CHEMICAL, CYTOLOGICAL AND GENETIC ANALYSIS OF A SURFACE MUTANT OF *RHIZOBIUM LEGUMINOSARUM* BIOVAR *VICIAE*

Thesis
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ABSTRACT

Following transposon mutagenesis of *Rhizobium leguminosarum* biovar *viciae*, a mutant altered in its colony morphology was isolated This mutant induced the formation of structures, superficially resembling root nodules on the bacterium's symbiotic host plant *Vicia hirsuta* These structures were shown to be true nodules but were blocked in nodule development and as a consequence failed to reduce atmospheric nitrogen

An analysis of the surface polysaccharides produced by the mutant revealed that it failed to produce both the immunodominant O-antigen moiety of the wild-type lipopolysaccharide molecule and a periplasmic β-glucan The size distribution of the acidic exopolysaccharide differed markedly between the mutant and the wild-type strain even though these exopolysaccharides were structurally indistinguishable. The mutant's outer membrane was shown to be disorganized, presumably as a consequence of the absence of part of the lipopolysaccharide molecule which is thought to play a role in the maintenance of outer membrane integrity Electron microscopic examination of the bacterial cells revealed that they possessed normal morphologies

The infection phenotype of the mutant strain of *Rhizobium leguminosarum* biovar *viciae* on *Vicia hirsuta* seedlings was normal indicating that neither β-glucan nor lipopolysaccharide molecules are involved at the infection stage of nodule development Microscopic examination of sectioned material revealed that in the majority of cases the infection thread was limited to the nodule periphery On a few occasions the infection thread did penetrate the central tissue of the nodule and in some of these cases bacteria were released The peribacteroid membrane of these bacteroids was normal but the bacterial cell envelope had an altered topography which is consistent with the outer membrane of this mutant being disorganized The plant cells into which the bacteria were released senesced rapidly. It is argued that lipopolysaccharide molecules play a role in the later stages of nodule development, most probably in bacteroid development

The *Rhizobium leguminosarum* biovar *viciae* mutant could be complemented by the introduction of a wild-type cosmid gene library The gene responsible for the defect was shown to be chromosomally located, unlike many of the genes involved in the symbiosis which are plasmid encoded The mutation was at a novel genetic locus and the very unusual phenotype exhibited by this mutant has not been described before

GENERAL INTRODUCTION

> The numerous and fundamental differences between eukaryotic and prokaryotic organisms have only recently recognised in the past few years In fact, the basic divergence in cellular structure which separates the bacteria and the blue green algae from all other organisms, probably represents the greatest single evolutionary discontinuity to be found in the present day living world
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> R Y STANIER, E ADELBERG, and M DUODEROFF 1970

The prokaryote / eukaryote dichotomy is indeed a profound distinction and yet Rhizobium bacteria stimulate a process in leguminous plants involving a complex series of developmental steps which leads to the formation of root nodules Nodules are specialized organs not homologous to lateral roots, which are functionally organised to facilitate metabolic and gaseous exchange between the host plant and the bacteria (Appleby, 1984) In this symbiosis bacteria reduce (fix) atmospheric nitrogen into ammonia, which they export to the plant, the plant reduces carbon dioxide into sugars during photosynthesis and translocates it to the roots where the bacteria use it as fuel

The legumes, family Leguminosae, is one of the three largest families of the Angiosperms with 550 genera and 13,000 species All sorts of plant bodies are represented herbs, both annuals and perennials, shrubs, vines, trees and the family is distributed world-wide The Leguminosae had its origin in an evenly mild humid terrestrial environment in the Cretaceous period of the Phanerozoic aeon (about 138 million years ago) (Norris, 1959) The success and diversity of the Leguminosae is no doubt due to their ability to grow independently of often scarce soil nitrogen Only one non-legume plant, Parasponia, has been shown to form symbiotic root nodules with *Rhizobium* There is also considerable specificity of individual species of *Rhizobium* for particular groups of leguminous plants Table 1

Initiation of Nodule Formation

In symbioses involving temperate legumes the developmental steps leading to the initiation of nodule formation can be divided as follows attachment to root hairs, root hair curling, formation of infection threads within root hairs, growth of the threads toward the inner root cortex, initiation of a nodule meristem in the inner root cortex and nodule organogenesis (Newcomb, 1981)

Table 1 Rhizobium-Plant Associations

Rhizobium	Plant	Common Name
Rhizobium meliloti	*Melilotus,Medicago, Trigonella*	Alfalfa
Rhizobium leguminosarum biovar viciae	*Pisum, Vicia, Lens, Lathyrus*	Pea,Vetch
biovar *trifolii*	*Trifolium*	Clover
biovar *phaseoli*	*Phaseolis vulgarus*	Bean
Rhizobium fredii	*Glycine max*	Soybean
Bradyrhizobium japonicum	*Glycine max*	Soybean
Bradyrhizobium lupini	*Lupinus, Ornithopus*	Lupin
Rhizobium loti	*Lotus*	Lotus
Azorhizobium caulinodans		Sesbania
Bradyrhizobium spp		Parasponia

Attachment of *Rhizobium spp* to Root Hairs (Figure 1)

Attachment of *Rhizobium spp* to the developing root hairs of leguminous plants is considered to be an early step in the host specific interaction process, however the molecular mechanism of attachment is not understood It has been proposed that host plant lectins are involved in the attachment in a host specific manner Dazzo *et al* , 1976, Dazzo *et al* , 1984, Stacey *et al* , 1980) However an alternative model of attachment, (Smit *et al* , 1987), based on the presence of Ca^{2+}-dependent adhesin(s) and a cellulose fibril dependent aggregation of rhizobial cells on the surface of the root hair has been proposed However, it is likely that multiple mechanisms of attachment exist, some host specific and others more general (Kijne *et al* , 1988)

Invasion of the Root Hair

This event involves the penetration of the host plant's cell wall by the bacterium Normally uninfected root hairs are straight After inoculation of seedlings with their symbiotic partner, deformation either as curling or branching of the root hairs, occurs Curling is due to the displacement of cell wall growth from the tip of the growing

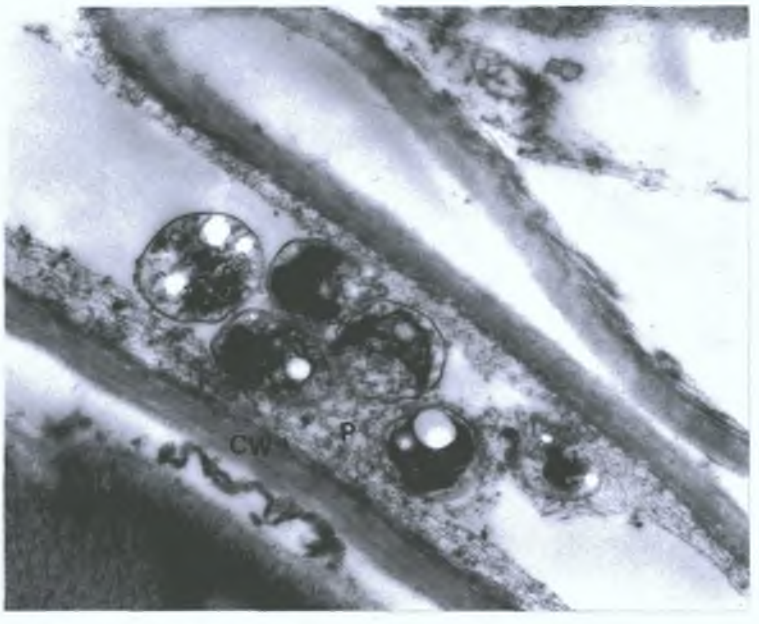


Figure 1. Transmission electron micrograph of *Rhizobium leguminosarum* biovar *viciae* in association with a hairy tare root hair. The fibrillar capsule is in contact with globular particles (P) on the outer periphery of the root hair cell wall (CW).

root hair to the outside of the curl (Hac^+ phenotype) and can result in a curvature of more than 360°, called a shepherd's crook (Callaham and Torrey, 1981), Figure 2. The first indication of infection is the appearence of a bright refractile spot at the root hair cell wall. The infection thread appears to originate from this spot and be continuous with it. The presence of an infection thread is indicative of an Inf^+ phenotype.

The Infection Thread

Callaham and Torrey,(1981), while studying the *R.leguminosarum* biovar *trifolii* / clover symbiosis confirmed that the pocket created by the root hair curling or branching was the site of infection thread formation. At this site in a curled root hair the electron dense root

hair cell wall is discontinuous and is replaced by a matrix, while the infection thread cell wall is continuous with a newly deposited root hair cell wall. This redirected cell wall growth results in envagination of the root hair cell wall and forms the tubular infection thread. Cellulolytic and pectinolytic enzymatic activity have been demonstrated in *Rhizobium* cultures (Hubbell *et al.*, 1978; Martinez-Molen *et al.*, 1979). Direct evidence for the involvement of these activities in root hair invasion has not been demonstrated. Callaham and Torrey,(1981), also examined the infection process in a slightly curled root hair. A single rhizobial cell was attached by its capsule to an area of cell wall which appeared to be degraded. This suggests that the positioning of the *Rhizobium* cell may be crucial in

Figure 2. A shepherd's crook induced on *Vicia lathyroides* following inoculation with *Rhizobium leguminosarum* biovar *viciae* VF39. The single arrow indicates the refractile spot, the double arrow the infection thread within the root hair.

order to prevent the lysis of the root hair cell. The *Rhizobium* cells are surrounded by mucopolysaccharide which is thought to be of plant origin (Bradley, 1988). The infection thread grows towards the base of the root hair by a process of degradation and deposition (Bakhuizen *et al.*, 1988).

The infection thread proceeds towards the inner cortical cell layers Prior to penetration of the infection thread into the root cortical cells, these cells change remarkably The microtubules rearrange, an additional cell wall is deposited and a cytoplasmic bridge is formed by which the infection thread will pass The infection thread passes through the prepared cells by the same mechanism of degration and deposition as for root hairs or alternatively it may pass between these cells In the latter case the *Rhizobium* cells and the cell matrix occupy the intercellular spaces and utilize the pre-existing cell walls as a thread wall In cross section intercellular infection threads take on the shape of intercellular spaces and do not appear circular as in the case of intracellular infection threads

Initiation of Nodule Meristem

It has been established that cortical cell divisions may be the earliest response to bacterial inoculation, and that this may occur in the absence of root hair curling or infection, (Calvert *et al* , 1984 Hirsch *et al* , 1984, Finan *et al* , 1985) The earliest divisions occur in the inner cortex and these divisions may be several layers away from the tip of the infection thread, Figure 3 Truchet *et al* ,(1980), noted that *R meliloti* leucine auxotrophs gave rise to a rare phenotype, where the bacterial cells were not released from the infection thread (Bar^-) They proposed that a nodule inducing principle (NOIP) existed, whereby cortical cell division stimuli from *Rhizobium* could traverse plant cell walls and membranes, because the Bar^- phenotype could be complemented by addition of leucine to the plant medium The leu^- mutant-induced nodules ,however, are monosomatic rather than polyploid (effective nodules are polyploid) From this observation, Truchet *et al* , (1980) suggested that differentation of the central tissue requires the presence of rhizobia in the cytoplasm The term "central tissue inducing principle" was coined for this signal molecule, which cannot pass from cell to cell These cortical cell divisions give rise to a nodule primordium Infection threads grow into this primordium, where bacteria are released from the infection thread tips (Libbenga and Bogers, 1974)

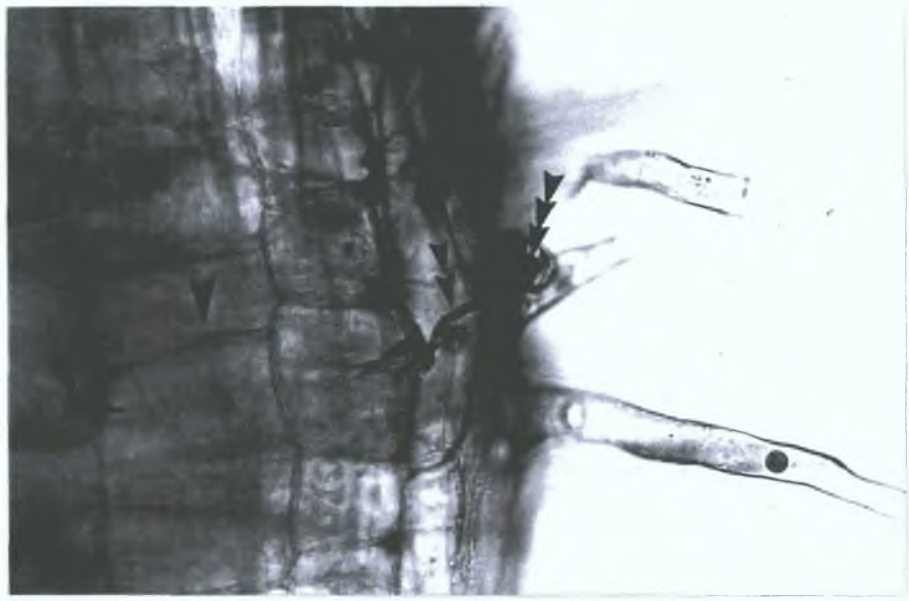


Figure 3. De-differentation of cortical cells. Cortical cell about to divide in the middle cortex (arrow), prior to the penetration of these cells by the infection thread (double arrow). The triple arrow indicates a shepherd's crook.

Classification of Root Nodules

Legume root nodules are classified into two groups:

(a) Indeterminate nodules: These nodules are cylindrical or elongated club shaped, such as those nodules of Alfalfa, Trifolium, Pisum, and Vicia.

(b) Determinate nodules: These nodules are spherical or oval shaped such as nodules of Glycine max, Arachis and Phaseolis.

Indeterminate Nodules

Pea nodules harvested five days after inoculation show mitosis occuring in the inner and middle cortex. This mitotic activity gives rise to the nodule primordium. At the apical part of this primordium, the cortical cells de-differentiate into the more primitive meristematic cells. This apical meristem is pushed outward by the increase in size of those plant cells caused by bacterial multiplication after being released from the infection threads. The direction of growth of the infection thread is then reversed in that it now follows the apical meristem

growing out of the root This gives rise to a continuous infection zone immediately adjacent to the apical meristem and is responsible for the cylindrical shape of indeterminate nodules The cells derived from the distal part of the meristem differentiate to become the nodule cortex

Determinate Nodules

Young soybean nodules contain a central zone of cytoplasm rich cells Surrounding this central zone are vacuolated cells which differentiate to form the nodule cortex, the nodular vascular bundles and schlerenchyma cells After the rhizobia are released from the infection thread into the central zone, the cells stop mitosis and undergo an increase in size This change in volume causes the nodule to grow radially giving rise to spherical nodules

Bacterial Release from the Infection Thread

Bacteria escape from the infection thread by a process of endocytosis *Rhizobium* release occurs at unwalled sites in the lateral position of the infection thread, and they become free in the plant cell cytoplasm but are surrounded by the peribacteroid membrane, a structure derived from the host plasma membrane (Bradley *et al* , 1988) Usually only one bacterium is surrounded by a peribacteroid membrane immediately after release

Early Symbiotic Development

After the bacteria are released from the infection thread they are called bacteroids In the early stages of development bacterial numbers increase dramatically Intially, the bacteroids are restricted to the outer regions of the host cytoplasm but eventually they are distributed throughout the cell In pea only one or two bacteria are surrounded by the peribacteroid membrane In contrast, in soybean nodules as many as six are enclosed in the peribacteroid membrane and in mung beans this number may as high as sixteen Increases in the numbers of proplasts, amyloplasts and mitochondria occur and these organelles are situated towards the cell periphery with the Rhizobia towards the centre of the cell

Late Symbiotic Development of Infected Cells

During this phase of development *Rhizobium* cells change in size, morphology, and cytology, Figure 4. For clover bacteroids, it has been estimated that the bacteroids increase to forty times the cell volume of the vegatative cells (Gourret and Fernandez-Arias, 1974). In vetch, alfalfa, and broad bean the bacteroids assume Y and X shapes, and p-hydroxybutyrate deposits accumulate during the nitrogen fixing stages (Goodchild and Bergersen, 1966). It should be noted that after Rhizobium cells have differentiated into bacteroids they are incapable of reverting to the free living form after the death of the nodule. Although bacterial colony forming units can be recovered from individual plant cell protoplasts (Gresshoff *et al.*, 1977), these probably represent an undifferentiated subpopulation (Zhou *et al.*, 1985).

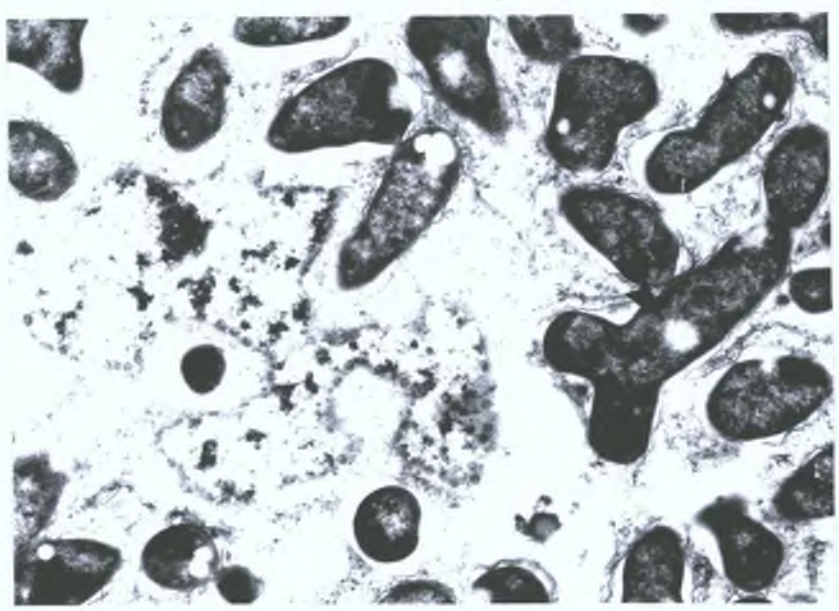


Figure 4. Late symbiotically developed bacteroids showing characteristic shapes associated with nitrogen fixation. The arrow indicates polyhydroxybutyrate inclusions.

Senescence of Infected Cells

In effective pea nodules the lifespan of an infected cell has been estimated to be between 7 and 10 days (Newcomb, 1976), however. this

time may be reduced by various environmental parameters which reduce the photosynthetic rate Vance *et al* , (1980) showed that the host cell cytoplasm degenerated prior to the bacteroids, and multivesicular bodies are involved in this degeneration

Histological Features of Indeterminate Nodules

The histological features which characterise indeterminate nodules are shown in Figure 5 These are an apical meristem, a central nitrogen fixing tissue, peripheral vascular bundles interconnected with the plant vascular system, and an outer endodermis and a cortex which surrounds the nodule

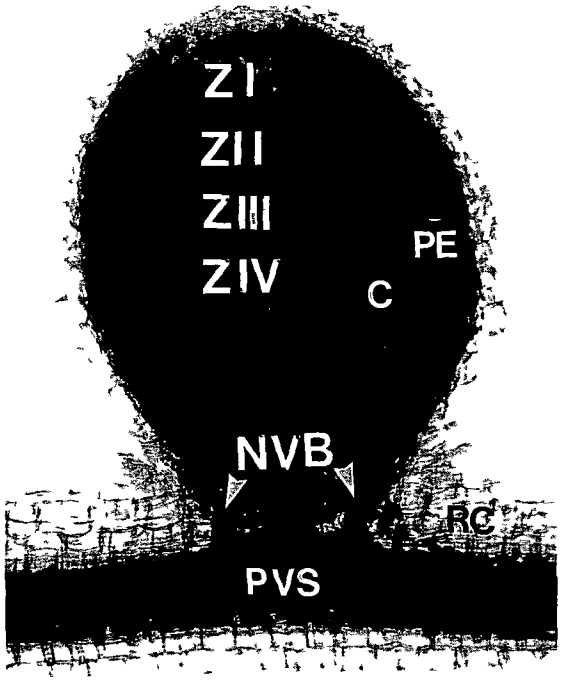


Figure 5 Mature *V hirsuta* nodule elicited by *R leguminosarum* biovar *viciae* VF39 Peripheral endodermis (PE), nodular vascular bundles (NVB), Apical meristem (AM), nodule cortex (C), meristimatic zone (Zone I), differentation zone (Zone II), fixation zone (Zone III), senescent zone (Zone IV),Plant vascular system (PVS), Plant root cortex (RC)

Rhizobium Genes Required for Nodulation

The particular host-range specificity of for example, the three biovars of *R leguminosarum*, is determined by genes on a large indigenous plasmid termed the symbiotic plasmid (pSym) When pSym is transferred

to a strain of *Rhizobium* with a different host range, or even to *Agrobacterium tumefaciens*, it confers on the recipient the ability to nodulate the host legume which is usually nodulated by the strain from which the pSym originated (Johnson *et al* , 1978, Hooykaas *et al* , 1981, Lamb *et al* , 1982) In one such plasmid, from a strain of *R leguminosarum* biovar *viciae*, a 10 kilobase region was shown to confer the ability to nodulate peas when transferred to a strain of *Rhizobium* which lacked the symbiotic plasmid or to a strain of *A tumefaciens* (Downie *et al* , 1983, Govers *et al* , 1986*a*) *Rhizobium* nodulation (*nod*) genes have also been mapped to large indigenous symbiotic plasmids in *R meliloti* and *R leguminosarum* biovar *trifolii* (Rosenberg *et al* ,1981), Fisher *et al* , 1985) A map of the nodulation regions of *R meliloti* and *R leguminosarum* is shown in Figure 6

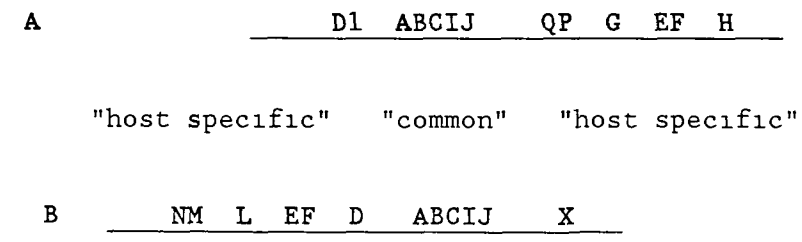


Figure 6 Map of the nodulation genes of (a) *Rhizobium meliloti*, and (b)*Rhizobium leguminosarum*

Mutations in either *nod*A, *nodB* and *nod*C give rise to both a Hac$^-$ (no deformation of root hairs), and a Nod$^-$ phenotype However these mutations may be complemented by homologous genes from *Rhizobium* strains with different host specificities Because these genes are functionally interchangable they are termed " common nodulation genes" Therefore these genes are unlikely to determine host range specificty

Rhizobium strains carrying mutations in *nod*FE, *nod*H, and *nod*LMN, elicit abnormal root hair reactions on their usual hosts and sometimes elicit root hair deformation and even curling on hosts they usually ignore (Debelle *et al* , 1986, Djordjevic *et al* , 1985) Thus whereas the common *nod*ABC transcription unit is absolutely required for root hair curling (Jacobs *et al* , 1985), it is the the *nod*FE and *nod*H

transcription units which control its specificity (Debellé *et al* , 1985)

Recently it has been shown using bioassays that *nod*ABC, and *nod*H and *nod*Q, are involved in the production of extracellular Nod signals (Lerouge *et al* , 1990) The major alfalfa-specific signal, Nod Rm-1 has been purified and its structure has been determined Figure 7

