasion response was not observed when high-molecular-weight succinoglycan was applied on roots. Similar results were also observed by Urzainqui and Walker (1992). Suppression of Fix- phenotype of an Exo- mutant in *R. meliloti* strain RM41was observed by LPS or a modified KPS (Putnoky et al. 1990, Williams et al. 1990, Reuhs et al. 1995). This suppression was due to the presence of *lpsZ* and some other chromosomal *lps* genes in this strain.

In class II EPS-defective mutants of TAL1145, infection of the root cortical cells may be facilitated by one or more of the components similar to those mentioned in other *Rhizobium* spp. Normal later steps of nodule development follow in these mutants like the wild-type resulting in normal, nitrogen-fixing nodule formation. Earlier studies have also shown that a defect in EPS production does not affect the nodule development past the invasion stage (Kapp et al. 1990, Zhan and Leigh 1990). Expression of genes involved in EPS synthesis has been suggested to decrease or to be completely inhibited after invasion

(Latchford et al. 1991, Reuber et al. 1991). High transcription activity of the *exo* genes affected in class II mutants was observed throughout the nodules of all three hosts. The importance of this transcription activity with no obvious symbiotic defect shown by the mutants is not clearly understood. Streeter et al. (1992, 1993) observed that certain strains of *Bradyrhizobium japonicum* deposited nodule polysaccharides in the symbiosomes of infected cells of soybean nodules. The bacteroids were found to be embedded in a fibrillar matrix in the symbiosomes of the aquatic legume *Neptunia natans*. This matrix filled the entire space between bacteria and the peribacteroid space and resembled the EPS network observed surrounding the rhizobia grown in carbon-rich medium under in vitro conditions (Subba-Rao et al. 1995). Extensive nodule polysaccharide production by TAL1145, similar to these rhizobia, was not observed in *Leucaena* nodules under the electron microscope. However, the gene(s) affected in class II mutants may be involved in the synthesis of a small amounts of nodule polysaccharide as indicated by their transcription activity in the nodules.

The EPS-defective mutants belonging to both class I (with mutations mostly in exoK) and class III (*exoH* mutants) that have reduced quantity of EPS production showed invasion defects during symbiosis with *L. leucocephala,* as observed in mutants of other *Rhizobium* spp. on indeterminate-nodule-forming hosts. The *exoH* mutants of *R. meliloti* also form empty nodules to the indeterminate-nodule-formning host *M. sativa.* However, mutantion in *exoK* gene of *R. meliloti* showed no nodulation defects. However, unlike other *Rhizobium* spp. EPS-defective mutants (in *exoK* and *exoH* genes) also formed empty nodules on determinate-nodule-forming hosts. These results suggest that *exo* gene

products of TAL1145 have a different role during symbiosis inspite of their structural similarities to the *exo* genes of other *Rhizobium* spp. Bacteria in determinate nodules spread primarily by the division of the infected cells while in indeterminate nodules, they spread by continuous infection-thread penetration. The identical symbiotic phenotypes observed in both indeterminate- and determinat-nodulating hosts shown by class I and class III EPS-defective mutants and the presence of some infected cells in nodules formed by several mutants suggests that infection thread penetration and/or lack of bacterial release from the infection thread may be responsible for the invasion defects of these mutants.

The invasion defects of class I and class III mutants as compared to normal infection and nitrogen-fixation shown by class II in this study further supports that the hypothesis that the characteristic low-molecular-weight EPS fraction or some other oligosaccharide substituted for the function of EPS during symbiosis in class II mutants. The fraction with a similar chemical composition may be absent in class I and class III mutants resulting in the nodule invasion defect observed in these mutants. Battisti et al. (1992) showed that only an oligosaccharide fraction containing a highly charged tetramer when applied to alfalfa roots was able to overcome the invasion defect of *exo* mutants of *R. meliloti* while smaller or less charged fractions were not active. Chemical characterization of the low-molecular-weight EPS produced by all three classes of mutants of TAL1145 is necessary to confirm that a similar oligosaccharide component is required for invasion of the nodules in TAL1145.

These results suggest that EPS of TAL1145 may be different from other *Rhizobium* species. The genes involved in EPS-synthesis in this strain do not show functional relatedness with *R. meliloti* or *Rhizobium* sp. strain NGR234 where EPS synthesis has been more extensively studied. Also, the functions of EPS during symbiosis in this tree-legume-nodulating *Rhizobium* strain differ from that observed in fast-growing *Rhizobium* spp. that produce copious amounts of exopolysaccharide and nodulate mainly herbaceous legumes. Some differences in the role of LPS of TAL1145 in symbiosis with indeterminate hosts may also exist but they are not as prominent as those observed in EPS.

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