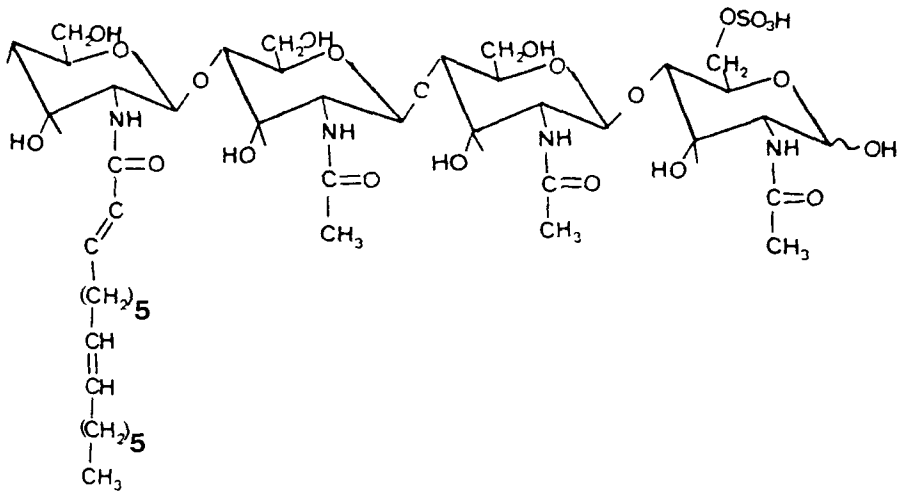


Figure 7 The proposed structure of Nod Rm-1 (Lerouge *et al* , 1990)

Regulation of Nodulation Genes

It has been shown that the only nodulation gene actively transcribed in bacterial cells grown on normal medium is *nod*D (Innes *et al* , 1985, Mulligan and Long, 1985, Rossen *et al* , 1985) When *Rhizobium* are exposed to root exudates of their hosts then transcription of the other nodulation genes is induced This induction is dependent on the presence of the regulatory *nod*D gene product The inducer molecules present in the root exudates have been shown to be particular flavones and flavanones, Figure 8 Peters *et al* , (1986), have shown that luteolin was the most potent inducer of transcription of the

R meliloti nodulation genes Redmond *et al* , (1986), identified the inducer 7,4'-dihydroxy flavone which is present in the exudate of clover seedlings, and activates the transcription of the *nod* genes of *R leguminosarum* biovar *trifolii* Similarly, Firmin *et al* ,(1986), have shown that several flavones and flavanones, all of which have hydroxyl or glycosidic substitutions in the 7 position of the A ring and at the 4' position of the B ring are inducers of the *nod* genes of *R leguminosarum* biovar *viciae* In soybean the natural inducers include an isoflavone, daidzein (Kosslak *et al* , 1987) This particular isoflavone, certain flavonols and one coumarin have been shown to inhibit induction of *nod* gene transcription by root exudate or by defined flavonoid inducers (Firmin *et al* , 1986 Djordjevic *et al* , 1987) This raises the possibility that the host legume determines the level of nod gene transcription by modulating the concentrations of the inducer and the anti-inducer molecules It has been shown that different *nod*D genes confer distinctive patterns of *nod* gene responses to a variety of pure flavonoids or plant root exudates (Spaink *et al* , 1987, Horvath *et al* , 1987, Bassam *et al* , 1988)

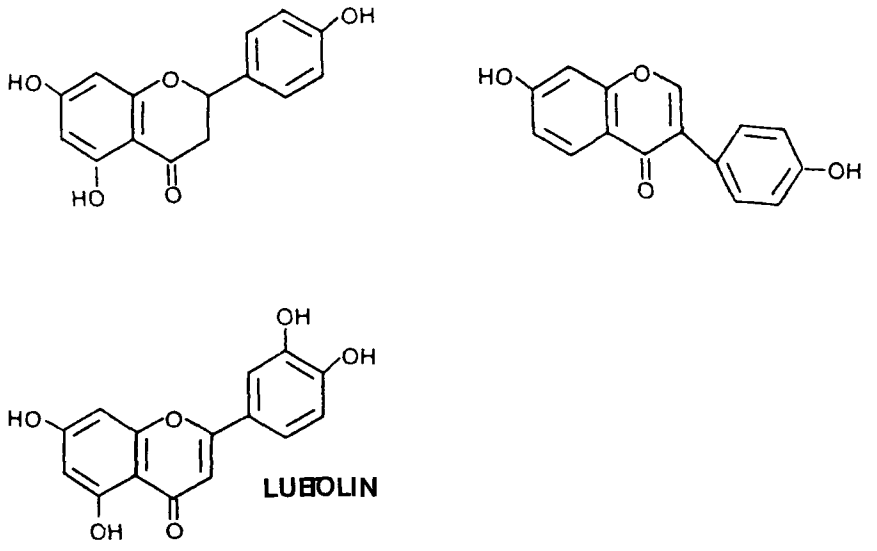


Figure 8 Compounds active as *nod* gene inducers

An important difference between *R meliloti* and the various *R leguminosarum* biovars is that in *R meliloti* there are three copies of the *nod*D gene, whereas, in for example, *R leguminosarum* biovar *viciae*

there is only one copy Because *R melıloti* forms effective symbioses with plants of at least three genera, *Medıcago*, *Trıgonella* and *Melılotus*, ıt may be that three different *nod*D genes evolved to optimize the interaction with specıfic flavones from the three species (Honma and Ausubel, 1987) Therefore multiple *nod*D genes may define one level of host specificity in the ınteraction of *R melıloti* with its host legumes

The *nod*D gene from *R legumınosarum* bıovar *vıcıae* has been shown to be autoregulatory, ın that ıt represses its own transcrıption (Rossen *et al* , 1985), *nod*D from *R melıloti* is not autoregulatory (Mulligan and Long , 1985),and is controlled by a separate locus *syr*M (Mullıgan and Long, 1989)

The *nod*D gene product binds promoters of the inducible *nod* genes (Hong *et al* , 1987) These inducıble promoters are characterızed by a long (50 base pairs), highly conserved sequence the "nod box" The nodD protein displays a footprınt on the *nod* gene promoters of about 55-60 base pairs, whıch corresponds very closely to the extent of the nod box (Long 1989b) Also there is evıdence that the *nod*D protein bınds directly with the inducer flavonoids (Hong *et al* , 1987)

Plant Genes Involved in Nodule Formatıon

Classical genetıc analyses of plant mutants have shown that plant genes play important roles ın all steps of the symbiotic process, from preınfectıon stage up to and ıncludıng the assimilatıon of ammonıa in the mature nitrogen fixing nodule (LaRue *et al* , 1985) Molecular biologıcal techniques have been used to identify nodule specıfic proteıns (ıe not occuring ın root), called nodulins, and to clone nodulin genes as well as to examine theır expression (Verma *et al* , 1986, Govers *et al* , 1987a)

Nodulın genes are classified on the basis of theır tıme of appearance in the developıng nodule Early nodulins are expressed at the stage when the nodule structure wıth all its defınıng characteristıcs ıs being formed (Govers and Bisseling, 1987) Late nodulin transcripts are not detected at thıs time (Govers *et al* , 1987b) Therefore ıt ıs lıkely that early nodulıns are involved ın nodule organogenesis whilst

late nodulins, detected at the onset of nitrogen fixation, are involved in the maintenance of nitrogen fixation

Early Nodulins

Early nodulins are involved in root hair deformation, infection and nodule morphogenesis Ngm-75 is the best characterized early nodulin and is encoded by the cDNA clone pENOD2 (Govers *et al* ,1986a) Northern blot analysis showed that pENOD2 cross hybridizes with nodule mRNA from pea,(Govers, *et al* ,1986a), vetch (Moerman *et al* , 1987) and alfalfa (Govers *et al* , 1987b), suggesting that the ENOD2 gene product plays a similar role in all legumes The Ngm-75 protein is a hydroxyproline rich protein which is thought to be a cell wall protein because of its homology to the extensins, a group of plant proteins that accumulate in the plant cell wall of dicotyledonous plants in response to wounding

Recently, Truchet *et al* ,(1989b) have shown that *Rhizobium* is not absolutely required for nodule morphogenesis They showed that structures resembling nodules which are suppressible by nitrate, express ENOD2, and that these structures possess all the histological features of indeterminate nodules As no root hair deformation was observed on the structures, ENOD2 is involved in nodule morphogenesis only Other nodulins involved in nodule morphogenesis and which do not play a role in infection are ENOD13 and ENOD55 (Frassen *et al* , 1987) Scheres *et al* , (1990), have shown that the ENOD12 nodulin protein is involved in the infection process ENOD12 is expressed not only in cells where the infection thread is migrating but also in cells that are about to receive an infection thread This implies the presence of soluble signals that emanate from the bacterial cells in the infection threads are involved in the induction of ENOD12 The common and host specific *nod* genes are essential for the production of these compounds (Scheres *et al* , 1990) Surprisingly, ENOD12 gene transcripts were detected in stem and flower tissue In most other studies on nodulin gene expression, the analyses have been restricted to root and nodule tissue Therefore, nodulins may be involved in developmental processes in other parts of the plant

Late Nodulins

Leghaemoglobin is the most abundant and the most extensively studied of the late nodulins (reviewed by Appelby, 1984) Leghaemoglobin is localized in the host plant cytosol and it comprises 25% of the total soluble protein of the nodule It is involved in the regulation of free oxygen concentration within the plant cell, therefore, it supports nitrogen fixation

The second most abundant nodulin in soybean is Ngm-35 This protein is the 33kd subunit of uricase (Verma *et al* , 1986), a key enzyme in ureid biosynthesis used in soybean in ammonia assimilation Another enzyme involved in ammonia assimilation is glutamine synthetase (GS) Although there are many GS isozymes in plant tissue Cullimore *et al* , (1984), showed that a nodule specific GS, n-GS, is produced in French bean nodules Other enzymes which show nodule specific forms are Ng-100, a subunit of sucrose synthetase, phosphoenolpyruvate carboxylase (Deroché *et al* , 1983), choline kinase (Mellor *et al* , 1986), xanthine dehydrogenase (Verma *et al* , 1986),purine nucleosidase (Larsen and Jochimsen, 1987) and malate dehydrogenase (Appels and Haaker, 1988) Although these enzymes may be nodulins, it has yet to be proven that the nodule specific forms are encoded by genes which are only expressed in the nodule The nodule specific forms may be the result of post transcriptional modification of proteins which are normally expressed by plant genes (Govers and Bisseling, 1987) In soybeans at least three nodulins are associated with the peribacteroid membrane (Verma *et al* , 1986) It has been proposed that these three proteins Ngm-23, -24, and -26 function in membrane transport but this has not been proven

Induction and Regulation of Nodulin Gene Expression

It has been demonstrated that nodulin gene expression is not induced by anaerobosis or in response to pathogen attack It can therefore be concluded that *Rhizobium* deliver the specific signals (Govers and Bisseling, 1987, Govers *et al* ,1985,1987b), and that only a 12kb fragment encoding the nodulation genes of *R leguminosarum* biovar *viciae* as well as some chromosomal genes and not *nif* or *fix* genes are required for induction of nodulin genes In effective nodules having normal root

nodule morphology, ENOD2 is expressed at normal levels but late nodulin mRNA levels are much reduced

Nitrogen Fixation

Nitrogen fixation is an anaerobic energy requiring process restricted to prokaryotes The enzyme system responsible for the fixation of atmospheric nitrogen is called nitrogenase The nitrogenase complex is composed of two dissociating proteins, one termed nitrogen reductase or Fe^{2+} protein and the other Molybdenum-Iron (Mo-Fe) protein Nitrogenase components from one particular organism will often cross react with components from other organisms to give a functional enzyme (Eady and Postgate,1974) This indicates that nitrogenase may have arisen only once in evolutionary development Since nitrogenase activity can be detected in *Clostridia spp* , Margulis (1981) has suggested that the emergence of nitrogenase preceded the origin of photosynthesis. Carr (quoted in Margulis, 1981), proposed that the enzyme complexes required for nitrogen fixation first evolved to oxidize ethylene to acetylene during the Archean aeon (3 9 to 2 4 billion years ago), before the transition to a more oxidizing atmosphere Such an origin may explain why the two components of nitrogenase are rapidly and irreversibly inactivated by oxygen, thus confining enzymatic nitrogen fixation to an anaerobic environment

Nif and *Fix* genes

The structure and regulation of nitrogen fixation, (*nif*), genes has been extensively studied in the enteric, free-living bacterium *Klebsiella pneumoniae* Since this organism fixes nitrogen for its own use, it is not surprising that its *nif* genes are regulated by the level of ammonia and for the reasons outlined above, by oxygen *Rhizobium spp* fix nitrogen only when they are in symbiotic association with a host legume, where they are not nitrogen stressed (Appelby, 1984) It is unlikely, therefore, that the *Rhizobium nif* genes are activated by

ammonia limitation In *Rhizobium spp* , genes which are required for nitrogen fixation but which do not have homologues of the *K pneumoniae nif* gene system, are termed *fix* genes Those with homologues retain the *nif* notation *Rhizobium* strains carrying mutations in *nif* or *fix* genes are able to elicit nodule formation, but these nodules are unable to fix nitrogen

Both *nif* and *fix* genes in *Rhizobium meliloti*, (the most extensively studied system), are located on the Sym plasmid (Jacobs *et al* , 1985) The structural genes for nitrogenase form an operon *nif*HDK (Ruvkun *et al* , 1982) A divergent operon contiguous with *nif*HDK contains four coding sequences *fix*ABCX, Figure 9

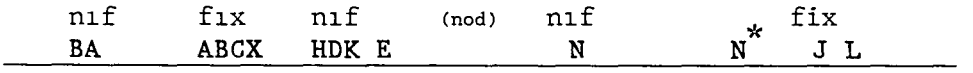


Figure 9 The organisation of *nif* and *fix* genes in *Rhizobium meliloti* *fix*N in *Rhizobium meliloti* is probably a multigene family (David *et al* , 1988)

Next to *fix*ABCX is a homologue of the regulatory *K pneumoniae nif*A gene whose product is required for activation of the *nif* operon (Szeto *et al* , 1984, Weber *et al* , 1985) Upstream of *nif*A is located *nif*B, which along with *nif*N and *nif*E, code for a protein involved in the synthesis of the molybdenum iron cofactor (FeMoco) which is required for the activity of the nitrogenase molybdenum-iron protein Another gene involved in the synthesis of this cofactor, *nif*E, has been mapped directly upstream of *nif*K (Weber *et al* , 1985) Another *fix* gene, *fix*F maps close to one of the *nod*D genes (Anguilar *et al* , 1985), and may be functionally related to the *nif* cluster since it undergoes the *nif* characteristic regulation by *nif*A

Regulation of *Nif* and *Fix* genes

In *K pneumoniae*, *nif* genes are subject to two levels of control in

response to ammonia and oxygen, one global and one *nif* specific The *nif* specific level of regulation is mediated by the *nifLA* operon The *nifA* gene product is a transcriptional activator, which is required with the *ntrA* gene product for the expression of all *nif* operons, except its own *NifL* antagonises the action of *nifA* when oxygen or a source of fixed nitrogen is present (reviewed by Gussin *et al* , 1986)

The global *nif* regulation, (the *ntr* system), controls the expression of nitrogen assimilatory genes in enteric bacteria Under conditions of nitrogen starvation, the *ntrC* product in conjunction with *ntrA*, activate the *nifLA* operon, along with other genes involved in nitrogen assimilation A homologue of *nifA*, but not of *nifL*, has been found in *R meliloti* (Szeto *et al* , 1984, Weber *et al* , 1985a) As in *K pneumoniae* it activates *nifHDKE*, *fixABCX*, *nifB* and *fixF* However, symbiotic expression of *nifA* is independent of *ntrC* in *R meliloti*, since *ntrC* mutants of *R meliloti* are Fix^+ (Szeto *et al* , 1987) *NifA* in *R meliloti* is induced by microanaerobiosis (Ditta *et al* , 1987) *FixLJ* activate *nifA*, which in turn activates the *nif* operons, and this has been shown to occur via a cascade system of regulation (David *et al* , 1988) The sequence of *fixLJ* shows that *fixL* and *fixJ* belong to a family of two component regulatory systems widely spread amongst prokaryotes (Nixon *et al* , 1986, Ronson *et al* , 1987) Kahn *et al* , (1987), proposed that FixL senses a symbiotic signal and transduces it to FixJ, which in turn activates the *fix* and *nif* operons This signal may be oxygen or an as yet unknown symbiotic signal

It should be noted that the regulatory pattern of *fix* and *nif* genes in other *Rhizobium spp* may differ from that of *R meliloti* (Pawlowski *et al* , 1987)

Role of NtrA in *R meliloti*

The *R meliloti* NtrC and NifA proteins and the promoters they activate (Buichema *et al* , 1985), show strong homology with their enteric counterparts, which suggests that *R meliloti* also contains a *ntrA* gene No such gene had been identified, until it was observed that *dctA*, a structural gene required for the transport of dicarboxylic acids, contains a *ntrA*-like promoter sequence (Ronson *et al* , 1987) Two

positive regulatory genes *dct*B and *dct*D are required for activation of *dct*A transcription The C-terminus of the DctB protein is homologous with the C-terminus of NtrB, and the N-terminus of the DctD protein is homologous with the N-terminus of the NtrC protein In *K pneumoniae* NtrB is a kinase / phosphatase that phosphorylates the NtrC in response to nitrogen limitation, and dephosphorylates it in response to an increase to nitrogen availablity, (Ninfa *et al* , 1986) These observations suggested that NtrA might be required for the expression of *dct*A, (Ronson *et al* , 1987) An *ntr*A-like gene was cloned using a selection strategy based on this supposition This *ntr*A-like gene was called *rpoN* and was also shown to be required for nitrate assimilation (Ronson *et al* , 1987) *Rpo*N showed 38% homology with the *K pneumoniae* *ntr*A gene

As succinate auxotrophs are Fix^- (Bolton *et al* , 1986, Engel *et al* , 1987) whereas sucrose auxotrophs and *Rhizobium spp* auxotrophic in other plant sugars are Fix^+ (Dilworth and Glenn, 1984), organic acids are thought to be the carbon source provided by the plant to the bacteroids The discovery that *rpoN* controls *dct* gene expression and that *rpoN* is a homologue of *ntr*A, a gene required for *nif* expression in *K pneumoniae*, may couple the import of carbon-skeletons with nitrogen fixation

The Involvment of Surface Polysaccharides in the Symbiosis

The interaction of surface polysaccharides of *Rhizobium spp* with the host cell undoubtedly plays a role in the establishment of the symbiotic state *Rhizobium spp* produce a variety of cell surface polysaccharides which exhibit biological activity These include (a) extracellular polysaccharides (EPS) andcapsular polysaccharides (CPS), (b) lipopolysaccharides (LPS) and (c) β-2 glucans

(a) **Extracellular Polysaccharides(EPS) and Capsular Polysaccharides(CPS)**

Prokaryotic extracellular polysaccharides range in the complexity of their chemical structures from homopolysaccharides, with one or more type of linkage, to heteropolysaccharides containing several different

monosaccharides, some of which may be present in more than one molar equivalent. In addition various acyl groups may be present, the most common being o-acetyl groups or pyruvate in the form of ketals attached to the 3 and 4 position of a neutral sugar residue.

Synthesis of EPS

Nucleoside diphosphate sugars are the precursors for exopolysaccharide synthesis. The mechanism of synthesis of sugar nucleotides and the general mechanism of their interconversion has been reviewed by Lenoir, (1964).

A C_{55} polyisoprenoid alcohol (bactoprenol) participates in the biosynthesis of EPS, as well as in the synthesis of all related polymers such as LPS and peptidoglycan, in which there is a regular repeating oligosaccharide unit (Sutherland 1977a). A scheme for the biosynthesis of a repeating unit of an EPS is shown in Figure 10.

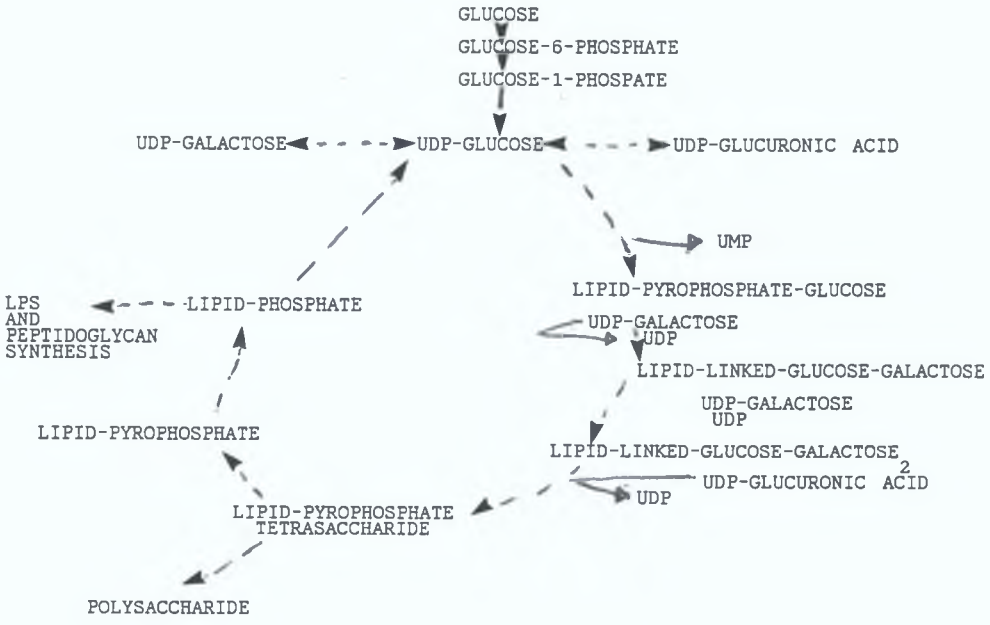


Figure 10. Pathway for the biosynthesis of exopolysaccharide (Sutherland, 1977b).

Both prokaryotes and eukaryotes contain isoprenoid lipids which are involved in glycosylation mechanisms. They provide a means of

converting the hydrophilic sugars, by their attachment to nucleoside diphosphate molecules, to lipophilic compounds capable of carrying the sugar into and through the cell membrane On the basis of the localisation of isoprenoid alcohols at the cytoplasmic membrane, and the requirement for nucleoside diphosphate sugars, (some of which are produced within the cytoplasm and others through the modification through membrane-bound enzymes), the cytoplasmic membrane appears to be the most likely site of EPS synthesis (Sutherland 1969) Based on work performed on an autoagglutinable *Klebsiella aerogenes* mutant, Norval and Sutherland (1969), have shown that a distinct system of priority for the available isoprenoid alcohols exists, whereby bacterial cells ensure that peptidoglycan synthesis occurs first, then LPS synthesis and finally EPS synthesis

The reason some bacteria form slime and some form distinct capsules and still others produce both is not known The only definitive study on a comparison of CPS and EPS produced by the same strain has been performed by Hollingsworth *et al* ,(1984) They demonstrated that CPS differed from EPS in the rate of depolymerisation of the polysaccharides by a bacteriophage enzyme and in the amount of non carbohydrate substitutions However, neither this study nor any other study have answered the question as to why capsular polysaccharide remains attached to the bacterial cell and to which chemical moiety on the cell surface it is attached to

Three Dimensional Structure of Bacterial Polysaccharides

The complete 3D structure of only two bacterial polysaccharides is known Although a great diversity of bacterial polysaccharides does exist general structural features can be assesed by reference to these two solved structures

Klebsiella K8 serotype capsular polysaccharide, consists of a tetrasaccharide repeating sequence made up of three neutral sugar residues in its backbone and one charged glucuronic acid residue in the side chain (Sutherland, 1970) The molecules pack into an orthorhombic

unit cell, in a left-handed 4_3 helix incorporating two hydrogen bonds (Isaac, 1985).

The chemical sequence of *E.coli* serotype 29 capsular polysaccharide has been determined by Choy *et al.*, (1975). It consists of a hexasaccharide repeat with four residues in the backbone and two in the side chain. The two charged groups are the carboxyl of the glucuronic acid residue of the backbone to which the side chain is attached, and a pyruvate group attached to the β-D-glucose residue. Crystallisation of the *E.coli* serotype was first accomplished by Moorhouse *et al.*,(1977).

The structure consists of an orthorhombic unit cell consisting of a two fold helix and a second antiparallel chain passing through the centre of the unit cell. None of the backbone linkages are stabilised by H-bonding, however the side chains are involved in both inter- and intra-chain bonding.

Based on these structures, some generalisations with regard to the structures of bacterial polysaccharides can be made (Isaac, 1985). As a general rule charged groups are deposited at the periphery of the molecule where they can best interact with counter ions and water molecules, and where they may be able to finger-print biological recognition. Many of the hydrogen bonding systems prevalent in simple homopolysaccharides and polydisaccharides are also present in the far more complicated heteropolysaccharide geometries. Such information may be useful in predicting the molecular structures of polysaccharides which cannot be crystalized.

The examination of the molecular conformation of heteropolysaccharides is of particular significance since each has unique properties derived from its specific combination of sugar linkages. The biological reactions of bacteria are thought to be dependent on the individual conformations of both CPS and EPS, for example antigenic specificity is thought to be dominated by the steric arrangement of the non -carbohydrate components (Dudman and Wilkinson, 1965).

The Role of *Rhizobium spp.'* EPS and CPS in the Infection of Legumes

Increases in infection thread formation and in the number of nodules

have been reported following treatment of roots with oligosaccharide fragments from EPS, or complete CPS from *R leguminosarum* biovar *trifolii* (Abe *et al* , 1984) Pre-treatment of white clover seedlings with as little as 2 5μg of CPS or EPS oligosaccharide fragment prior to inoculation with *R leguminosarum* biovar *trifolii* resulted in a concomitant 70% to 106% in infection threads (Abe *et al* , 1984) The EPS oligosaccharide fragments increased the number of infection threads whereas the intact EPS did not, indicating that the molecular weight of the EPS may be important for biological activity Alternatively the 3d-structure of the intact EPS may mask biologically active epitopes which are present in the repeating unit The native CPS also increased the number of infection threads, which suggests that it is the CPS that is the naturally occuring structure involved in the infection process (Abe *et al* , 1984)

Sanders *et al* , (1978), used mutational analysis to establish a correlation between lack of EPS and the loss of nodulation capability Napoli and Albersheim, (1980), extended this work by isolating mutants with reduced quantities of EPS but which retained a morphologically distinct capsule These mutants were capable of nodulation, abeit at a reduced effeciency Mutants which did not produce a capsule were unable to nodulate peas, and were blocked at an early step in the infection process in that they were Had^-, Hac^-, and Inf^- Napoli and Albersheim (1980), concluded that a correlation may exist between the presence of capsules and the infectivity of *Rhizobium leguminosarum* biovar *viciae* Derivatives of *R leguminosarum* strains which have lost their symbiotic plasmids produce normal amounts of EPS, which shows that the genes for EPS and CPS synthesis are not on the symbiotic plasmid However, Gardiol *et al* , (1986), demonstrated that a gene neccessary for the pyruvylation of CPS was resident on one of the indigenuos plasmids in *R leguminosarum* biovar *viciae*, and Gardiol and Dazzo (1986), demonstrated that this pyruvylation of CPS occured at the lipid bound saccharide stage

The elaboration of the capsule in *Rhizobium spp* differs from that of

E coli in that in the latter the capsular material is secreted from distinctive points around the cell (Bayer and Thurow, 1977) , the rhizobial capsule is secreted from only one pole on the cell, Figure 11

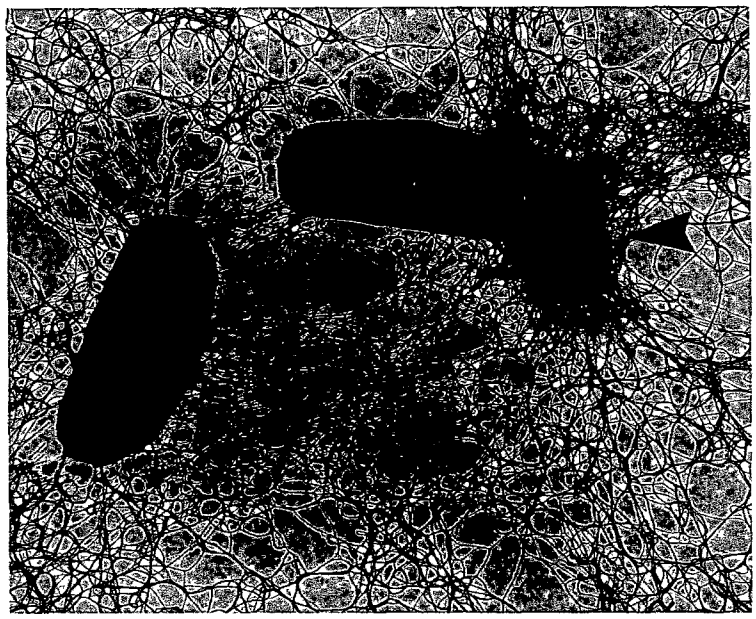


Figure 11 Ruthenium Red stained *Rhizobium leguminosarum* biovar *viciae* VF39 showing polar elaboration of the bacterial capsule (single arrow), and the positively stained EPS

Contrary to the above evidence, Sanders *et al* , (1981), demonstrated that no correlation existed between EPS and nodulation ability in *Rhizobium spp*, as different mutant strains defective in EPS synthesis, were variously unable to nodulate Borthukar *et al* , (1986) further complicated the topic by demonstrating that the same mutation which abolished a strain of *R leguminosarum* biovar *viciae*'s ability to produce EPS, and resulted in its inability to nodulate peas, did not affect a strain of *R leguminosarum* biovar *phaseoli*'s ability to nodulate beans

It should be noted, before discussing *R meliloti* exopolysaccharide mutants that *R meliloti* strains unlike *R leguminosarum* and *B japonicum* strains do not elaborate capsules (J Vasse, personal communication) Finan *et al* , (1985), reported that surface symbiotic mutants of *R meliloti* uncoupled plant from bacterial differentiation These mutants induced nodules on alfalfa that were Hac^- and Inf^-, and that these nodules were devoid of bacteroids Leigh *et al* , (1985), characterised these mutants as Exo^-, as their deficiency was in the wild-type EPS The mutants fell into several complementation groups *exoA, exoB, exoC, exoF, exoL, exoM, exoP, exoQ,* and *exoT* Mutations in any of these loci resulted in an Exo^- phenotype, which produced no EPS-1 (Long *et al* , 1988) Not surprisingly several of these genes products were shown to be membrane proteins (Long *et al* , 1988) Two genes which affect acidic substitution of the EPS, *exoH* for succinylation (Leigh *et al* , 1987), and another gene involved in the pyruvylation of the EPS (Muller *et al* , 1988), result in delayed root hair curling and an infection thread that aborts in the nodule root cortex, giving rise to empty nodules

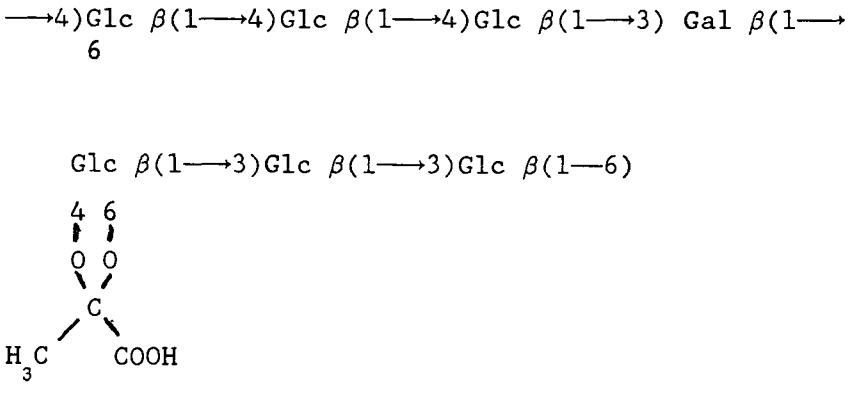
Mutations in *exoG, exoJ,* and *exoN,* diminish the production of EPS-1 but do not affect the symbiotic properties of these strains *ExoG* mutants which do not produce high molecular weight EPS, do form effective nodules, but do so at a considerably reduced efficiency relative to the wild-type strain (Glazebrook and Walker,1989)

Two unlinked chromosomal loci *exoR* and *exoS*, whose products play a negative role in the regulation of EPS-1 synthesis have also been described (Doherty *et al* , 1988) *Exo* genes are expressed during nodulation (Keller *et al* , 1988)

A regulatory mutation *exp*rl01, has the remarkable effect of derepressing the synthesis of a second, structurally different EPS termed EPS II (Glazebrook and Walker, 1989) The structures of *R meliloti* EPS I and EPS II are shown in Figure 12

The production of EPS-II requires at least seven genes, *expA*, *expB*, *expD*, *expE*, *expF*, *expG*, and the previously identified *exoB* locus (Long *et al* , 1988) EPS-II can substitute efficiently for EPS-I in nodulation of *Medicago sativa*, but it must be made at high levels On four other plant species nodulated by *R meliloti* 1021 mutants expressing only EPS-II, empty ineffective nodules were induced *Exo*$^-$ mutants of the broad host *Rhizobium* strain NGR234 have been shown to be Hac$^-$, Inf$^-$, Noi$^+$, but are Fix$^-$ on most tropical legumes (Chen *et al* , 1985) The ability of these mutants to form nitrogen fixing nodules could be restored to some degree by the addition of purified EPS or oligosaccharide from the parent strain, which suggests a positive role for EPS in infection (Djordjevic *et al* , 1987a)

EPS I



EPS II

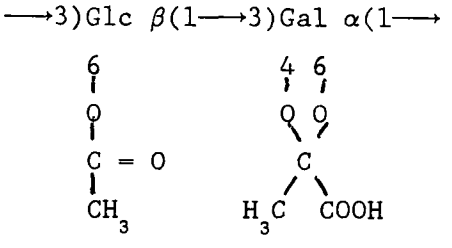


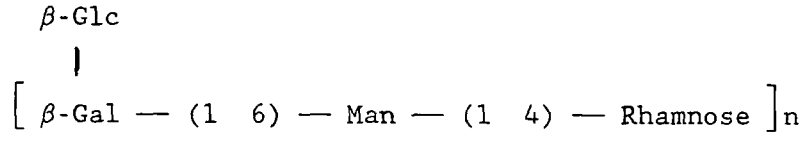
Figure 12 Structures of the repeating units of EPSI (Åman *et al* ,1981) and EPSII (Her *et al* ,1990)

However, *exo* mutants of *R meliloti* were unable to be rescued by addition of purified EPS (Leigh et al 1987) Five of the genes involved in the synthesis of the EPS from *Rhizobium* NRG234 have been identified (Chen *et al* , 1987)

(b) Lipopolysaccharides

The outer surface of the outer membrane of Gram-negative bacteria is covered by a lipopolysaccharide which is chemically extremely complex (Rietschel *et al* , 1972) In enteric bacteria, the outermost layer consists of long projecting polysaccharide chains, with specific repeating units that have antigenic properties and are called O-antigens Specific antibodies can be raised against these structures and so varied are the structures that 1000 different serotypes of *Salmonella* are known These are classified into 17 principle groups

(a)



(b)

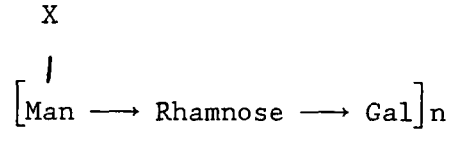


Figure 13 Repeating sequence from serotypes of *Salmonella* where n may be ≃ 50 Group E has the repeating sequence shown in (a) and groups A, B, and D contain the repeating sequence shown in (b), where X is an 3,6,-dideoxyhexose, paratose in type A, abequose in type B, and tyvelose in type D

At the inner end of the O-antigen is a shorter polysaccharide chain whose structure is less varied, however it contains two sugars only found in bacterial lipopolysaccharide, heptose and ketodeoxyoctonate (KDO) Figure 14 KDO is usually used as a biochemical marker for LPS

The arrangment of these sugars in the *Salmonella* LPS core is shown in Figure 15 The lipids typically contain D-glucosamine, fatty acids, phosphate and often ethanolamine in various proportions The core oligosaccharide bearing the O-antigen is attached to the lipid-A via two molecules of N-acetylglucosamine in a $\beta 1 \rightarrow 6$ linkage The disaccharides are not linked together in a conventional way but by pyrophosphate bridges Furthermore the free positions of the disaccharides are esterified with fatty acyl residues that penetrate the outer membrane and which serve to anchor the entire LPS molecule

Mutants of *Salmonella*, known as R forms, because they grow as rough forms on agar plates have been described (revieved by Hitchcock and Brown, 1983) These forms are usually non-pathogenic It is thought that the great diversity in LPS O-antigens amongst *Salmonella*, is a mechanism whereby this bacterium can bypass the host defence mechanism because the O-antigen is not recognised as dangerous

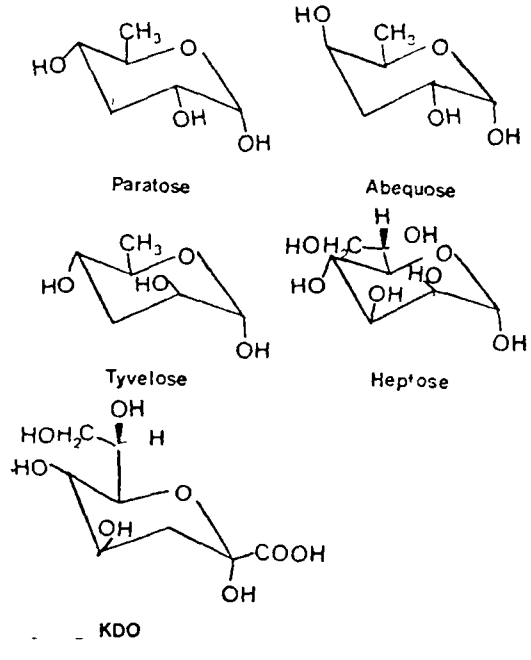


Figure 14 Some of the unusual sugars found in bacterial LPS

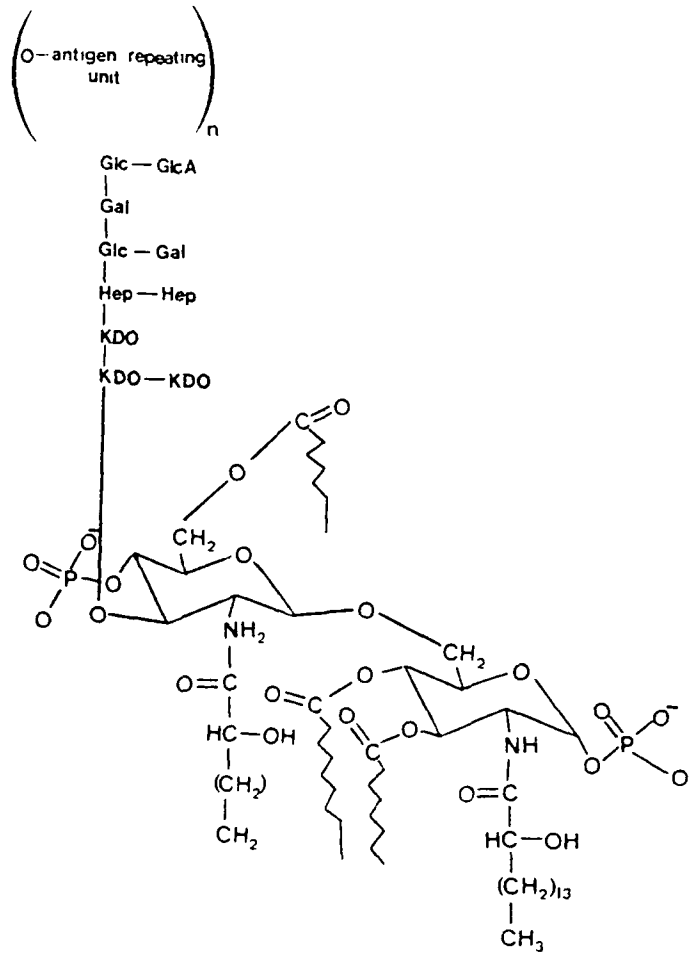


Figure 15 Chemical structure of the *Salmonella* core LPS region

LPS mutant chemotypes can be differentiated on the basis of their mobility on SDS-PAGE The wild-type strain gives rise to a ladder-like profile caused by heterogeneity in the length of the repeating O-antigen The mutant chemotype SR does not posses the ladder-like pattern as it lacks the O-antigen The other chemotypes can be distinguished on the basis of the mobility of the fastest migrating band on SDS-PAGE The more severe the defect in the LPS core region, the faster the rate of migration of the leading band, (Hitchcock and Brown,1983)

Biosynthesis of LPS

The biosynthesis of the heteropolysaccharide componants of LPS involves an isoprenyl carrier lipid and it closely resembles that of EPS LPS biosynthesis differs from that of EPS in that during the assembly of LPS monosaccharides are added at the non-reducing end of the growing chain, (Kanegasaki and Jann, 1979)

Pleiotrophic Effects of LPS Mutations

Kupla and Leive, (1972), and Wu, (1972), first described how LPS mutants gave rise to "light" outer membranes The term "light" refers to the fact that the the outer membranes from LPS mutants are of a lower density than those from the wild-type on sucrose density gradients The decrease in density is not only due to the loss of carbohydrate moieties but also to the loss of outer membranes The LPS defect or the loss of outer membranes does not affect drastically the survival of the bacteria, although they may have slightly slower growth rates (Ames *et al* , 1974) Rough mutants may lack flagella, differ in phage susceptibility, differ in intrinsic antibiotic resistance, differ in their ability to form mating pairs during bacterial conjugation, may have an increased sensitivity to lysozyme, dyes and detergents as well as large polycylic aromatic compounds, compared to the wild type (Monner *et al* , 1971)

LPS of *Rhizobium spp*

Initial compositional studies indicated that LPS from *Rhizobium spp* consists of the same components as those from enteric bacteria, (Carlson *et al* , 1978, Zevenhuizen *et al* , 1980)

LPS from *Rhizobium* species has been shown to have the same general characteristics as lipopolysaccharides from enteric bacteria, namely a core oligosaccharide attached to a lipid moiety and a higher molecular weight heterogenous region (Carlson, 1984) Other than these general characteristics *Rhizobium* LPSs appear to be quite different to those of enteric bacteria The PAGE results indicate that the larger molecular

form of these *Rhizobium* LPSs appears as a single band rather than as multiple bands observed with LPS extracted from enteric bacteria In addition this higher molecular weight form has a greater electrophoretic mobility than that of enteric bacteria which suggests that the complete form of the *Rhizobium* LPS has a short O-antigen compared to that of enteric bacteria In addition, the chemical complexity of the *Rhizobium* species O-antigen suggests that it is composed of a complex oligosaccharide and not of a repeating unit (Carlson, 1984)
Further chemical analysis revealed that LPS composition varied greatly between strains, (Carlson, 1984)

The core region of the LPS from strains of *R leguminosarum* all have molecular weights of 600-700 daltons and are largely composed of galacturonic acid with smaller amounts of mannose, galactose and KDO, (Noel *et al* , 1986, Carlson, 1984, Carlson *et al* , 1987a)
Two KDO containing core oligosaccharides have been isolated from the LPS of *R leguminosarum* biovar *trifolii* ANU843 The major core oligosaccharide has been demonstrated to be a trisaccharide consisting of two terminal galacturonic acid residues α-linked to the 4- and 7-positions of KDO, (Carlson *et al* , 1988) The second core region is a tetrasaccharide composed of galactose, galacturonic acid, mannose and KDO, (Hollingsworth *et al* , 1988) Both of these structures are shown in Figure 16

The Role of *Rhizobium spp* LPS in the Symbiosis
Because of the chemical diversity of LPS, initial studies on *Rhizobium* LPS were concerned with determining whether or not this molecule played a role in the recognition between the micro- and macro-symbiont Pre-treatment of clover and cowpea seedlings with low concentrations of LPS prior to inoculation with their respective bacterial hosts, resulted in an increase in infectivity and degree of nodulation, (Abe *et al* , 1984, Dazzo *et al* , 1984) Such a pre-treatment also inhibits the subsequent binding of the symbiont bacteria (Kato *et al* , 1980, Caeano-Anolles and Favelukes,1986),

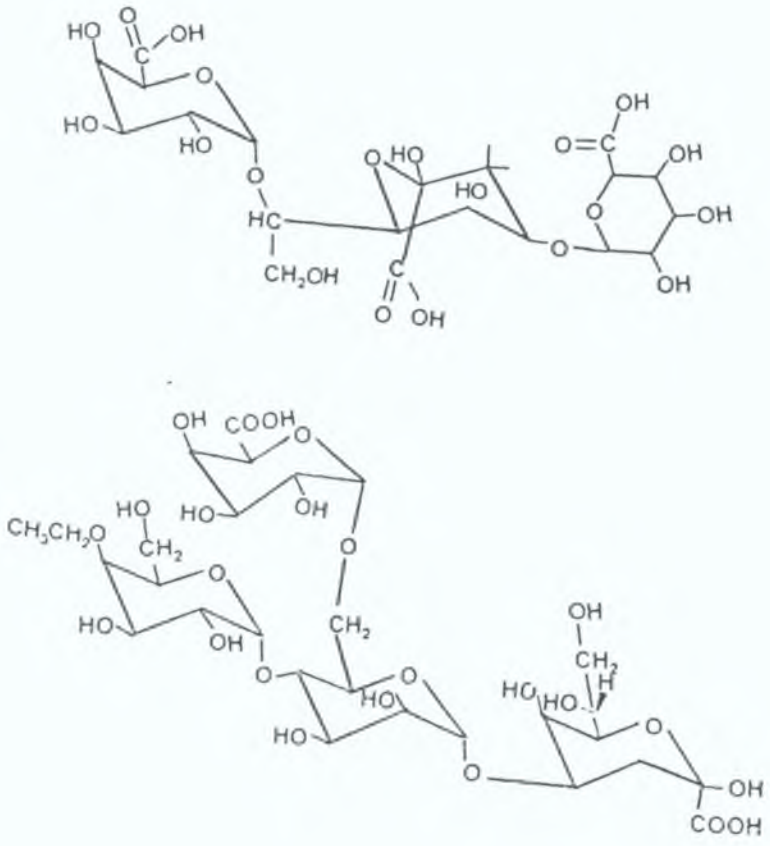


Figure 16. Structures of the two main oligosaccharides from the core region of *Rhizobium leguminosarum* biovar *trifolii* ANU843 (Carlson *et al.*, 1988, Hollingsworth *et al.*, 1989).

A physical association between the peribacteroid membrane and the bacterial cell surface could account for the concomitant division of the peribacteroid membranes with the intracellular bacteroids observed by Robertson and Lyttleton, (1984). The new membrane material for the enlarging peribacteroid membranes is supplied by membrane vesicles derived from the Golgi apparatus (Brewin *et al.*, 1986, Robertson *et al.*,1978 a,b). Variation in the strengths of such associations may

account for observed differences in the numbers of bacteroids contained within a single peribacteroid membrane envelope (Bradley *et al* , 1986) Bradley *et al* , (1986) suggested that a morphological analysis of nodules induced by LPS mutants of *Rhizobium* could help answer the question as to whether LPS plays a role in the interaction with the peribacteroid membrane LPS antigen is known to be present in the cell wall of the plant, perhaps as a result of fusion between the bacterial cell surface and the plasma membrane (Bradley *et al* , 1986) or it may have arrived there as a result of secretion through the bacteroid membrane after the endocytotic event has taken place

The biochemical and immunochemical composition of LPS have been shown to differ between the bacteroid and the free-living forms of the same strain of *R leguminosarum* biovar *viciae* (Bradley *et al* , 1988, Brewin *et al* , 1986) A monoclonal antibody, AFRC MAC 203, which recognises an epitope associated with the LPS expressed in the nodule but not in free-living cultures under standard growth conditions, (Bradley *et al* , 1988) Another monoclonal antibody, MAC 57, recognises a similiar LPS associated antigen which is constitutively expressed The nodule induced surface antigen associated with LPS was not expressed either in rhizobia in infection threads or in bacteria that were recently released into the host cell cytoplasm The antigen was expressed by membrane enclosed bacteroids, including forms that had not yet expressed nitrogenase, (VandenBosch *et al* , 1989) This antigen could be induced in free-living cultures by exposure of the cultures to low oxygen tensions, (Kannenberg and Brewin, 1989) This induction occured before the *nif*A-dependent activation of nitrogenase which suggests that the expression of MAC 203 antigen and nitrogenase are triggered by different physiological signals, (VandenBosch *et al* , 1989) Mutants which constitutively express the MAC 203 antigen have been isolated following Tn5 mutagenesis These mutants were symbiotically normal, but they lacked the LPS epitopes that were recognised by another monoclonal antibody MAC 281 This suggests that the corresponding epitopes may be interconverted or may share a common precursor, (Wood *et al* , 1989) These mutants did not map to genomic sites associated with *nif*A or to

any known genes on the symbiotic plasmid This suggests that an unknown oxygen regulatory control system, different from the oxygen sensitive clusters already described, may exist in *Rhizobium* (Wood *et al* ,1989)

Genetic analysis of LPS in *Rhizobium spp*

A direct genetic analysis of LPS from *R leguminosarum* biovar *phaseoli* has been undertaken, (Noel *et al* , 1986) Transposon mutagenesis yielded a nodule development minus (Ndv^-), mutant, which formed aborting infection threads that were restricted to the nodule cortex These mutants lacked the O-antigenic side chain, termed LPS-I, (Carlson *et al* , 1987b) All of these mutations were chromosomal and were clustered around two distinct regions of the chromosome, (Cava *et al* , 1989) Two LPS mutants of *R leguminosarum* biovar *trifolii* ANU 83 were isolated by Brink *et al* ,(1990) One of these mutations lacked not only the O-antigen but part of the core oligosaccharide as well Genetic regions from *R leguminosarum* biovar *phaseoli* were used to complement these mutants, one of which expressed the *R leguminosarum* biovar *phaseoli* O-antigen and the other which expressed the *R leguminosarum* biovar *trifolii* O-antigen Therefore the *R leguminosarum* biovar *phaseoli* genetic region specifies a function that is conserved between these *R leguminosarum* strains, whereas the other specifies a strain specific LPS structure A possible explanation for these observations is that the core region of *R leguminosarum* strains is conserved and may be complemented by DNA from other biovars, the O-antigen however, is strain specific and may only be complemented by DNA from that particular strain, (Brink *et al* ,(1990) Analagous results have been obtained on transfer of genetic regions coding for LPS core (*rfa)* or O-antigenic (*rfb)* between enteric bacteria, (Makela and Stocker, 1981)

Russa *et al* ,(1982), demonstrated a correlation between the occurence of an indigenous plasmid and alterations in LPS structure Similarly, quantative differences in LPS composition have been shown between nodulating and non nodulating strains of *Rhizobium leguminosarum* biovar

trifolii which only differ in a 40kb deletion on the pSym plasmid (Carlson *et al* , 1987a) Hynes and McGregor, (1990), have shown that an indigenous plasmid,(VF39c), of *R leguminosarum* biovar *viciae* does encode for genes involved in LPS assembly The cured VF39c strain could be complemented by clones from *R leguminosarum* biovar *phaseoli*, which suggests that this plasmid encodes for genes involved in the synthesis of the core region of the LPS molecule

DeMaagd *et al* , (1989), reported the isolation of Tn5 mutants defective in the production of the O-antigenic oligosaccharide of LPS and claimed that these mutants were defective in bacterial release, (Bar^-) These authors claimed to have obtained a correlation between the surface hydrophobicity of these mutants and bacterial release from the infection thread This author disputes the basis for this correlation, because some bacteria were actually released from the infection thread, this phenotype cannot be assigned as Bar^- Notwithstanding this, the data reported by deMaagd *et al* , (1989), shows that these mutants differ from those reported for *R leguminosarum* biovar *phaseoli*, (Noel *et al* , 1986) in that the infection thread does penetrate the central tissue of the nodule

Priefer,(1989), also isolated Tn5 mutants of *R leguminosarum* biovar *viciae* affected in LPS assembly These mutations were shown to be clustered on the chromosome and gave rise to plant cells that were, in fact, infected

Although the role of LPS in the symbiosis is not clear, mutational analysis has shown that normal LPS is essential for the formation of effective symbiosis, involving *R leguminosarum* This has been shown not to be the case for the *R meliloti* / alfalfa symbiosis, (Clover *et al* ,1989), as *R meliloti* mutants defective in LPS form Fix^+ nodules on alfalfa E Signer and G Walker have stated (quoted by Long, (1989b), that" it is important to keep in mind that the information carried in a surface polysaccharide is not necessarily exclusive to that one type of molecule Other complex surface componants may have equivalent information, mounted in a distinct molecular setting" Recently, Williams *et al* , (1990), reinforced this point by showing that the

R meliloti exo$^-$ mutations could be suppressed by a *lpsZ*$^+$ mutation, a gene involved in the biosynthesis of LPS in *R meliloti* The *lpsZ* mutation does not restore EPS production, but instead alters the composition and structure of the LPS molecule so that it may substitute for EPS in nodule development

(c) β-D-(1——→2)-Linked D-Glucans

β-D-(1——→2)-linked glucans (β-glucans), are cyclic oligosaccharides consisting of between 20 and 30 glucose residues These molecules are found in the periplasm and are sometimes secreted from the cells of bacteria which belong to the family *Rhizobiaciae*

The integration into the plant genome of a piece of *Agrobacterium tumefaciens* Ti plasmid results in crown gall tumours on dicotyledonous plants, (Zambryski *et al* , 1989) This process not only requires genes on the Ti plasmid but also genes on the chromosome called chromosomal virulence loci (*chv*) Both *chv*A and *chv*B are required for the attachment of the pathogen to the host plant's cells (Douglas *et al* , 1982) These avirulent *A tumefaciens* do not produce β-glucans, Homologues of the *chv* loci have been described in *R meliloti* Strains carrying mutations in these genes have the same phenotype as the Exo$^-$ mutants of *R meliloti* in that neither infection threads nor bacteroids are observed in the small white nodules elicited by these mutants, (Dylan *et al* , 1986) These genes have been assigned *ndv*A and *ndv*B genotypes after the defect in nodule development

The *ndv*B gene encodes an inner membrane protein of 319 kilodaltons that is involved in the synthesis of β-glucan, (Ielpi *et al* , 1990), while the *ndv*A gene is homologous to the *E coli* hemolysin export protein, HlyB, and is postulated to be involved in the export of β-glucan to the cell exterior, (Stanfield *et al* , 1988)

Recently Dylan *et al* , (1990a), reported that β-glucan was necessary in *R meliloti* for the ability to osmoadapt Second site pseudo-revertants were isolated which partially regained their ability to osmoadapt but did not regain their ability to synthetise β-glucan These revertants regained their symbiotic ability, which raises the possibility that

this oligosaccharide is not involved in nodule development, (Dylan *et al* , 1990b)

When exogenous β-glucan was added to alfalfa seedlings along with wild type *R meliloti*, an enhancement of the kinetics of nodulation as well as a doubling of the number of nodules eventually produced was observed, which supports the theory that β-glucan is involved in nodule development, (Dylan *et al* , 1990b) These results are in agreement with those reported by Abe *et al* , (1984) for the *R leguminosarum* biovar *trifolii* / clover symbiosis The β-glucan produced by *R meliloti* are neutral, however, acidic cyclic β-D-(1$\longrightarrow$2)-glucans containing methylmalonic acid or pyruvate or both, have been isolated from *Agrobacterium radiobacter, R leguminosarum* biovar *phaseoli* and *R leguminosarum* biovar *trifolii*, (Hisamatsu *et al* , 1987), but their involvement in the symbiosis has not been examined by mutational analysis

MATERIALS AND METHODS

Table 2 Bacterial Strains

Strain	Phenotype/Genotype	Source or Refererence
R leguminosarum biovar *viciae*		
VF39	Nod^+Fix^+ Isolated from *V faba* nodules	University of Bielefeld
$VF39Sm^r$	Spontaneous Sm^r derivative of VF39	This study
$VF39Sm^r$ derivatives		
JB45	Noi^+Fix^- (Tn5-Mob)	This study
JB45c1	Nod^+Fix^+ JB45 carrying an RP_4-pSUP205 cosmid cointegrate	"
JB45c2	Nod^+Fix^+ JB45 merodiploid with a pSUP205 cosmid integrated in the chromosome	"
JB57	Nod^+Fix^+ (Tn5-Mob) underproducer of EPS	"
JB10	Nod^+Fix^+ (Tn5-Mob) overproducer of EPS	"
JB18	Nod^+Fix^+ (Tn5-Mob) overproducer of EPS	"
JB14	Nod^+Fix^+(Tn5-Mob) *Cys*	
JB20	Nod^+Fix^+ (Tn5-Mob) *trp*	"
JB33	Nod^+Fix^+ (Tn5-Mob) *phe*	"
JB37	Nod^+Fix^+ (Tn5-Mob) *pro*	"
JB38	Nod^+Fix^+ (Tn5-Mob) *his*	"
VF39-51	Noi^+Fix^-(LPS-I^-)(Tn5)	U Priefer

Table 2 (continued)

Strain	Phenotype/Genotype	Source or Reference
R leguminosarum biovar *viciae*		
336E	Ery^r Nal^r	M Hynes
336E-26	336E (pSym Tn5-Mob)	M Hynes
151(VF39c)	carries only the VF39c plasmid from VF39	U Priefer
3855	128C53 Sm^r contains pRL6J1	Brewin *et al* , (1982)
biovar *phaseoli*		
8002	wild-type strain Nod^+Fix^+ on *Phaseolis vulgaris*	Johnston *et al* ,(1978)
Rhizobium meliloti		
2011	wild-type strain Nod^+Fix^+ on *Medicago sativa*	Rosenberg *et al* , (1981)
Escherichia coli		
CSH56	F^- *ara* Δ*lac pro sup*D NalA *thi*	Miller, (1972)
S17-1	Rec^- derivative of 294 with RP_4-2Tc Mu Km Tn7 in the chromosome *hsd*R *pro* Tp-Sm res^- mod^+	Simon, (1984)
JA221	F′ *rec*A1 *leu*B6 *trp*Δ5 *hsd*MT $hsdR^-$ *lac*Y *xyl*	Clark, (1981)
CC118	*araD* 139 Δ *(ara,leu)* 7697 Δ*lac* X 74 *phoA* Δ20 *galE galK thi rpsE rpoE* $argE_{am}$ recA1	J Beckwith
LE392	*supF supE hsdR galK trpR metB lacY tonA* host for λTn*phoA*-1	J Beckwith

Table 3. Plasmids and Bacteriophages

Plasmid	Relevent characteristic	Source or Reference
pME206	RP_4 derivative, deletion in Tc gene. Ap Km.	Priefer, (1990)
RP_{4-4}	Km^s derivative of RP_4 Tc Ap.	R.Simon
pSUP5011	pBR325 derivative,Tn5-Mob Cm Tc Km.	Simon, (1984)
pSUP205	pBR325 derivative Mob,cos, Cm,Tc.	Simon *et al.*,(1983)
pSUP202	Ap^r Tc^r Cm^r Mob.	Simon, *et al.*, (1983)
pUC19	Ap^r *lacZ*.	Norrander *et al.*,(1983)
pBR322	Ap^r Tc^r.	Bolivar *et al.*,(1977)
Recombinant plasmids		
pCos4	38-kilobase DNA of *R. leguminosarum* cloned in pSUP205.	U.Priefer
pJB-452	7-kilobase DNA containing the kanamycin resistance determinant from Tn5-Mob and part of the gene into which Tn5-Mob had inserted into in JB45 cloned into pUC19.	This study
pDJB201	pSUP202::Tn*phoA* Ap^r Cm^r Tc^r.	"
pDJB202	pSUP202::Tn*phoA* Ap^r Cm^r Tc^r.	"
pDJB203	pSUP202::Tn*phoA* Cm^r Tc^r.	"
pDJB204	pSUP202::Tn*phoA* Ap^r Cm^r.	"
Bacteriophages		
λc*Its*	Lambda mutant,*cI*857.	Miller, (1972)
λTn*phoA*-1	*b*221 *cI*857 $P_{am}3$ with Tn*phoA* in or near *rex*.	J.Beckwith

Bacterial Strains and plasmids

The bacterial strains and plasmids bacteriophages used in this study are listed in Table 2 and Table 3, respectively

Bacterial Growth Media

Media on which bacteria were grown was sterilized by autoclaving at 121°C for 15 min Complex media were solidified with 1 5% Oxoid No 3 agar and minimal media were solidified with 1 5% Bacto (Difco) agar

Complex Media for Growth of *Rhizobium spp*

For routine culturing of *Rhizobium spp* , Tryptone/Yeast extract medium (Beringer, 1974) was used

TY medium

Tryptone	5g/l
Yeast extract	3g/l
$CaCl_2,6H_2O$	1 3g/l

For long term storage of *Rhizobium spp* Yeast-mannitol (Nuti *et al* ,1977) was employed

YM medium

K_2HPO_4	0 5g/l
$MgSO_4,7H_2O$	0 2g/l
NaCl	0 1g/l
Yeast extract	0 4g/l
Mannitol	10g/l
pH = 6 9	

For selective growth of *R meliloti* over *R leguminosarum* strains, RGMC medium (Simon, 1984) was used

RGMC

Tryptone	4g/l
Yeast extract	0 4g/l
NaCl	3 2g/l
Glucose	0 4g/l
$CaCl_2$	0 12g/l
$MgCl_2$	0.4g/l

Minimal Medium for the Growth of *Rhizobium spp*

Vincent's Minimal Medium (VMM) was used to culture *Rhizobium spp* It was also used to characterize *Rhizobium* auxotrophs, as well as being a convenient medium upon which to visually screen for mutants of *R leguminosarum* biovar *viciae*, altered in surface polysaccharides

VMM Soln 1

K_2HPO_4	1 0g/l
KH_2PO_4	1 0g/l
KNO_3	0 6g/l
Mannitol	10g/l

Soln 2

$CaCl_2$	0 1g/100ml
$MgSO_4,7H_2O$	0 25g/100ml
$FeCl_2$	0 01g/100ml

Soln 3

Biotin	10mg/10ml
Pantothenic acid	10mg/10ml
Thiamine	10mg/10ml

Solutions 1 and 2 were autoclaved separately Solution 3 was dissolved in 10ml of water and filtered through a 0 45μm filter All three solutions were combined to form VMM

Vincent's Glutamate-mannitol medium (VGM)

VGM medium was used for the production of EPS from *Rhizobium spp* This medium was identical to VMM except that 1g glutamate was used instead of potassium nitrate as a nitrogen source

Bishop's medium

In order to induce the production of capsules by *R leguminosarum* biovar *viciae* cells were grown on Bishop's solid medium (Bishop, 1976)

Mother soln

K_2HPO_4	0 23g/l
$MgSO_4,7H_2O$	0 10g/l
Sodium glutamate	1 10g/l
Mannitol	10g/l

Microelements soln	$CaCl_2$	0 5g/l
	H_3BO_3	14 5mg/l
	$FeSO_4, 7H_2O$	12 5mg/l
	$CoSO_4, 7H_2O$	7 0mg/l
	$CuSO_4, 7H_2O$	0 5mg/l
	$MnCl_2, 4H_2O$	0 43mg/l
	$ZnSO_4, 7H_2O$	10 8mg/l
	$NaMoO_4$	12 4mg/l
	pH = 5 0	

Vitamin soln	Riboflavin	2mg
	Para amino benzoic acid	2mg
	Nicotinic acid	2mg
	Thiamine	2mg
	Pyridoxamine	2mg
	Panthothenic acid	2mg
	Inositol	2mg
	Na phosphate buffer pH = 7	100ml

The mother solution and the microelement solution were autoclaved separately The vitamin solution was filter sterilized Complete Bishop's medium was constructed by adding 1ml of each of the microelements and vitamin solutions to 1 liter of mother solution

Complex Medium for the Growth of *E coli* Strains

Luria Bertoni (LB) medium was routinely used to culture *E coli* strains

LB·	Tryptone	10g/l
	Yeast extract	5g/l
	NaCl	10g/l

Minimal Medium for the Growth of *E coli* strains

M9	Na_2HPO_4	6g/l
	KH_2PO_4	3g/l
	NaCl	0 5g/l
	NH_4Cl	1g/l
	pH = 7 4	

1M $MgSO_4$	2ml/l
20% Glucose	10ml/l
1M $MgCl_2$	0 1ml/l

The above solutions were sterilized separately by filtration (glucose) or by autoclaving Vitamins and amino acids were sterilized by filtration and were used at concentrations of 10μg/ml or 30μg/ml respectively

Growth Conditions

Rhizobium strains were grown in broth culture at 30°C on a rotary shaker and plates were incubated at 30°C in an inverted position

E coli strains were grown in a shaking water bath at 37°C and plates were incubated in an inverted position at 37°C

Plant Growth Medium

Seedlings were grown in Jensen's N-free medium

Jensen Medium (Jensen, 1942)

Solution 1

$MgSO_4$	0 2g/l
K_2HPO_4	0 2g/l
$CaHPO_4$	1 0g/l
NaCL	0 2g/l
$FeCl_3$	0 1g/l

Solution 2

$CuSO_4,2H_2O$	0 35g/l
$MnSO_4,4H_2O$	6 1g/l
$ZnSO_4,7H_2O$	0 97g/l
H_3PO_3	12 7g/l
$NaMoO_4,2H_2O$	3 98g/l

Solution 1 was thoroughly mixed and undissolved material was allowed to sediment overnight The supernatant was decanted and 12g of Bacto agar

was added per liter, prior to autoclaving Solution 2 was autoclaved separately and 2 5ml of this solution was added to 1 liter of solution 1

Fåhraeus Medium (Fåhraeus, 1957)

$CaCl_2,H_2O$	0 1g/l
$MgSO_4$	0 12g/l
KH_2PO_4	0 1g/l
Na_2HPO_4	0 15g/l
Fe citrate	0 005g/l
(A-Z solution)	trace

A-Z solution consisted of trace quantities of $CuSO_4$, $MnSO_4$, ZnO_4, H_3BO_3, and $NaMoO_4$

Buffers and Solutions

TE Buffer

Tris-Hcl	10mM
Na_2-EDTA	1mM
pH = 8 0	

TES Buffer

Tris-HCl	10mM
Na_2-EDTA	1mM
NaCl	50mM
pH = 8 0	

STET Buffer

Sucrose	8%
Triton-X-100	5%
EDTA	50mM
Tris-HCl	50mM

Tris-Borate Buffer

Tris-base	90mM
H_3BO_3	90mM
Na_2EDTA	2 5mM
pH = 8 3	

Denaturing Solution

NaCl	1 5M
NaOH	0 5M

Neutralization buffer

Tris-HCl	1M
NaCl	1 5M
pH = 7 0	

20 x SSC

NaCl	3M
Na-citrate	0 3M

20 x SSPE

Na_2-EDTA	20mM
NaOH	0 1M
NaCl	3 5M
NaH_2PO_4	0 2M
pH = 7 0	

50 x Denhardt's Solution

BSA	1%(w/v)
Ficoll400	1%(w/v)
Polvinylpyrrolidone	1%(w/v)

DNase Dilution Buffer

Tris-HCl	50mM
$MgSO_4$	10mM
Mercaptoethanol	1mM
BSA	50μg/ml
pH = 7 5	

10 x Nick Translation Buffer

Tris-HCl	500mM
$MgCl_2$	50mM
Mercaptoethanol	100mM
BSA	100μg/ml
pH = 7 8	

Prehybridization Fluid for Hybridization to Southern Filters:

6 x SSC

0.5% SDS

5 x Denhardt's solution

100μg denatured salmon sperm DNA

Hybridization Fluid for Hybridization to Southern Filters:

6 x SSC

0.5% SDS

5 x Denhardt's solution

100μg denatured salmon sperm DNA

0.01M EDTA

^{32}P-labeled denatured DNA

Prewashing Solution for Colony Blotting:

Tris-HCl (pH=8)	50mM
NaCl	1M
Na_2-EDTA	1M
SDS	0.1% (w/v)

Prehybridization Solution for Colony Blots:

50% Formamide

5 x Denhardt's solution

5 x SSPE

0.1% SDS

Denatured salmon sperm DNA (100μg/ml).

Genetic Transfer

M1.

Conjugation:

For all matings cells were grown to late log phase in LB medium in the case of *E.coli* and in TY medium in the case of *Rhizobium* strains. These media were supplemented with the appropriate antibiotic when required. Donor and recipient (0.7ml of each) were mixed in a microfuge tube, centrifuged and resuspended in 100μl of fresh TY broth. The suspension was placed on a 0.45μm membrane filter on a TY agar plate and incubated overnight at 30°C (Simon, 1984). The filters were removed

aseptically to 4 ml of sterile water and the cells were resuspended by vortexing for 1 min For *E coli* x *Rhizobium* crosses, the *E coli* donors were counter selected by use of chromosomally encoded drug resistance markers in the recipient strains For *R leguminosarum* x *R meliloti* matings the *R leguminosarum* donors were counter selected by use of RGMC medium, a medium which fails to support the growth of *R leguminosarum* strains

For analyses, cells from matings were diluted on medium with or without antibiotic as required The frequency of the selected markers was determined by dividing the titre of cells from the selective plates by the titre of cells from the non-selective plates If the frequency of the selected markers in a mating was significantly higher than the frequency observed spontaneously in control experiments, the frequency of the selected markers was considered to be the frequency of plasmid transfer per recipient cell

M2

Transformation

Strains which were transformed routinely were *E coli* JA221, *E coli* DH83 and *E coli* S17-1 Competent cells were prepared by the method of Dayert and Ehrlich (1979)

An *E coli* culture grown overnight was diluted 1 in 100 and grown to an A_{600} of 0 3 The culture was chilled on ice for 30 min before sedimenting the cells at 4000 x g at 4°C for 4 min The pellet was resuspended in half its original volume in ice cold 50mM $MgCl_2$ Cells were again recovered by centrifugation and resuspended in half the original volume of ice cold 50mM $CaCl_2$ After a one hour incubation on ice the suspension was again centrifuged and the pellet was resuspended in one fiftieth of the original volume in ice cold 50mM $CaCl_2$ The cells were now considered competent and would remain so for up to 24 hours

Uptake of plasmid DNA was accomplished by addition of 20µl of DNA dissolved in TE buffer to 200µl of cold competent cells and allowing the transformation mix to stand on ice for one hour prior to a two min heat shock at 42°C. An outgrowth for one hour at 37°C allowed

expression of plasmid encoded drug resistance before plating on selective medium

M3.

Isolation of Bulk Small Plasmid DNA

The cleared lysate procedure (Cannon *et al* , 1974) followed by CsCl-EtBr density gradient centrifugation was used to isolate large amounts of plasmid DNA

A 10ml culture grown under antibiotic selective pressure was used to inoculate 200ml of LB broth and growth was allowed to continue to stationary phase Cells were harvested by centrifugation at 5000 x g at 4°C for 10 min The pellet was resuspended in 1 5ml of 25% sucrose in 0 2M Tris (pH 8) and the suspension was transferred to a 50ml polypropylene centrifuge tube After 5 min incubation on ice, 0 4ml of a freshly prepared lysozyme solution (20mg/ml) was added and the mixture was incubated for a further 5 mins on ice 0 8ml of ice cold 0 25M EDTA (pH 8) was added and mixed gently After 10 min , 3 2ml of lytic mix (2% v/v Triton X-100 in 0 05M Tris/0 05 EDTA, pH 8) was added and mixed gently After 20 mins incubation on ice, cell lysis could be detected by the increase in viscosity of the solution The membrane/chromosome components of the cell were then separated from the lysate by a clearing spin at 26000 x g for 40 min

Caesium chloride (6 9g) was added to the cleared lysate and this solution was transferred to a quick-seal polyallomer ultracentrifuge tube Ethidium bromide (180μl of a 10mg/ml aqueous solution) was added and the weight of the solution was brought to 14 1g by the addition of 10mM EDTA All air was removed from the tube by the addition of liquid paraffin and the tube was heat sealed with a Beckman heat sealer

The density gradient was formed by centrifugation at 150000 x g at 20°C using a 70Ti Beckman fixed angle rotor in a Beckman ultracentrifuge Model LH-M Separation of covalently closed circular plasmid DNA from the open circular or linear chromosomal DNA was achieved after 20 hours centrifugation The DNA was visualised by use of a Desega ultra-violet lamp, Model UVIS, and the plasmid DNA was recovered by puncturing the side of the centrifuge tube with a syringe Ethidium bromide was

removed by extracting several times with an equal volume of isopropanol saturated 20 x SSC CsCl was removed by dialysis against TE buffer (4 x 1 litre changes at 4°C)

Rapid Plasmid Preparation

M4

Alkaline Extraction Procedure (Ish-Horowicz and Burke 1981)

An overnight bacterial culture (1 5ml) which had been grown under selective pressure was transferred to a microcentrifuge tube and the cells were recovered by centrifugation for 2 min The supernatant was discarded and the pellet was resuspended in 100μl of 50mM glucose, 25mM Tris, pH 8, 10mM EDTA The tube was placed on ice for 5 min , after which time 200μl of a 0 2M NaOH, 1% SDS solution was added with gentle mixing After 10 min incubation on ice lysis was evident by the increase in viscosity One hundred and fifty microliters of pre-cooled 5M K-acetate pH 4 5 was added and the tube was placed on ice for a further 10 min The precipitate was removed by centrifugation for 1 min Two volumes of ethanol were added to the supernatant and the suspension was held at room temperature for 2 min DNA was recovered by centrifugation and the pellet was washed twice with 70% ethanol The pellet was dried under vacuum and resuspended in 50μl of TE buffer

M5

Rapid Boiling Method. (Holmes and Quigley, 1981)

A patch of growth from an overnight culture of *E coli* was scooped from a selective antibiotic plate and suspended in 300μl of STET buffer in a microcentrifuge tube Freshly prepared lysozyme (20μl of 10mg/ml in TE) was added and the tube was incubated at room temperature for 10 min The tube was placed in boiling water for 1 min and then centrifuged for 10 min The supernatant was removed and the DNA was precipitated by the addition of an equal volume of isopropanol The suspension was placed at -20°C for 20 min and then centrifuged for 5 min The isopropanol was removed and the pellet was washed with 70% ethanol The pellet was dried under vacuum and the DNA dissolved in 50μl of TE buffer

M6.

Characterization of Plasmids

Plasmids were characterized by the "in gel" lysis method developed by Eckhardt(1978) and modified by Hynes *et al* (1986) A single colony from an overnight culture of *E coli*, grown on selective medium, was emulsified in 20μl of Eckhardt solution containing lysozyme (1mg/ml) This suspension was immediately loaded onto a 0 8% agarose gel which contained 1% SDS in a horizontal gel appratus The gel was run at 10V for 30 min initially, after which time the turbid suspension in the wells had cleared due to cell lysis The voltage was increased to 100V until such time as the tracker dye (which was loaded in an unused well) reached the base of the gel

Rhizobium cultures were grown to an A_{600} of 0 3 and 400μl of culture was centrifuged for 2 min in a microcentrifuge The pellet was resuspended in 100μl of a 0 03% sarcosyl (SERVA) solution Cells were again centrifuged and all of the supernatant was removed The pellet was resuspended in 20μl of Eckhardt solution containing 1mg/ml lysozyme and this suspension was loaded on an 0 8% agarose gel containing 1% SDS

The gel was run as above After electrophoresis, the gel was placed in a bath of ethidium bromide (5μg/ml) for 30 min The DNA was visualised over a U V transilluminator (U V P Inc , San Gabrielle, California, U S A) Gels were photographed using a Polaroid M P 4 Land camera equipped with a red filter (25A KODAK) loaded with Polaroid 667 positive film

M7.

Isolation of Total DNA from *Rhizobium* Strains (Priefer *et al* , 1984)

Early stationary phase cultures of *Rhizobium spp* (10ml) were sedimented in a bench top centrifuge (Heraeus Christ labofuge 6000) at 5000 x g for 5 min The cells were washed with 10ml of TES buffer and resuspended in 5ml of TE buffer Lysozyme (0 5ml of a 2mg/ml solution in TE) was added and the suspension was incubated at 30^{o}C for 30 min A sarkosyl/pronase solution (0 5ml of 10% sarkosyl in TE buffer

containing 5mg/ml pronase) was added and the suspension was incubated at 37°C for 1 hour, after which time lysis was evident Sodium acetate (0 7ml of 3M Na-acetate pH=5 2) was added and the lysate was extracted with 2 5ml of Kirby mix (a phenol/chloroform mix in equal volumes) for 15 min The phases were separated by centrifugation at 2000 x g for 2 min The aqueous phase was removed to a corex tube and the suspension was centrifuged at 20000 x g for 15 min The supernatant was removed and extracted again with Kirby mix, followed by a chloroform extraction The DNA was precipitated by addition of an equal volume of isopropanol The total DNA was recovered by centrifugation at 5000 x g for 5 min and washed twice with 70% ethanol The pellet was dried under vacuum and resuspended in 200μl of TE buffer

M8

DNA Manipulation

Restriction enzymes and DNA modifying enzymes were obtained from BRL and were used according to the manufacturers instructions

M9

Transfer of DNA to Nitrocellulose Filters by Southern Transfer

Restricted total DNA was electrophoresed in a 0 8% agarose gel overnight, stained and photographed before blotting The gel was placed in denaturing solution and placed on a shaking table for 1 hour at room temperature The denaturing solution was poured off and replaced with neutralizing solution until the surface of the gel was below pH 8 5 (as indicated by litmus paper) The gel was transferred to a sheet of Whatman 3MM filter paper which had been soaked in 20 x SSC and which was connected to a reservoir of 20 x SSC via two wicks Nitrocellulose (Schliecher & Schull), cut to the exact size of the gel and soaked in 2 x SSC, was placed on top of the gel Two sheets of Whatman 3MM filter paper which were also soaked in 2 x SSC separated the nitrocellulose from the paper towels which served to instigate the transfer Transfer

was allowed to continue for 20 hours, after which time the nitrocellulose filter was removed and air dried and baked for 2 hours at 80°C

M10

Preparation of Radioactive Probes

Nick translation (Rigby *et al* , 1977)

The nick translation reaction consisted of

10x Nick Translation buffer	4μl
DNA probe (0 1μg/μl)	10μl
dGTP (10mM)	1μl
dCTP (10mM)	1μl
dTTP (10mM)	1μl
(α-^{32}P)dATP (800Ci/mM)	1μl
DNAse (0 1μg/ml)	0 5μl
Polymerase 1	5 units

The reaction was allowed to proceed at 16°C for 1 hour Labelled DNA was separated from unincorporated nucleotides by gel filtration through a Sephadex G-50 column (Maniatis *et al* ,1982)

M11

Random Primer Labelling (Feinberg and Vogelstein, 1983)

The random priming reaction consisted of

5x Random primer buffer	5μl
Mixture of unlablled dNTPs	20μM each
Denatured DNA linear template	1-25ng
Acetylated BSA (1mg/ml)	2μl
[α-^{32}P]dATP (3000Ci/mmole)	5μl
Klenow enzyme	5units

Sterile water was added to give a final volume of 50μl The reaction was allowed to proceed at room temperature for 1 hour, and then terminated by heating at 100°C for 2 min and subsequently chilling in an ice bath EDTA was added to a final concentration of 20mM and this mixture was used directly in a hybridization reaction

M12

DNA/DNA Hybridization

The filter onto which the DNA was transferred was soaked in 6 x SSC for 2 min It was then transferred to a heat-sealable bag containing pre-hybridization fluid (0 2ml per cm^2 of nitrocellulose filter) and incubated at 68°C After 4 hours the pre-hybridization fluid was replaced with the same volume of hybridization fluid The DNA probe was denatured by boiling for 2 min and was added to the hybridization mix Hybridization was continued for 16 hours at 68°C After this time the filter was removed from the bag and submerged in 2 x SSC containing 0 5% SDS for 5 min at room temperature The filter was then washed in 2 x SSC containing 0 1% SDS for 15 min After this time the filter was placed in 0 1 x SSC which contained 0 5% SDS and incubated at 68°C for 2 hours The buffer was changed and incubation was continued at 68°C for a further 30 min The filter was then removed and air dried, before being placed in clingfilm and inserted into a cassette equipped with phosphotungstate intensifying screens along with Kodak X-ray film After exposure, the film was developed and fixed using Kodak DX-80 developer and Kodak FX-40 X-ray liquid fixer

M13

Isolation of DNA Fragments from Agarose Gels

Gene clean procedure

The DNA fragment of interest was excised from an agarose gel and weighed Two to three volumes of a sodium iodide stock solution was added to the agarose in an microfuge tube and the slurry was heated to between 45°C and 55°C The NaI solution was prepared from a saturated solution (6M NaI), which was filtered through Whatman number 1 filter paper, this solution was brought to 0 12M Na_2SO_3 by the addition of solid sodium sulphite Silica 325 mesh glass beads (2μl), which were obtained from Stratech Scientific Ltd , were added to the slurry and the mixture was incubated at room temperature for 5 min The glass beads were sedimented in a microfuge for 5 seconds The glass pellet was washed three times with ice cold "new" wash (50% ethanol, 10mM TRIS pH 7 5, 0 1M NaCl, 1mM EDTA) The DNA was recovered from the beads by

gently resuspending the pellet in 20µl of TE buffer and heating the suspension at 45-55°C for 5 min The glass beads were removed by centrifugation in a microfuge for 30 seconds The supernatant, which contained the DNA was stored at 4°C

M14

In Situ Hybridization of Bacterial Colonies

Binding of DNA fragments liberated from colonies to nitrocellulose

Nitrocellulose filters were placed on petri dishes containing selective medium The clones to be screened were replica plated onto the filters and were grown at 37°C until the colonies had reached 2mm in diameter The filters were removed from the plates and placed colony side up on Whatman 3MM paper soaked in the following solutions 10% SDS (5 mins), denaturing solution (5 mins), neutralizing solution (5 mins) and 2 x SSPE (30 mins) The filter was air dried and then baked at 80°C for 2 hours

M15

Hybridization to Nitrocellulose Filters Containing Replicas of Bacterial Colonies

The baked filters were floated on a solution of 6 x SSC and then submerged for 5 min at room temperature The filters were transferred to a glass petri dish For 10 circular, 82mm-diameter filters, 33ml of pre-washing solution was added and incubated at 42°C The pre-washing solution was discarded and was replaced with 22ml of colony blot pre-hybridization solution After incubation at 42°C for 4-6 hours with agitation, the denatured ^{32}P-labelled probe was added Hybridization was allowed to continue for 12 hours at 42°C The hybridization solution was discarded and the filters were washed 3-4 times for 5-10 min in 500ml of 2 x SSC and 0 1% SDS at room temperature The filters

were then washed in 100ml of 1 x SSC and 0 1% SDS at 68°C for 1 hour The filters were air dried and placed in saran wrap, the filters were exposed to X-ray film as described for Southern transfer filters

M16

Ethyl Methanesulphonate (EMS) Mutagenesis

Rhizobium leguminosarum biovar *viciae* VF39Sm^r was grown in TY broth to late stationary phase The culture was transferred to a water bath at 37°C for 15 min This treatment was found to enhance the mutagenic effect of EMS, possibly by inactivating a DNA repair mechanism The cells were harvested by centrifugation and the pellet was washed twice with TS buffer (10mM Tris-HCl pH 7 6, containing 0 85% NaCl) The pellet was resuspended in half the original culture volume of TS buffer One fiftieth the volume of ethyl ethanesulphonate was added, and this cell suspension was incubated with agitation for 1 5 hours at 30°C An equal volume of 20mM sodium thiosulphate was added to this suspension and the cells were recovered by centrifugation The pellet was resuspended in the original volume with fresh TY broth, and an out growth at 30°C was allowed for 4 hours

The percentage survival was determined by dividing the titre of viable cells recovered after EMS mutagenesis by the number of cells present prior to mutagenesis

M17

Derepression and Release of Alkaline Phosphatase Activity in *R leguminosarum* biovar *viciae*

This procedure was a modification of the procedure described by Glenn and Dilworth (1979)

R leguminosarum biovar *viciae* strains were grown in VGM medium until the cultures had reached mid-exponential phase of growth (A_{600} 0 4) The cells were harvested by centrifugation at 5000 x g for 5 min and washed twice with sterile saline The pellet was resuspended in the original culture volume with derepression medium the medium was identical to VGM medium except that all phosphates were replaced with

5g/l PIPES, pH 6 8 The cultures were reincubated at 30°C with agitation Hourly samples (5ml) were centrifuged at 4000 a x g The supernatant was discarded and the cells were resuspended in 0 5ml of spheroplasting buffer, (20% sucrose, 30mM Tris-HCl, pH 8 0, 1mM EDTA, 500μg/ml lysozyme), and incubated at 30°C for 15 min The suspension was cleared by centrifugation and the supernatant was assayed for alkaline phosphatase activity

Alkaline phosphatase was assayed by measuring the rate of increase in A_{420} in a 1ml test solution containing 0 1M Tris-HCl, pH = 8 6, 0 4M $MgCl_2$, 0 4mg of *p*-nitrophenol phosphate per ml and 100μl of enzyme solution

M18

Hot Phenol-Water Extraction of Lipopolysaccharides

(Wesphal and Jann, 1965)

R leguminosarum biovar *viciae* strains were grown to stationary phase in TY broth The cells were harvested by centrifugation at 10000 x g for 15 min in a Beckman Model J2-15 centrifuge and washed twice with phosphate buffered saline (PBS) Bacteria in the final sediment were lyophilized and stored at -20°C

Freeze-dried cells having a dry weight of 5g were resuspended in 70ml water at 65-68°C Hot phenol (65-68°C) was added to the bacterial suspension and the mixture was stirred vigorously for 15 min at 65°C The emulsion was cooled to 10°C on ice and then centrifuged at 10000 x g for 10 min The upper aqueous phase was saved and the phenol was re-heated to 65°C and back extracted with water at 65°C Following centrifugation, both water layers were combined and dialysed against distilled water for 3 days The dialysed fraction was concentrated to 5ml by rotary evaporation at 40°C and centrifuged at 3000 x g for 10 min to remove insoluble material Half the organic material in the supernatant was LPS The major contaminant was RNA The solution was therefore treated with RNAse at a final concentration of 0 1mg/ml for 1 hour prior to centrifugation at 100000 x g for 1 hour in a Beckman ultracentrifuge Model L8-M The pellet was thoroughly resuspended in saline and the centrifugation was repeated five times The sedimented

material was dissolved in distilled water and lyophilized

M19

Preparation of Antisera directed against LPS

In separate experiments, 50μg of isolated LPS was injected intraperitoneally into two balb/c female mice On day 9 after inoculation, one of the mice was killed by a sharp blow on the head and the blood was collected from the thoracic cavity The blood was allowed to clot for 1 hour at 4°C before being centrifuged in a microfuge for 1 min The supernatant, the serum, was taken off and diluted 1 5 with 0 5% BSA in phosphate buffered saline (PBS), aliquoted into 200μl lots and stored at -20°C This serum fraction was enriched in IgM antibodies and was designated the IgM fraction

A booster inoculation was administered on day 10 to the second mouse On day 25 the blood was collected and processed as above This fraction was enriched in IgG antibody and was designated the IgG fraction

M20

Immunodiffusion

Micro double diffusion tests were performed as described by Crowle (1973) The diffusion plates were prepared with 10mM sodium phosphate buffer (pH 7 0) containing 137mM NaCl The diffusion gels were allowed to develop for 48 hours at 4°C before being submerged overnight at room temperature in a solution of 10mM sodium phosphate buffer (pH 7 0) containing 0 5M NaCl The diffusion gels were then placed in agitated distilled water for 30 min and stained in a solution composed of water-isopropylalcohol-acetic acid (13 5 2), containing 0 05% Coomassie blue (Sigma) The gels were destained for 1 hour in a solution composed of water-isopropylalcohol-acetic acid (8 1 1)

M21

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide slab gel electrophoresis in the presence of SDS was

performed using the discontinuous system described by Laemmli (1970) Recipes for the preparation of the gels (10%, 13% and 15%) and the stacking gel (3%) are given in Table 4 The electrode buffer contained Tris (0 025M), glycine (0 192M) and SDS (0 1% w/v) pH 8 3 Samples were solublized by heating for 3 min at 100°C in solubilization buffer (0 1M dithiothreitol, 2% (w/v) SDS, 0 08M Tris-HCl pH 6 8, 10% (v/v) glycerol, 0 2% (w/v) bromophenol blue,), and the samples applied to the gel Electrophoresis was carried out at 35mA constant current, in the case of LPS samples and at 150V constant voltage in the case of protein samples Staining of proteins was for two hours with a 0 5% (w/v) solution of coomassie blue dissolved in acetic acid-methanol-water (1 10 8 v/v/v) The same solvent mixture was used for destaining

TABLE 4 Recipes For The Preparation of Polyacrylamide Slab Gels

Stock Solution	10%	13%	15%	3% Stacking gel
Acrylamide/Bisacrylamide*/ml	7 0	9 1	10 15	1 25
Tris-HCl(1 87M,pH 8 8)/ml	4 2	4 2	4 2	—
Tris-HCl(0 5M,pH 6 8)/ml	-	-	-	0 602
TEMED/ml	0 02	0 02	0 02	0 05
SDS (10% w/v)/ml	0 12	0 12	0 12	0 05
Ammonium persulphate (10%w/v)	0 10	0 10	0 10	0 04
Water/ml	9 4	7 25	6 2	3 75

* Acrylamide (30% w/v), methylene bisacrylamide (0 8% w/v) in water

M22

Silver Staining for the Detection of LPS in Polyacrylamide Gels

LPS in polyacrylamide gels was visualized by silver staining following periodic acid oxidation of the LPS (Tsai and Frasch, 1982)

The LPS in the gel was fixed overnight in a solution composed of 40% ethanol and 5% acetic acid The LPS was oxidized by replacing the fixing solution with 0 7% periodic acid in 40% ethanol and 5% acetic acid for 5 min The gel was washed three times with 500ml, per wash, of distilled water The water was discarded and 150ml of freshly prepared staining reagent was added and agitated vigorously for 10 min The staining reagent (150ml) was prepared by adding 2ml of ammonium hydroxide to 28ml of 0 1M sodium hydroxide Five millliliters of 20% silver nitrate (w/v) was added to the solution while it was being stirred vigorously Water (115ml) was added to make 150ml of staining reagent Three ten min washes were performed with 500ml water, each wash was performed before the addition of formaldehyde developer (200ml) which contained 50mg citric acid and 0 5ml of 37% formaldehyde per liter Development was terminated by the addition of 7% acetic acid Gels were photographed with a Fuji STX-2 camera using 32-ASA Panatomic-X black and white film over a tungsten light box

M23

Determination of 2-Keto-3-Deoxyoctanic Acid

The 2-keto-3-deoxyoctanoic acid (KDO) of LPS was determined by the method of Weisbach and Hurwitz (1958) as modified by Osborn (1963)
When oxidized with periodate KDO yields formyl pyruvic acid which reacts with thiobarbituric acid to yield a chromagen with an absorption maximum at 550nm Deoxy sugars other than 2-keto-3-deoxy sugar acids produce different products which form chromagens in this assay which absorb light maximally outside the 545-550nm spectral region
The lyophilized polysaccharide containing 0 1 to 0 25μmol of KDO was dissolved in 0 04M H_2SO_4 and was heated to 100°C for 20 min so as to release the KDO from the polymer The hydrolysate was adjusted to 0 2ml with 0 04M H_2SO_4 and 0 25ml of 0 025M HIO_4 (in 0 25MH_2SO_4) was added and the reaction was allowed to stand at room temperature for 20 min Two millliliters of 0 2M sodium arsenate (dissolved in 0 5M HCl) was added and the reaction was incubated for a further 2 min at room temperature Two milliters of a 0 3% aqueous thiobarbituric acid solution were added, mixed and heated to 100°C for 20 min The reaction

was cooled and the absorbance was measured at 540nm The concentration of KDO in the samples was determined from a standard curve constructed using purified KDO (Sigma)

Isolation and Characterization of Extracellular Polysaccharides (EPS) from *Rhizobium spp*

M24

Preparation of crude EPS

Rhizobium spp were grown in 250ml of Vincent's-glutamate-mannitol medium for 6 days The cultures were centrifuged at 10000 x g for 10 min The supernatants were filtered firstly through Whatman GF/C filters and then through 0 45μm filters The supernatant was concentrated to 100ml by rotary evaporation at 40°C and dialysed for 48 hours against distilled water After dialysis, the solution was freeze dried, to yield a white powder of crude EPS

M25

Cetylpyridinium Chloride (CPC) Precipitation of Acidic EPS

Crude EPS was dissolved in 10mM Na_2SO_4 to give a final concentration of 2mg/ml Ten per cent v/vof 0 3% CPC in 10mM Na_2SO_4 was added dropwise This suspension was incubated at 37°C for 1 hour and the precipitate was collected by centrifugation at 4500 x g for 5 min The precipitate was washed 3 times with distilled water and redissolved in 10% NaCl so as to give a final concentration of 2mg/ml EPS Two volumes of acetone were added and the precipitate was collected by centrifugation This precipitate was redissolved in 10% NaCl (the same volume as before) and was dialysed against 1% NaCl (2L) The 1% NaCl was changed twice and the EPS was then dialysed against distilled water for 24 hours This preparation, after freeze-drying was pure EPS

Determination of Hexose

M26

Phenol Sulphuric Acid Assay (Dubois *et al* , 1956)

This assay depends on the dehydration of mono- and polysaccharides to furfural derivatives in the presence of H_2SO_4 These derivatives condenses with phenol and the coloured product is measured spectrophotometrically

A millilitер of analyte and 1ml of phenol (a 5% aqueous solution) were added to a test tube and vortexed Five milliliters of 95% H_2SO_4 were added and the solution was incubated at 30°C for 10 min The absorbance was read at 490nm on an LKB 2000 spectrophotometer The concentration of hexose in the analyte was determined by reference to a standard curve which was constructed with glucose

M27

Anthrone Assay

The principle of this assay is the same as that of the phenol sulphuric assay, however anthrone, an aromatic amine, condenses with the with the furfural derivative to produce a chromagen with an absorption maximum of 625nm

One millilиter of EPS dissolved in distilled water was placed on ice and 5ml of ice cold anthrone reagent (200mg anthrone dissolved in 5ml of ethanol and brought to 100ml with 75% sulphuric acid) were added The reaction was left on ice for 5 min and heated to 100°C for 10min before being returned to the ice for a further 10 min , prior to reading the absorbance at 625nm The concentration of the hexose was determined by reference to a standard curve constructed with glucose

M28

Determination of Acidic EPS in Culture Supernatants

A 10ml culture of *Rhizobium* grown in VMM medium for eight days in a Lukham cell mixer, model CM100, was centrifuged at 7,000 x g for 10

min The supernatant was transferred to a corex tube and 0 5ml of 0 3% CPC was added so as to precipitate the acidic EPS The EPS was recovered by centrifugation at 7,000 x g The supernatant was discarded and the pellet was resuspended in 1ml of 10% NaCl The CPC was removed by centrifugation at 10,000 x g at 4°C for 15 min The supernatant was assayed for hexose using the anthrone method as described in section M27

M29

Size Fractionation of EPS Produced by *Rhizobium spp*

For the production of EPS, cultures were grown on Vincent's glutamate-mannitol medium (250ml) for 6 days Distribution of extracellular soluble carbohydrates of *Rhizobium spp* according to molecular weight was demonstrated by gel filtration on a BioGel A5m column (2x150cm, BioRad Laboratories, Richmond, California,U S A) This column, which had a fraction range between 10000 and 500000, molecular weight, was equilibrated with a solution containing 100mM NaCl and 50mM sodium phosphate pH 7 0

The void volume (V_o) of the column was determined from the elution volume of dextran blue (molecular weight $2x10^6$) The elution volume (V_t) was determined by the volume taken to elute phenol red (molecular weight 354) The bed volume of the column (V_s) was calculated from equation 1

$$V_t - V_o = V_s \qquad \text{(eqn 1)}$$

The sample volume was 1-5% of the bed volume so that maximum resolution was attained Fractions (7ml) were collected with a Broma 2112 redirac fraction collector and were assayed for carbohydrate using the phenol sulphuric assay procedure

M30

Hot Water-Ethanol Extraction of Glucan from *Rhizobium spp*

(Zevenhuzuizen and van Neerven, 1983)

For the production of *B*-glucan, cultures (250ml) were grown in

Vincent's glutamate-mannitol medium for 6 days *B*-glucan was obtained by suspending washed cell pellets in 10ml of water, adding 30ml of ethanol, heating the mixture at 80°C for 30 min and removing the debris by centrifugation at 48000 x g The water-ethanol extract was dried by rotary evaporation, redissolved and loaded onto a Sephadex QAE A50 anion exchange column The column was equilibrated with 5mM Na_4HCO_3 followed by a gradient (5mM to 1M) of Na_4HCO_3 using a flow rate of 10ml per hour Fractions (5ml) were collected using an LKB Broma 2112 redirac fraction collector and were assayed for carbohydrate using the phenol sulphuric acid assay procedure

M31

Proton Magnetic Resonance Spectroscopy

For nuclear magnetic resonance (NMR) the high molecular weight CPC isolated fraction had firstly to be depolymerized by ultrasonic irradiation (u i) as even dilute solutions of this fraction were too viscous to yield NMR spectra

An MSE, soniprep 150, ultrasonic disintegrator with a tapered probe (3mm in diameter) was used The temperature of sample was maintained between 2°C and 9°C by using an ethanol-ice bath Sonication treatments of polysaccharides were performed under a stream of N_2 so as to minimize oxidation (Szu *et al* , 1986)

The low molecular weight saccharide fractions from the BioGel A5m column were pooled, freeze dried, redissolved in water and desalted by passage through a Sephadex G-10 column

Samples containing phenol-sulphuric positive material from QAE A50 chromatography were pooled freeze dried and desalted by passage through a Sephadex G-25 column Samples were shown to be salt free by conductivity measurments using a metrohm conductivity meter

All samples were exchanged several times with D_2O before finally being dissolved in 99 98% D_2O (Sigma) Proton magnetic resonance was performed on an instrument (350Hz) in the Chemistry Department, University College Galway The chemical shift standard was tetramethyl silane (TMS), and the probe temperature was 75°C

M32

Examination of Bacterial Cell Surface Polysaccharides by Electron Microscopy

Bacterial cells were contrasted by the Glutaraldehyde-Ruthenium-Uranyl acetate (GRU) procedure (Mutaftschiev *et al* , 1982) This procedure required the use of FORMVAR coated grids and these were prepared by dipping a clean glass slide into FORMVAR (0 05% Ethylene dichloride) The slide was removed and air-dried The edges of the glass slide were scored with a forceps to facilitate the removal of the FORMVAR film The slide was dipped in water and the FORMVAR coat was released Grids which had been washed in acetone were placed, dull side up, on the FORMVAR coat The FORMVAR coated grids were removed from the water by placing a strip of parafilm on the coat and lifting it free of the water These grids were then ready for use

Bacterial cells were streaked on Bishop's medium and incubated at 30°C for 5 days Bacteria were resuspended in Bishop's liquid medium which had been autoclaved and filtered through 0 22μm filters, so as to give a final concentration of 10^7-10^8 colony forming units per ml Two FORMVAR coated grids were placed on a 200μl drop of bacterial suspension Twenty microliters of ruthenium red (a 1% aqueous solution, centrifuged and filtered through a 0 22μm filter) and 20μl of a glutaraldehyde solution (1% glutaraldehyde in 0 2M cacodylate buffer, pH 7 2) were mixed in a drop The grids were removed from the bacterial suspensions, dried with filter paper and were placed in the drop of ruthenium red\glutaraldehyde solution for 20 to 30 seconds The grids were then removed and dried and were placed in a drop (40μl) of uranyl acetate solution (2%) so as to remove excess ruthenium red After a second wash in uranyl acetate, the grids were placed on filter paper to dry The grids were examined by transmission electron microscopy

Analysis of Outer Membrane Proteins from *Rhizobium spp*

M33

Isolation of Outer Membranes from Cells Grown in Complex Medium

(deMaagd and Lugtenberg, 1986)

Logarithmic-phase *Rhizobium* cells were obtained by overnight growth in 400ml of TY broth to an A_{600} of 0 5-0 6 All further procedures were carried out at 4°C After being harvested the cells were resuspended in 10ml of 50mM Tris-HCl, (pH 8 5), 20% (w/v) sucrose, 0 2mM dithiothreitol supplemented with 0 2mg of both DNAse I and RNAse I per ml The cells were broken by three passages through an X-press which had been precooled to -30°C in an ethanol bath The broken cell suspension was treated with 0 2mg of lysozyme per ml for 30min , subsequently diluted with two volumes of 50mM Tris-HCl pH 8 5 and centrifuged at 900 x g for 20 min to remove cell debris The supernatant was then centrifuged at 260,000 x g for two hours and the resultant pellet consisted of cell envelopes Cytoplasmic envelopes were dissolved using 0 1% Triton-X-100 and the outer membranes were harvested by ultra centrifugation at 260,000 x g for two hours The outer membranes were washed twice with water and resuspended by manual use of a Potter homogeniser Samples were solubilized and subjected to PAGE as described in section M21

M34

Isolation of Outer Membranes from Cells Grown in Minimal Medium

Cells were grown to mid-log phase in 400ml of culture medium and harvested by centrifugation at 10,000 x g for 10 min The resulting pellet was washed 3 times with PBS and the cells were resuspended in 20ml of Tris-HCl containing 0 3% NaCl pH 8 0 Cells were disrupted by sonication (9 x 30 seconds, with a 1 min rest interval) in an MSE, Soniprep 150 sonicator The lysate was centrifuged at 5,000 x g for 10 min , the supernatant was decanted and centrifuged at 30,000 x g for 40 min in a Beckman L-8M ultracentrifuge To prepare outer membranes the cell envelopes were treated with 1 5% sarkosyl (w\v) in distilled water at room temperature for 1 hour to dissolve the inner membrane This treatment was repeated at least twice Outer membranes were resuspended

in PAGE solubilization buffer and electrophorezed and stained as described in section M 20 Densitometer scans were performed using Hoefer Scientific Instruments GS300 scanning densitometer

Plant Tests

Vicia hirsuta seeds were obtained from John Chambers, Kettering, Northamptonshire, U K *Vicia lathyroides* and *Vicia sativa* seeds were kind gifts from Donal Sinnot of the National Botanic Gardens, Glasnevin, Dublin 9

M35

Surface Sterilization of Seeds

Seeds were sterilized by immersing them for 30 min in concentrated sulphuric acid, rinsed in tap water and immersed in commercial bleach for 30 min To remove all traces of the bleach 4 washes with sterile water were performed The seeds were swollen by incubation for 4 hours in sterile water and germinated on TY agar plates For *V sativa* and *V lathyroides* the TY plates were incubated upside down for at least 4 days at 4°C, followed by 2 days incubation at 24°C in the dark *V hirsuta* did not require this cold treatment for good germination Germinated seeds were transferred to Jensen agar slants (Vincent, 1970)

M36

Inoculation of Seedlings

Seedlings were inoculated with a bacterial suspension (10^7/ml), immediately after transfer of the seedlings to Jensen agar slants

Growth Conditions

Tubes containing the seedlings were placed in racks so that the root systems were covered and incubated at 22°C with a day length of 16 hours The light intensity at table level was 1500 lux (OSRAM 30W bulbs ref L77 30W)

M37

Observation of Infection

Root infection was observed by bright-field microscopy after staining with methylene-blue (Vasse and Truchet, 1984) Roots were stained for 15 min in a humid chamber with a 0 01% (w/v) methylene-blue in filtered N-free Fårhaeus medium (Fårhaeus, 1957) Staining or washing of the plant material was achieved by allowing solutions to flow gently between the slide and cover slip

Discrimination Between Root Nodules and Non-Nitrogen Fixing Root Derived Structures

M38

The Clearing Method

To date the unequivocal method to distinguish a root nodule from a non-nitrogen fixing root derived structure was microscopy of sectioned material A fast and simple method for such discrimination has been developed by Truchet *et al* , (1989a) The method is based on the observation of cleared material by bright field microscopy The method described here is a modification of the method developed by Truchet *et al* (1989) for *R meliloti*/alfalfa interactions

Before clearing, the whole root system of *V hirsuta* was fixed in glutaraldehyde (2 75%) in 0 2M cacodylate buffer, 0 65 osmol Kg^{-1}, pH 7 2, containing 2 5mM $CaCl_2$ Osmomolarity was measured using a Hiermann Roebling micro-osmometer Fixation was for 15 min under vacuum followed by 15 min at atmospheric pressure with a change of fixative After fixation the root system was washed (3 x 5min) with distilled water The roots were immersed in a solution of sodium hypochlorite which titrated 24 chlorometric degrees This solution was prepared just before use from a concentrated commercial preparation which was diluted with distilled water Plants were cleared for 22 min under vacuum followed by a change in clearing solution followed by a further 8 min at atmospheric pressure The clearing solution was removed and the plant was rinsed with distilled water The specimen was mounted between a slide and cover-slip and observed by bright field microscopy on an Olympus Vanox microscope

M39

Specimen Preparation for Electron Microscopy

Fixation

Fixative 0 2M Cacodylate containing 2 5mM $CaCl_2$
Glutaraldehyde 2 7%
pH 7 2

osmolarity between 650 and 750 milliosmoles

The entire root system was placed under vacuum in fixative for 15 min and was agitated intermittently The root system was removed from the fixative and the nodules were excised The nodules were placed under vacuum for a further 15 min The nodules were then removed and placed in fresh fixative for 1 hour at atmospheric pressure

Wash

Samples were washed with 0 3M cacodylate containing 2 5M $CaCl_2$(3 x 30min washes)

Postfixation

This was performed in 0 2M cacodylate
2 5M$CaCl_2$
pH 7 2
\+ Osmium 2%

The nodules were incubated at room temperature in this solution for 1 hour in the dark

Dehydration

Water was removed by sequential washes with solutions containing increasing concentrations of ethanol These were

1/4 - 3/4 -30min
1/2 - 1/2 -30min
3/4 - 1/4 -1 hour or overnight
9/10 - 1/10 -1hour
absolute EtOH 3 x 1 hour

Replacement of ethanol with propylene oxide was accomplished by 2 x 15

min washes with propylene oxide, and care was taken not to allow the nodules to dry out

Polypropylene oxide was replaced by EPON by sequential addition of EPON / polypropylene oxides as follows

1/4	-3/4	-propylene oxide	-30min
1/2	-1/2	-propylene oxide	-45min
3/4	-1/4	-propylene oxide	-1hour or overnight
Pure EPON		3 x 45 min	

The following constitutes EPON

MNA - Methylnorborneu-2-3-dicarbon aurhydrid Merck (12153)

Epikote 812 - Glycidether 100 Merck (12153)

DDSA - Dodecenylbernsteinaureanhydride

Accelerator - 2,4,6 Tris(dimethylaminoethyl) phenol Merck (12388)

EPON A 62ml Epikote 812 and 100ml DDSA

EPON B 100ml Epikote 812 and 89ml MNA

EPON To make EPON, 2/3 of solution A was mixed with 1/3 of solution B for 30 min The accelerator was added dropwise to the mixture of A and B at high agitation to a final concentration of 2%

Embedding

Individual nodules were placed in separate plastic capsules containing 2 drops of EPON Nodules were orientated so that longitudinal sections would be obtained, as *Vicia spp* form indeterminate nodules After orientation, the capsules were filled with EPON and placed at 60°C for 2 days

M40

Sectioning

Prior to sectioning the excess EPON and the capsule were removed from around the sample This was accomplished by cutting out a pyramid around the sample with a razor blade, using an ocular microscope to facilitate viewing of the manipulation

Glass knives

Glass knives were made on an LKB 7800 knife maker according to the manufacturer's instructions, and were used at a cutting angle of 4°

Diamond knives

These knives were obtained from Histodiatome and were used at the cutting angle recommended by the manfacturer (usually 5° or 6°)

Semi-thin sections

Sections(99μm) were prepared using a Reichert-Jung ultra cut microtone equipped with a glass knife

Thin sections

Sections (14μm) were obtained with a Reichert-Jung ultra cut microtone equipped with a diamond knife

Staining of Sections for Microscopy

For light microscopy, semi-thin sections were placed on glass slides and were fixed to the slide by heating over a flame Sections for electron microscopy were placed on FORMVAR grids prior to staining

M41.

Fuschin Red-Methylene Blue Staining for Light Microscopy

Fuschin basic (4% aqueous solution) was centrifuged at 2,000 x g for 5 min A drop of the supernatant was added to the fixed section for 1 min The section was then washed with distilled water and allowed to dry Methylene blue (2% aqueous sol' tion) was centrifuged for 5 mins at 2,000 x g and 6 drops of the supernatant were added to 5 drops of 0 01M NaOH One drop of alkaline methylene blue was added to the section for 5 seconds and the section was washed with distilled water The section was viewed under a Olympus Vanox microscope

M42.

Lead Citrate - Uranyl acetate Staining for Electron Microscopy

(Venable, 1965)

For visualization of the sample under the electron beam, sections were stained with a lead citrate solution containing 0 04g lead citrate and 0 1ml of 10M NaOH in 10ml distilled water The solution was made a day in advance of use and was filtered through a 0 22μm filter The filtered solution was stored in at 4°C in a bottle with a tightly fitting lid, as CO_2 will cause the lead to precipitate The uranyl acetate solution contained 5g of uranyl acetate per 100ml of water This solution was stored in the dark at 4°C Prior to use, the solution was filtered and diluted 1·1 with ethanol

M43

Nitrogenase Activity

The reduction of acetylene to ethylene was used to assay nitrogenase activity of nodules (Hardy *et al* 1968) Twenty six days after inoculation, test tubes containing the entire plant or individual excised root nodules were sealed with suba-seals Acetylene was injected (10% of the total volume) and after 3 hours incubation at 22°C in the light ethylene production was measured in a Perkin-Elmer Gas Chromatograph Model 780-L equipped with a flame ionization detector A poropak N column (1 5m x 3mm) was used to separate acetylene from ethylene The flow rate of the carrier gas (N_2) was 55cm^3/min The oven temperature was 175°C Samples were injected onto the column using a gas-tight syringe (AGB Scientific)

RESULTS I

Introduction

Bacterial surface componants are thought to play a role in the establishment of the *Rhizobium*/legume symbioses. In this study defined mutations were introduced to a strain of *Rhizobium leguminosarum* biovar *viciae* by the use of Tn5-Mob a transposon which includes the transfer origin from plasmid RP_4, (Simon, 1984). Mutations affecting the surface componants of this bacterium were selected by using a growth regime which amplified the production of EPS and therefore facilitated the detection of surface mutants based on altered colony morphologies. The symbiotic phenotypes of these mutants were investigated on *V.hirsuta* seedlings.

Introduction of Tn5-Mob to *Rhizobium leguminosarum* biovar *viciae*

Rhizobium leguminosarum biovar *viciae* $VF39^r$ was obtained from U. Priefer University of Bielefeld, F.R.G. A spontaneous streptomycin resistant mutant of this strain was obtained by plating aproximately 10^9 cells onto TY plates containing 200 μg/ml streptomycin. Spontaneous resistant clones arose at a frequency of 10^{-8}. One of these clones, $VF39Sm^r$ was purified and was used throughout this study.

The plasmid pSUP5011 was crossed from *E.coli* S17-1 to *Rhizobium leguminosarum* biovar *viciae* $VF39Sm^r$. The mating was performed on 0.45μm membrane filters as described in Ml. Transconjugants were selected on TY agar containing 200μg/ml streptomycin (Sm) and 60μg/ml neomycin (Nm) and were detected at a frequency of 1×10^{-4} per recipient cell.

Screening Tn5-Mob Mutants for Surface Defects.

Ten thousand Tn5-Mob mutants were replica plated using sterile cocktail sticks, onto TY and VMM media, both of which contained streptomycin and neomycin. The plates were incubated at $30^{\circ}C$ for two days and for three days at room temperature before the phenotypes were scored .

Rhizobium spp. when grown on a medium containing a high ratio of carbon to nitrogen source such as VMM, produce copious amounts of EPS. When grown on media such as TY which contain a high nitrogen to carbon source ratio little or no EPS can be detected.

Tn5-Mob mutants fell into four categories : 1. Transparent; 2. Overproducers of EPS; 3. Underproducers of EPS; 4.Auxotrophs.

1 Transparent Mutants

These mutants appeared as transparent colonies on VMM, Figure 17, and had a slower growth rate on VMM (as judged by the length of time taken for isolated colonies to appear) than the wild-type strain Microscopic examination of these colonies revealed that far fewer bacteria were present in transparent colonies compared to the wild-type strain Transparent mutants were unable to grow in VMM liquid medium These results suggested that the transparent mutants were in fact auxotrophs which were capable of growth on solid minimal medium because the nutrient for which they were auxotrophic was present at a low concentration in the Bactoagar used to solidify the medium Several of the transparent mutants were shown to be amino acid auxotrophs, Figure 17 The reason why most auxotrophs fail to grow on minimal medium and yet the transparent mutants do is not known Similar observations have been made regarding purine and pyrimidine auxotrophs of *Rhizobium leguminosarum* biovar *phaseoli* (Noel et al , 1988) Some of these purine auxotrophs were Fix^-, as *de novo* purine synthesis is required for effective symbiosis (Noel et al , 1988), these purine auxotrophs were initially identified for EPS mutants (VandenBosch *et al* , 1985) The transparent auxotrophs isolated during this study were all Fix^+

2 Mutants Overproducing EPS

Control of polysaccharide genes appears to be critical in *Rhizobium meliloti* as mutants which overproduce EPS have been shown to be Fix^-, (Doherty et al , 1988)

The wild-type strain *Rhizobium leguminosarum* biovar *viciae* VF39Sm produced 3,100 μg/mg of glucose equivalents per ml of culture supernatant of acidic EPS when grown on VMM, as described in M28 Two Tn5-Mob mutants JB10 and JB18 produced 4,000 μg/ml and 4,300 μg/ml glucose equivalents per ml of supernatant respectively However both these mutants gave rise to a Fix^+ phenotype when inoculated on *V hirsuta* seedlings

3 Mutants Underproducing EPS

Two Tn5-Mob mutants which produced less EPS than the wild-type strain were isolated One of these mutants gave rise to an effective symbiosis when inoculated onto *V hirsuta* seedlings The other underproducing

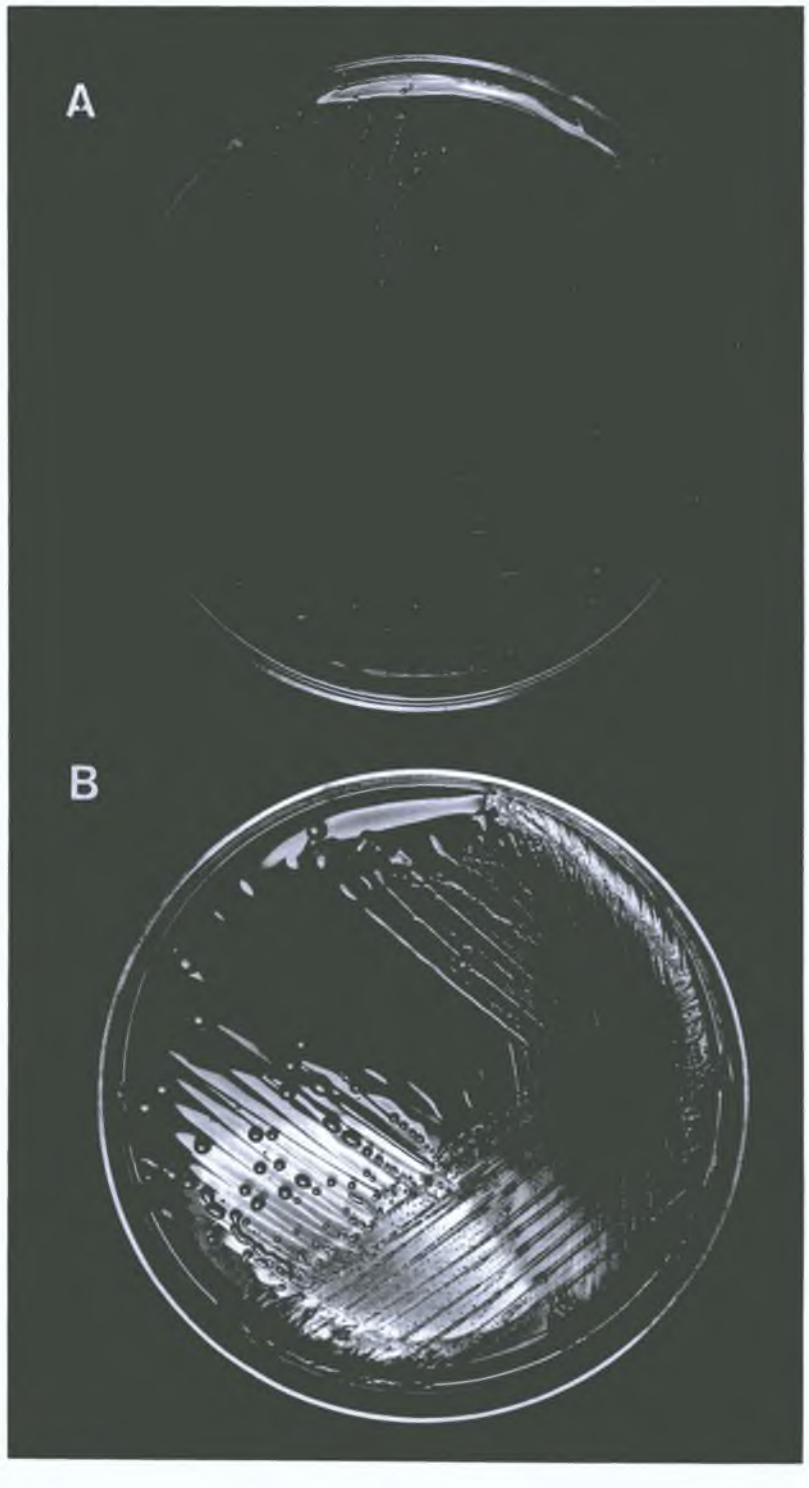


Figure 17. Restoration of the wild-type colony morphology upon incorporation of an amino acid into minimal medium. The transparent mutant type colony morphology exhibited by *R.leguminosarum* biovar *viciae* JB14 when grown on :(a) VMM ; (b) VMM supplemented with cysteine.

strain, JB45, not only had an altered morphology on VMM medium but has a rough colony morphology on TY plates When inoculated onto *V hirsuta* seedlings it gave rise to pseudo-nodules that were Fix^-

This strain flocculated when grown in media containing a high Ca^{2+} concentration such as TY, Figure 18, and was non-motile, Figure 19, both of which are characteristics of LPS mutants

Surprisingly no mutant totally devoid of EPS was isolated after screening 10,000 mutants which indicates that more than one genetic locus may be involved in the synthesis of EPS in *Rhizobium leguminosarum* biovar *viciae* $VF39Sm^r$

4 Auoxotrophic mutants

Auxotrophs appeared at a frequency of 0 6% which indicated that Tn5-Mob mutagenesis was a random mutagenesis

Construction of a Vector for the Delivery of Tn*phoA* to *Rhizobium spp*

Since surface Tn5-Mob mutants altered in their symbiotic ability were detected at a very low frequency the feasibility of using a transposon Tn*phoA*, (Manoil and Beckwith, 1985), which would allow more rapid screening of surface mutations in *Rhizobium leguminosarum* biovar *viciae* was investigated

The *phoA* gene codes for the *E coli* periplasmic alkaline phosphatase The important property of alkaline phosphatase is that it is only active when exported across the cytoplasmic membrane, (Manoil et al , 1990) If certain mutations or gene fusions are used to cause the alkaline phosphatase to be retained in the host cell cytosol, it is enzymatically inactive

A transposon derivative of Tn5 has been constructed which contains the *phoA* gene missing its promoter, its translational initiation start site, the DNA corresponding to its signal sequence and the first five amino acids of the protein, (Manoil and Beckwith, 1985) Therefore the Tn*phoA* system can be used to preferentially distinguish genes which code for envelope (the site of EPS and LPS synthesis) periplasmic and secreted proteins as such genes would be detected as ones yielding active fusions

Insertion of Tn*phoA* into genes coding for export signals can be

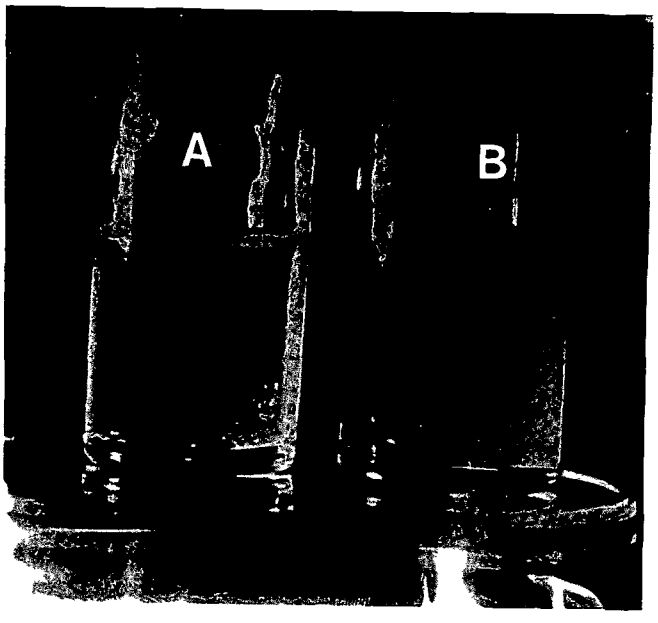


Figure 18 Growth characteristics of the wild-type strain VF39Sm^r and the mutant underproducing EPS, JB45 Strains grown in 10ml of TY medium (a) the mutant strain showed autoagglutation, (b) the wild-type strain shows normal turbid growth

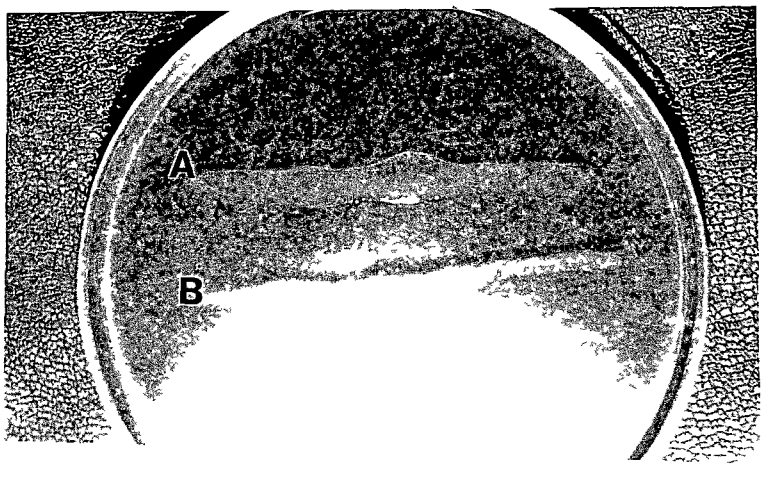


Figure 19 (a) Swarm behavior of the wild type strain when grown on motility agar, (b) the non-motile mutant JB45 when grown of TY agar solidified with 10g gelatin and 5g per liter agar

detected as blue colonies on media containing the alkaline phosphatase dye, XP (5-bromo-3-chloro-indoyl-phosphate). Therefore screening of surface mutants need only be carried out on blue colonies which would significantly reduce the number of mutants to be screened.

The bacteriophage λTn*pho*A-1 was used as the source of Tn*pho*A-1 in this study. Plasmid pSUP202 was used as the suicide plasmid which would deliver the Tn*pho*A to *Rhizobium spp*.
Plasmid pSUP202 was used to transform *E.coli* CC118 (*recA*$^-$, *phoA*$^-$, Km^s, λ^s), as described in section M2. Transformants were selected on LB Cm (30μg/ml). One of these colonies was purified and used to inoculate 10ml of LB broth containing 10mM $MgSO_4$ and Cm (15μg/ml). λTn*pho*A was added to 1ml of this culture which had been grown to early stationary phase, at a multiplicity of 1. The phage was allowed to adsorb to the cells for 15 min at 30°C after which time it was diluted 1:10 with LB broth and out-growth with aeration was for two hours at 30°C. Two hundred microliter aliquots of this culture were plated on selective antibiotic plates containing Cm (30μg/ml), Km (300μg/ml) and XP (40μg/ml). These plates were incubated at 30°C for two to three days after which both blue and white colonies were visible. Blue and white colonies were scraped off and plasmid DNA was prepared from this pool by the alkaline lysis procedure for rapid plasmid preparation, as described in section M4. .This preparation was used to transform *E.coli* CC118 and transformants were selected on LB Km (30μg/ml) and XP (40μg/ml). Individual colonies were purified and used to transform *E.coli* CC118 yet again. Transformants (blue and white) were purified and plasmid DNA was prepared by the alkaline lysis procedure. These plasmid preparations were used to transform *E.coli* S17-1 and for restriction enzyme digestion analysis. *E.coli* S17-1 cells containing pSUP202::Tn*pho*A insertions were selected on LB Cm (30μg/ml) plates. Transformants were purified and plated on M9 and M9 proline (the auxotrophic marker carried on the *E.coli* S17-1 chromosome). Growth was observed on M9 proline but not on M9 alone indicating that these clones were in fact *E.coli* S17-1 clones. Digestion of these plasmids with *Eco*R1 verified that these clones contained Tn*pho*A (data not shown).
Table 5 shows the properties of the varios pSUP202::Tn*pho*A vectors constructed during this study.

Table 5 Properties of pSUP202 Tn*phoA*

Plasmid	Colour on XP plates*	Ap^r	Cm^r	Tc^r	Km^r	Transfer frequency$^{\varphi}$
pDJB201	white	+	+	+	+	1×10^{-4}
pDJB202	white	+	+	+	+	2×10^{-5}
pDJB203	blue	-	+	+	+	1×10^{-5}
pDJB204	white	+	+	-	+	5×10^{-5}

*plasmids were harboured in *E coli* CC118 and strains were plated on LB agar medium which contained 40μg/ml XP

$^{\varphi}$the frequency of transfer of Tn*PhoA* was taken as being that of the transfer of Nm resistance to $VF39Sm^r$ after these plasmids had been introduced into *E coli* S17-1 and crossed with $VF39Sm^r$

Although alkaline phosphatase activity is induced in most Gram-negative bacteria by phosphate limitation, sufficient alkaline phosphatase activity is expressed on phosphate rich media to give rise to blue colonies on complex media containing XP (Taylor *et al* , 1989) When *Rhizobium leguminosarum* biovar *viciae* $VF39Sm^r$ was plated on TY-XP medium only a very weak blue colour was produced by these colonies, and the degree of colour was variable This was not found to be the case for *Rhizobium leguminosarum* biovar *viciae* 3855 which produced deep blue colonies on XP medium Following Tn*phoA* mutagenesis the degree of colour was again variable on XP containing medium, rendering it impossible to detect clones which gave rise to fusion proteins which were exported In an attempt to abolish the light blue colour produced by *Rhizobium leguminosarum* biovar *viciae* $VF39Sm^r$ chemical mutagenesis was undertaken using EMS as a mutagen as described in section M16 Following EMS mutagenesis some apparently white colonies were detected however on purification of these clones the variability in blue colour was again observed

In order to understand the reason why *Rhizobium leguminosarum* biovar *viciae* $VF39Sm^r$ did not give rise to blue colonies on XP medium and *Rhizobium leguminosarum* biovar *viciae* 3855 did, a quantative analysis

of the alkaline phosphatase activity in these strains was undertaken. Even after *Rhizobium leguminosarum* biovar *viciae* VF39^{r} cells were grown in phosphate limiting medium no alkaline phosphatase activity could be detected in permeablized cells, section M17 This failure to detect alkaline phosphatase activity was not due to a technical limation as *Rhizobium leguminosarum* biovar *viciae* 3855 expressed akaline phosphatase activity. Figure 20 shows that when *Rhizobium leguminosarum* biovar *viciae* 3855 was grown in normal phosphate-rich glutamate medium with mannitol as a carbon source, only a very low basal activity of alkaline phosphatase activity could be detected Cells grown in such medium to mid-exponential phase became derepressed for alkaline phosphatase activity one hour after centrifugation and resuspension in in a phosphate-free medium. After this time alkaline phosphatase activity increase rapidly, and reached a level 5 times the basal level of activity 4 hours after derepression The kinetics of derepression of alkaline phosphatase activity for *Rhizobium leguminosarum* biovar *viciae* 3855 are similar to those reported for other *Rhizobium leguminosarum* biovar *viciae* strains (Glenn and Dilworth, 1979)

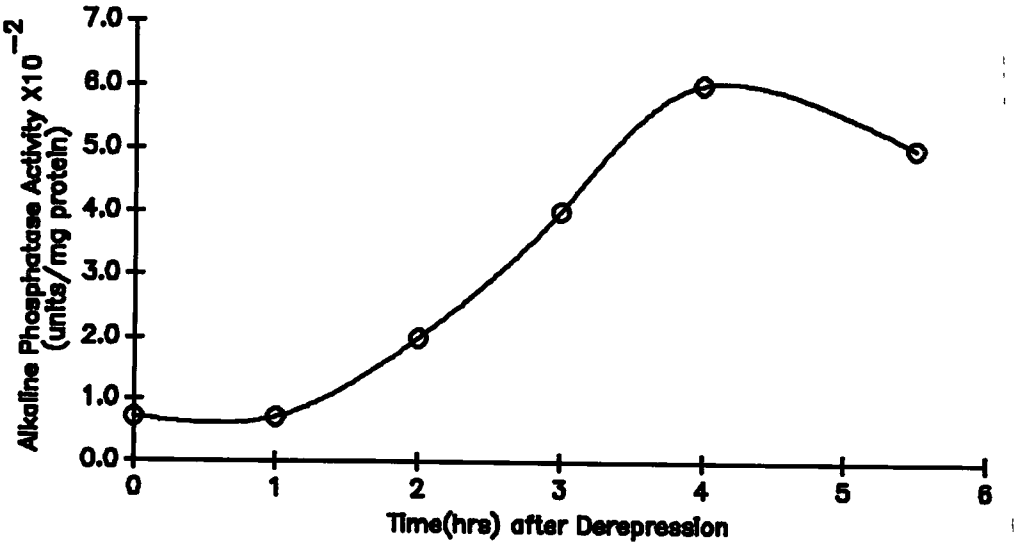


Figure 20 Derepression and release of alkaline phosphatase activity in *Rhizobium leguminosarum* biovar *viciae* 3855 At intervals, samples were removed from phosphate-deficient cultures and assayed for alkaline phosphatase activity as described in section M 17

In *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r endogenous phosphatase activity is not detectable as measured by its ability to cleave *p*-nitrophenyl phosphate, the faint blue colour detected on XP solid medium must be due to some other type of phosphatase(s) The removal of such phosphatase activity is a prerequisite for use of Tn*phoA* in the detection of active fusions and as this could not be accomplished the Tn*PhoA* system could not be used to isolate active fusions in *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r Nevertheless an efficient delivery system was constructed which will prove of use in mutagenesis of other soil bacteria in this laboratory

Positive Selection for LPS Mutants of *Rhizobium leguminosarum* biovar *vicia* VF39Sm^r Using Complement Fixation

LPS from *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r was extracted by the hot phenol-water method described in M18 Antiserum directed against this LPS was obtained by intaperitoneal injection of LPS preparations into Balb/c mice as described in M19 The IgM enriched fraction of these polyclonal antibodies was used in conjuction with rabbit complement to complement kill *Rhizobium leguminosarum* biovar *viciae* VF39Sm cells

Rhizobium leguminosarum biovar *viciae* VF39Sm^r cells were grown to mid-log phase in TY broth Cells from 1ml of this culture were sedimented in an Eppendorf centrifuge and washed twice with phosphate buffered saline (PBS) The cell pellet was resuspended in 100μl of PBS One hundred microliters of antiserum was added and allowed to adsorb to the cells for two hours at 4°C One hundred microliters of rabbit complement serum was added and the mixture was incubated at 37°C for one hour

Control experiments were 100μl of washed cells + 200μl PBS, 100μl washed cells + 100μl rabbit complement serum + 100ml of PBS

Complement on its own did not kill cells Complement and antiserum directed against LPS did kill the cells and only 0 004% of the cells survived Although the survivors probably represent spontaneous LPS mutants none of these cells failed to fix nitrogen on *V hirsuta* seedlings

Characteristics of *R leguminosarum* biovar *viciae* mutant JB45

It is known that more than one Tn5 transposition event may take place following Tn5 mutagenesis, (Priefer, 1990). Tn5-Mob also contains IS50 which is capable of independent transposition. It was therfore necessary to determine if the mutant phenotype of JB45 was due to a single transposition event

Total DNA from VF39Sm^r and JB45 was prepared as described in M 7 The DNA was restricted with *Eco*R1 and the resulting fragments were seperated by electrophoresis on an agarose gel The DNA was transferred to nitrocellulose by Southern transfer as described in M9

The plasmid pSUP5011 was used as a source of Tn5-Mob This plasmid was labbled with ^{32}P by nick translation as described in M10 and was then hybridized to the *Eco*R1 fragments on the Southern filter as described in M12

The result of this experiment is shown in Figure 21 Since Tn5-Mob contains no *Eco*R1 sites then if only one copy of this transposon is present in the mutant JB45's genome, only one fragment of the of the *Eco*R1 cut DNA of the mutant JB45 should hybridize to the the ^{32}P-labelled pSUP5011 Such was found to be the case The Tn5-Mob was carried on a 15 5 Kb *Eco*R1, Figure 21 This indicated that the JB45 phenotype resulted from a single transpositional event

Figure 21 Autoradiograph of hybridization between ^{32}P-labeled pSUP5011 and lane 1 unrestricted pSUP5011, lane 2 *Eco*R1 digested total DNA from JB45, lane 3 *Eco*R1 digested total DNA from VF39Sm^r.

Integration of the Tn5 carrier vector has been observed to occur at a frequency of 10% of the total number of transpositional events, (Priefer,1990). Therefore in order to determine if any of the carrier vector, pBR325, survived in the mutant JB45, total DNA isolated from the mutant strain was restricted with *Eco*R1 and transferred to Southern filters as described in M9. After hybridization no homology was detected between ^{32}P-labeled pBR322 (a vector which is related to pBR325) and *Eco*R1 digested DNA (data not shown). The mutant strain did not express any chloroamphenicol resistance which should be present if the delivery vector was retained in this mutant.
Therefore the mutant strain *R.leguminosarum* biovar *viciae* JB45 contains only copy of Tn5-Mob and none of the carrier vector survived in this strain.

Chromosomal Location of the Tn5-Mob Insertion in JB45

The wild-type strain *R.leguminosarum* biovar *viciae* VF39Sm^r contains six indigenous plasmids (Hynes and McGregor, 1990). It was of interest to determine whether the Tn5-Mob insertion was located on any of these plasmids or if it was located on the chromosome. Since Tn5-Mob contains the RP_4 origin of conjugal transfer and a neomycin resistance gene, it may be transfered to other strains if it is located on a plasmid and the transfer functions of RP_4 are supplied *in trans*. If therefore the Tn5-Mob insertion in strain JB45 was on a plasmid then it should have been possible to transfer this plasmid to other strains by supplying transfer functions and a suitable neomycin sensitive recipient strain. Such an experiment was undertaken and the results are shown in Table 6. Plasmids known to have Tn5-Mob located on them were used as positive controls and were readily transferable, Table 6. A strain known to have the Tn5-Mob located on the chromosome served as negative control and no transfer of indigenous plasmids was observed when this strain was crossed with *R.meliloti*. No transfer of indigenous plasmids from the mutant strain JB45 to *R.meliloti* was observed. It was therefore concluded that the Tn5-Mob insertion in strain JB45 was chromosomally located.

Table 6. Transfer of Tn5-Mob* Insertions from *Rhizobium leguminosarum* biovar *viciae* strains to *Rhizobium meliloti* 2011$^{\varphi}$

Donar Strain	Recipient Strain	Transfer Frequency
Rhizobium leguminosarum biovar *viciae* JB33	*Rhizobium meliloti* 2011	$< 1 \times 10^{-9}$
Rhizobium leguminosarum biovar *viciae* 336E (pSym336E Tn5-Mob)	*Rhizobium meliloti* 2011	4×10^{-7}
Rhizobium leguminosarum biovar *viciae* B151(VF39c)	*Rhizobium meliloti* 2011	1×10^{-6}
Rhizobium leguminosarum biovar *viciae* JB45	*Rhizobium meliloti* 2011	$< 1 \times 10^{-9}$

*The mobilizing strain was CSH56 RP_{4-4}

$^{\varphi}$*Rhizobium meliloti* recipients were selected on RGMC medium containing 200μg/ml Rifampicin

R leguminosarum biovar *viciae* Carries Tn5-Mob at a New Genetic Location

Priefer, (1989), reported that a cosmid, pCos4, which carried a 38 kilobase region of the wild-type *R leguminosarum* biovar *viciae* VF39 chromosome, complemented three different LPS mutants of this strain As JB45 had characteristics of LPS mutants (such as non-motility, and autogglutination during growth on medium containing high concentrations of Ca^{2+}) it was of interest to determine if pCos4 could complement mutant JB45 It was claimed that pCos4 could complement JB45 (Priefer,U , personal communication) This authour disputed this claim as the introduction of pCos4 (which does not replicate in *Rhizobium spp*) or *Eco*R1 fragments of pCos4 subcloned into plasmid pSUP104 (which does replicate in *Rhizobium spp*) did not complement JB45

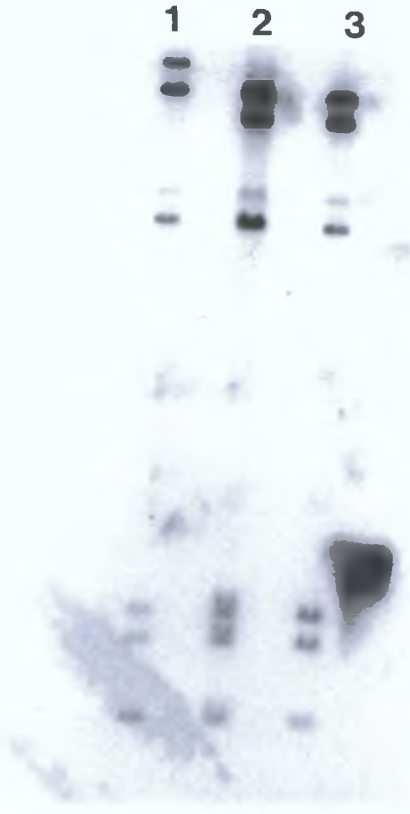


Figure 22. Autoradiograph of hybridization between ^{32}P-radiolabeled pCos4 and *Eco*R1 restricted total DNA from lane 1, *Rhizobium leguminosarum* biovar *viciae* VF39-51, lane 2, *Rhizobium leguminosarum* biovar *viciae* JB45, and lane 3, the wild-type strain *Rhizobium leguminosarum* biovar *viciae* VF39Sm^{r}.

Further genetic evidence that pCos4 does not complement JB45 came from Soutern blot analysis of *Eco*R1 digested total DNA from VF39Sm^r, JB45, and an LPS mutant, VF39-51 which pCos4 complements, (Priefer, 1989). Radiolabled pCos4 was hybridized to *Eco*R1 digested total DNA which had been transferred to nitrocellulose filters. Figure 22 shows that pCos4 hybridized to *Eco*R1 digested total DNA of the wild-type strain giving rise to a pattern identical to an *Eco*R1 digestion of pCos4.probed with ^{32}Plabeled pCos4. When total *Eco*R1 restricted DNA from the strain pCos4 complemented was probed a shift in the size of the E8 fragment was observed from 9.5kb⟶15.5kb. As Tn5 is 6kb in length this result indicates that the Tn5 insertion was in the E8 fragment. This result is in agreement with those of Priefer, (1990).

No such increase in size of the *Eco*R1 fragments was observed in *Eco*R1 digested total DNA isolated from the mutant strain VF39Sm^r.

This indicates that the the Tn5-Mob insertion in JB45 was not in the 38kb region of the wild-type chromosome cloned in pCos4, and therefore it was unlikely that pCos4 would complement JB45.

This point of contention was resolved when the further experiments were carried out by the Bielefeld group and it was agreed that pCos4 did not complement JB45 (Pühler,A., personal communication). Therefore JB45 carries a mutation in a previously undefined genetic locus.

Complementation of *R.leguminosarum* biovar *viciae* Mutant JB45.

A wild-type cosmid gene bank of *R.leguminosarum* biovar *viciae* VF39 was generously donated by Ush Priefer, University of Bielefeld, F.R.G. The bank was constructed in the mobilizable vector pSUP205 and was maintained packaged in λ phage particles.

The gene bank lysate was used to infect *E.coli* CSH56 (pME206) and clones containing the cosmids were solected on LB tetracyline plates. The cosmids were mated *en masse* into *R.leguminosarum* biovar *viciae* JB45. Transconjugants were selected on TY tetracycline plates. Four hundred transconjugants were streaked onto VMM plated containing tetracycline and neomycin. Two tetracycline resistant colonies were

found to have regained the wild-type colony morphology. When these clones were inoculated onto *V.hirsuta* seedlings both were found to have regained the ability to fix nitrogen.

One of these clones, JB45c1, was caplable of transfering its tetracycline resistance to *E.coli* via conjugation. This indicated that the clone carried a co-integrate of pME206 and a cosmid containing DNA that complemented the mutation in JB45.

The other clone, JB45c2 was unable to transfer its tetracycline resistance which indicated that the complementary cosmid had integrated into the genome of the mutant by homologous recombination.

The cointegrate carried by strain JB45c1 could not be resolved therefore further genetic analysis on this clone could not be undertaken.

Cloning of the Neomycin Determinant of Tn5-Mob Containing Part of the Gene Into which Tn5-Mob had Inserted.

Tn5 contains a single site for the restriction endonuclease *Bam*H1. The *Bam*H1 site does not lie within the neomycin resistance gene, (Jorgensen *et al.*, 1979). The Mob site of RP_4 which was contained on a Sau3a fragment was cloned into this site, (Simon, 1984) however, two *Bam*H1 sites were regenerated (O'Hara, 1989).

In an effort to clone part of the gene into which Tn5-Mob had inserted total DNA from the mutant JB45 was cut with *Bam*H1. The *Bam*H1 digestion was then partially digested with Sau3a to yield fragments of 12kb or less. These fragments were then ligated to plasmid pUC19 which had been cut with *Bam*H1, and the ligation was used to transform *E.coli* JA221. Transformants were selected on LB ampicillin (100μg/ml) and Kanamycin (50μg/ml). Analysis of one of these transformants showed that it contained a plasmid which contained an insert of 7kb in the *Bam*H1 site of pUC19. This plasmid was designated pJB-452.

Total DNA from the wild-type strain was restricted with various restriction enzymes and the fragment were seperated by agarose gel electrophoresis. The DNA was then transfered to nitrocellulose filters as described in section M9. Plasmid pWWC was restricted with *Bam*H1 and the two fragments were separated by agarose gel electrophoresis. The larger of the two fragments which contained the neomycin determinant of

Tn5 and part of the gene into which the Tn5-Mob had inserted was, cut from the gel and purified from the agarose using the "gene clean" procedure as described in M13 This fragment was radiolabled with α-^{32}P-labled ATP by random hexamer priming as described in M11 One nanogram of a 1kb ladder was also added to the reaction mix so that a size marker would be visible on the autoradiogram A sample of total DNA from JB45 cut with *Eco*R1 was included in the experiment so as to verify that part of the gene into which the Tn5-Mob had inserted into had been cloned This would be seen as a shift in size of the *Eco*R1 fragment of 7kb (the size of Tn5-Mob) in the mutant strain compared to the wild-type strain

Figure 23 shows the result of this experiment The fragment which had been cloned was part of the gene into which the Tn5-Mob has inserted because a shift of approximately 7kb was observed between the *Eco*R1 digest of the wild-type strain and that of the mutant

Attempts to Clone the Complete Gene Into Which Tn5-Mob had Inserted

Plasmid pJB-452 could not be used to probe the gene bank of *Rhizobium leguminosarum* biovar *viciae* as the lysate has deterioated Instead total DNA from the wild-type was restricted with *Pst*1 and the fragments were seperated by agrose gel electrophoresis The fragments between 4 and 5 kilobases were cut from the gel and the DNA was purified by the method described in M13 These fragments were ligated to pUC19 which had been digested with *Pst*1 The ligation was used to transform E *coli* JA221 as described in section M2 Five hundred of the chimeras which resulted from this transformation were transferred to nitrocellulose membranes, lysed and the DNA was fixed to the membranes as described in section M14 The *Bam*H1 fragment from pJB-452 was radiolabeled as described in cection M11 Hybridization to nitrocellulose replicas containing replicas of the bacterial colonies was performed as described in section M15 No hybridization to any of these replicas was detected

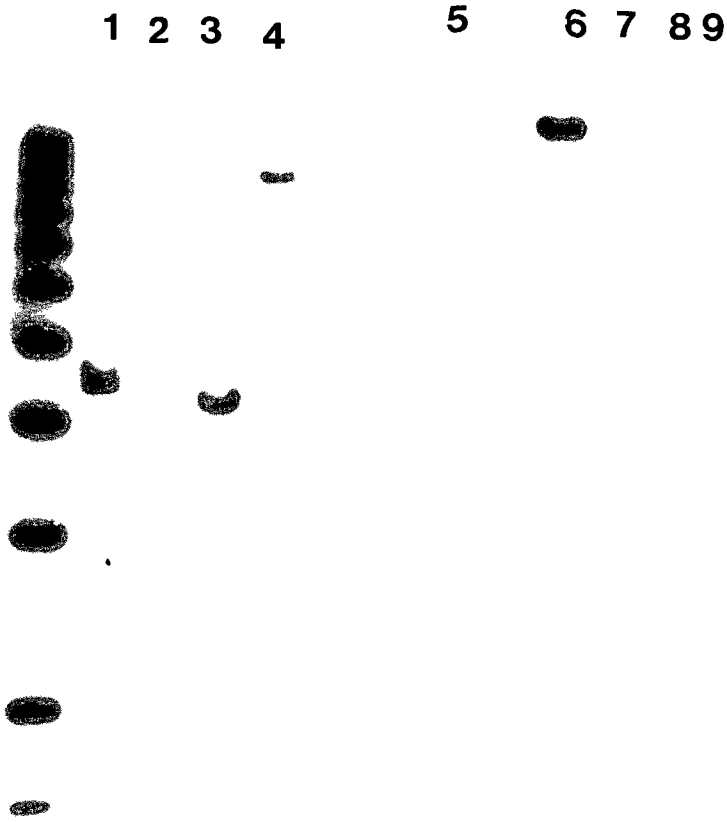


Figure 23 Autoradiograph of hybridization between an 7 kilobase ^{32}P-radiolabeled fragment encoding the kanamycin resistance determinant from Tn5-Mob and part of the gene into which Tn5-Mob had inserted and total DNA from the wild-type strain VF39Sm^{r} cut with various restriction endonucleases

Lanes 1, *Xho*I, 2, *Sal*I 3, *Pst*I, 4, *Hin*dIII, 5, *Hae*II 7, *Eco*RI
8, *Cla*I, 9, *Bam*HI (wild-type DNA)

Lane 6 Mutant DNA cut with *Eco*RI

DISCUSSION I

Tn5-Mob mutagenesis was used to generate defined mutations in *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r. Mutants with an altered colony morphology were identified visually. The simultaneous occurence of altered colony morphology and a symbiotic defect was only 0.01%. Similar results have been reported by Pühler *et al.*, (1988) who observed that following Tn5 mutagenesis of *Rhizobium leguminosarum* biovar *viciae* VF39 only 0.023% of mutants exhibited both altered colony morphology and a Fix$^-$ phenotype on *V.hirsuta* seedlings.In an effort to enhance the detection of surface mutants while still introducing defined mutations, a delivery system for Tn*phoA* to *Rhizobium leguminosarum* biovar *viciae* was constructed. Tn*phoA* allows for discrimination between insertions into genes which encode export signals and those which do not,thus facilitating the screening for surface mutation affecting symbiosis. Such a system has been used successfully to detect genes in *Rhizobium meliloti* coding for envelope proteins which are required for *Rhizobium meliloti* to effectively nodulate alfalfa plants (Long *et al.*, 1988). A delivery system was constructed which was as efficient as the delivery system used to transfer Tn5-Mob to *Rhizobium spp*. The system could not be used to select surface mutants of *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r because of this strain's anomalous behaviour on XP plates. However the system could be employed with strains such as *Rhizobium leguminosarum* biovar *viciae* 3855 once the indigenous alkaline phosphatase activity is removed by chemical mutagenesis.

An attempt was made to select LPS mutants by a positive selection procedure using serum complement in conjunction with antibodies raised against isolated LPS. Although the survivors of complement fixation may have been spontaneous LPS mutants none were Fix$^-$ on *V.hirsuta* seedlings. To prove that these survivors were altered in LPS it would have been necessary to obtain monoclonal antibodies directed against particular epitopes on the LPS molecule.

The surface mutant *Rhizobium leguminosarum* biovar *viciae* JB45 gave rise to poorly developed nodules that were Fix$^-$ on *V.hirsuta* seedlings. Before any attempt was made to correlate the surface defect(s) of this

strain and the particular block in the development of *V hirsuta* root nodules, it was necessary to determine if the mutant phenotype was the result of a single defined mutation Southern hybridization was used to demonstrate that an single transposition event of Tn5-Mob was responsible for the phenotype Complementaion analysis demonstrated that the mutation could be complimented by a cosmid gene library constructed from *Rhizobium leguminosarum* biovar *viciae* VF39 A cointegrate between RP_4 and this cosmid could not be resolved Attempts to resolve the cointegrate by transferring it to a Rec^+ background was not succesful Part of the gene into which the Tn5-Mob had inserted was cloned by virtue of the fact that Tn5-Mob carries a neomycin resistance determinate and cloning of this determinant allowed part of the gene into which Tn5-Mob had inserted to be cloned This gene was shown to be chromosomally located It has been demonstrated that some of the genes involved in LPS production are clustered on the chromosome (Priefer, 1989), whilst at least one other gene is located on the VF39c plasmid in *Rhizobium leguminosarum* biovar *viciae* VF39 (Hynes and McGregor,1990) The Tn5-Mob insertion in mutant JB45 was shown to be chromosomally located and did not form part of the LPS gene cluster discovered by Priefer (1989) Therefore the mutation in strain JB45 represents a mutation at a novel genetic locus

RESULTS II

Introduction

Mutations in genes which are responsible for the surface characteristics of *Rhizobium* cells may indicate if these genes play a role in the symbiotic association between legumes and *Rhizobium spp* In order that such mutational analysis be successful, an accurate phenotypic characterization of any particular surface mutant must be undertaken so that the surface defect can be correlated with the symbiotic defect

Mutants carrying single Tn5 insertions have been shown to give rise to multiple surface carbohydrate defects, (Leigh and Lee, 1988) In view of this and the structural role played by many surface carbohydrates, a survey of the various surface polysaccharides produced by both the Fix$^-$ strain isolated during this study, JB45, and the wild-type strain was undertaken The surface characteristics of both strains are discussed and a model is proposed to account for the pleiotrophic properties observed in the case of the mutant strain

PAGE and Immunochemical Analysis of LPSs

Lipopolysaccharides were extracted by the hot phenol-water method of Wesphal and Jann (1965), and purified by RNAse treatment followed repeated ultracentrifugation as described in M18 Polyacrylamide gel electrophoresis and silver staining of the LPS were performed as described in M21 and M22 respectively Gels were also stained for protein using coomassie blue as described in M21 to ensure that there was no protein contamination of the LPS samples

Figure 24 shows the results of PAGE analysis of the wild-type strain, VF39Sm^r, and that of the Tn5-mob mutant strain JB45 In addition, the LPS from the *E coli* serotype 026 B6 (sigma) was included for the purpose of comparison

The wild-type strain gave rise to two bands The slower migrating of these bands was designated LPS I The broadness of this band is thought to arise not because of the LPS molecules differing from one another by a single O-antigen, but rather because of differing aggregation states of the LPS molecules In *Rhizobium* species this heterogenous fraction is thought to be composed of a relatively short polysaccharide rather than a long repeating oligosaccharide (Carlson, 1983)

Figure 24 Electrophoresis of purified LPS in a 13% Polacrylamide gel, stained with silver as described in section M22

Lane 1 *E coli* serotype 026 B6, the arrow indicates the high molecular weight repeating O-antigen

Lane 2 LPS from the mutant strain *R leguminosarum* biovar *viciae* JB45,which shows the presence of the LPS II band only

Lane 3 LPS from the wild-type strain *R leguminosarum* biovar *viciae* VF39Sm^{r}, which shows the presence of LPS I and LPS II

Lane 4 Pre-stained protein molecular weight markers

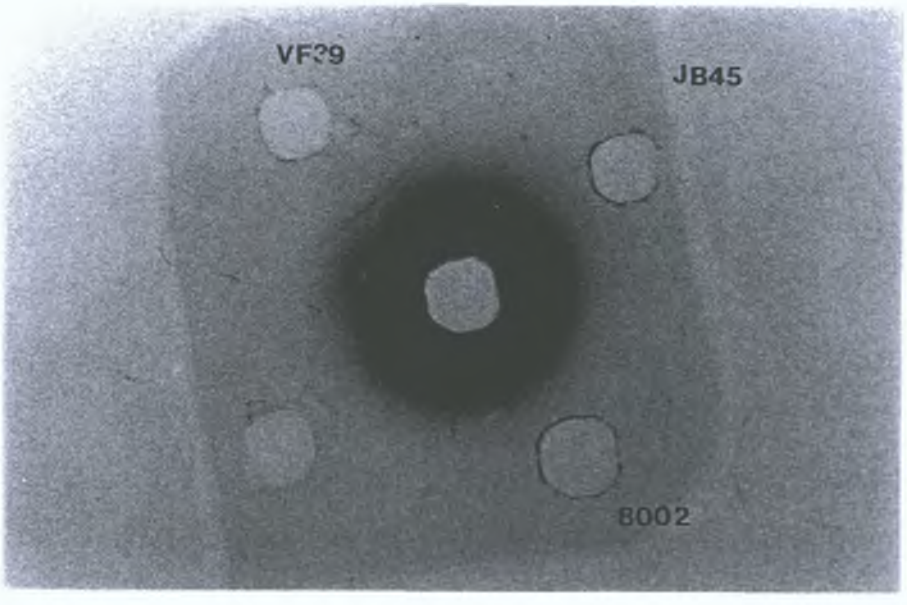


Figure 25. Microdouble diffusion gel. Undiluted mouse antiserum (centre well) formed against the LPS purified from *R.leguminosarum* biovar *viciae* VF39Sm^r, was diffused against lyophilized cells, 100μg/ml in phosphate buffered saline, of *R.leguminosarum* biovar *viciae* VF39Sm^r, JB45 and *R.leguminosarum* biovar *phaseoli* 8002. Each of the outer wells contained about 20μl of cell suspension of the indicated strain. The center well contained about 20μl of antiserum.

The lower molecular weight band most probably consists of the lipidA-core oligosaccharide (Carlson, 1984)
The mutant strain JB45 consists only of the fast migrating LPS II band
The LPS II band of the mutant strain appears to migrate slightly faster than that of the wild-type strain, VF39Sm^r, however, this slight difference in migration rate could not be amplified by examination of LPS profiles on 15% or 17% SDS-PAGE gels, (data not shown)
The O-antigen of the *E coli* serotype 026 B6 is much larger than that of *Rhizobium leguminosarum* biovar *viciae* strain used in this study The banding pattern of the apparently high molecular weight fraction is consistant with that of LPS molecules differing from one another by one O-antigen side chain repeat unit, (Petersen and McGroary, 1985) The lipid A-core oligosaccharide region of the *E coli* strain is also larger than that of the strain of *Rhizobium leguminosarum* biovar *viciae* used during this study

Polyclonal antibodies (IgG) were raised in mice against isolated wild-type LPS as described in section M19 The reaction between LPS antisera and lyophilized cells was detected by the formation of precipitin bands on double diffusion gels as outlined in section M20 The antiserum raised against the LPS isolated from strain *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r reacted with VF39Sm cells to form a precipitin band as expected, Figure 25 However no such reaction was observed with cells from the mutant strain JB45 Therfore the O-antigen of strain *Rhizobium leguminosarum* biovar viciae appears to be the immunodominant portion of the LPS molecule The O-antigen also appears to be biovar specific in that it does not react with cells of *Rhizobium leguminosarum* biovar *phaseoli* 8002, Figure 25

Quantity and Size Distribution of EPSs
Measurement of soluble acidic EPS produced by *Rhizobium* strains was performed as described in section M28
The wild-type strain,VF39Sm^r, produced 3,100 μg glucose equivalents per ml of culture, whereas the mutant strain, JB45, produced only 410 μg of glucose per ml equivalents Therefore JB45 produces only 13% of the amount of soluble EPS produced by the wild-type strain

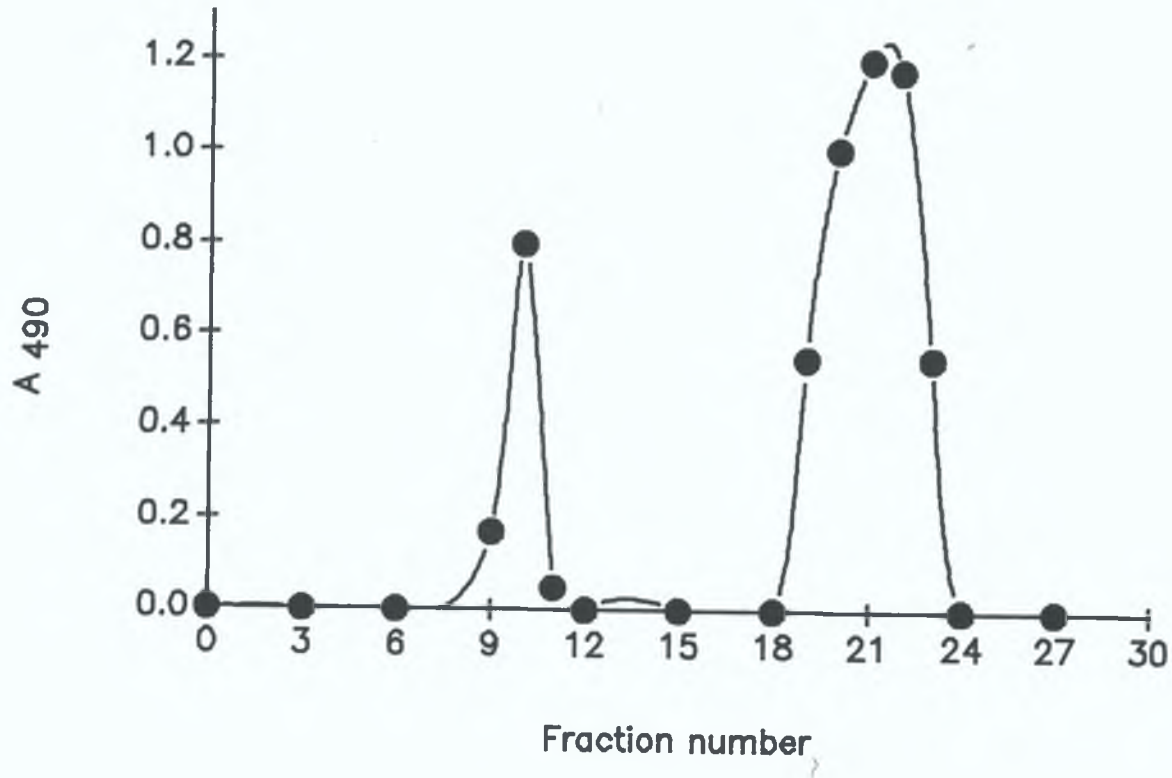


Figure 26. A BioGel A5m elution profile of the soluble exopolysaccharide of *R.meliloti* 2011. The column was run as described in section M29. The marks on the left and right indicate the void volume and the bed volume respectively.

The distribution of soluble extracellular carbohydrates of *Rhizobium leguminosarum* biovar *viciae* strains, VF39Sm^r and JB45, according to molecular weight was demonstrated by gel filtration of whole supernatants on a Bio-gel A-5m column as described in section M29 *Rhizobium meliloti* was included in this analysis as the EPS from *Rhizobium meliloti* is well characterized with regard to the number and size of the carbohydrate fractions present in its culture supernatants, (Zevenhuizen and vanNeerven, 1983, Amemura et al , 1983, Leigh and Lee, 1988)

After incubation of these strains in VGM medium for 6 days the following levels of phenol/sulphuric acid reactive material accumulated in the culture supernatants (in micrograms of glucose per millilIter equivalents) *Rhizobium leguminosarum* biovar *viciae* VF39Sm, 1,300, *Rhizobium leguminosarum* biovar *viciae* JB45, 180, *Rhizobium meliloti* 2011, 900

Following fractionation of *Rhizobium meliloti* 2011 culture supernatant on a Bio-gel A-5m column two phenol/sulphuric acid reactive fractions were resolved, Figure 26 This result is in agreement with those of other workers who reported that culture supernatants of *Rhizobium meliloti* strains contain, in addition to the succinoglycan polymer, a low molecular weight acidic fraction corresponding to the structure of the repeat unit of the succinoglycan polymer, (Zevenhuizen and vanNeerven, 1983) The high molecular weight fraction eluted close to the void volume, while the low molecular weight fraction eluted close to the salt volume Zevenhuizen and vanNeerven, (1983), estimated that the high molecular weight fraction had a molecular weight of several million daltons whereas the low molecular weight fraction had a molecular weight of $\leq 10^4$ daltons The low molecular weight fraction of the *Rhizobium meliloti* 2011 culture supernatant was seen to be the major fraction, Figure 26 This result is in agreement with those reported by Zenvenhuizen and vanNeerven, (1983), for strain SU47 and from those of Leigh and Lee, (1988), for strain 1002.

The elution profile of the wild-type *Rhizobium leguminosarum* biovar *viciae* strain,VF39Sm, was quite different to that of *Rhizobium meliloti* 2011, Figure 27(a) The parental *Rhizobium leguminosarum* biovar *viciae*

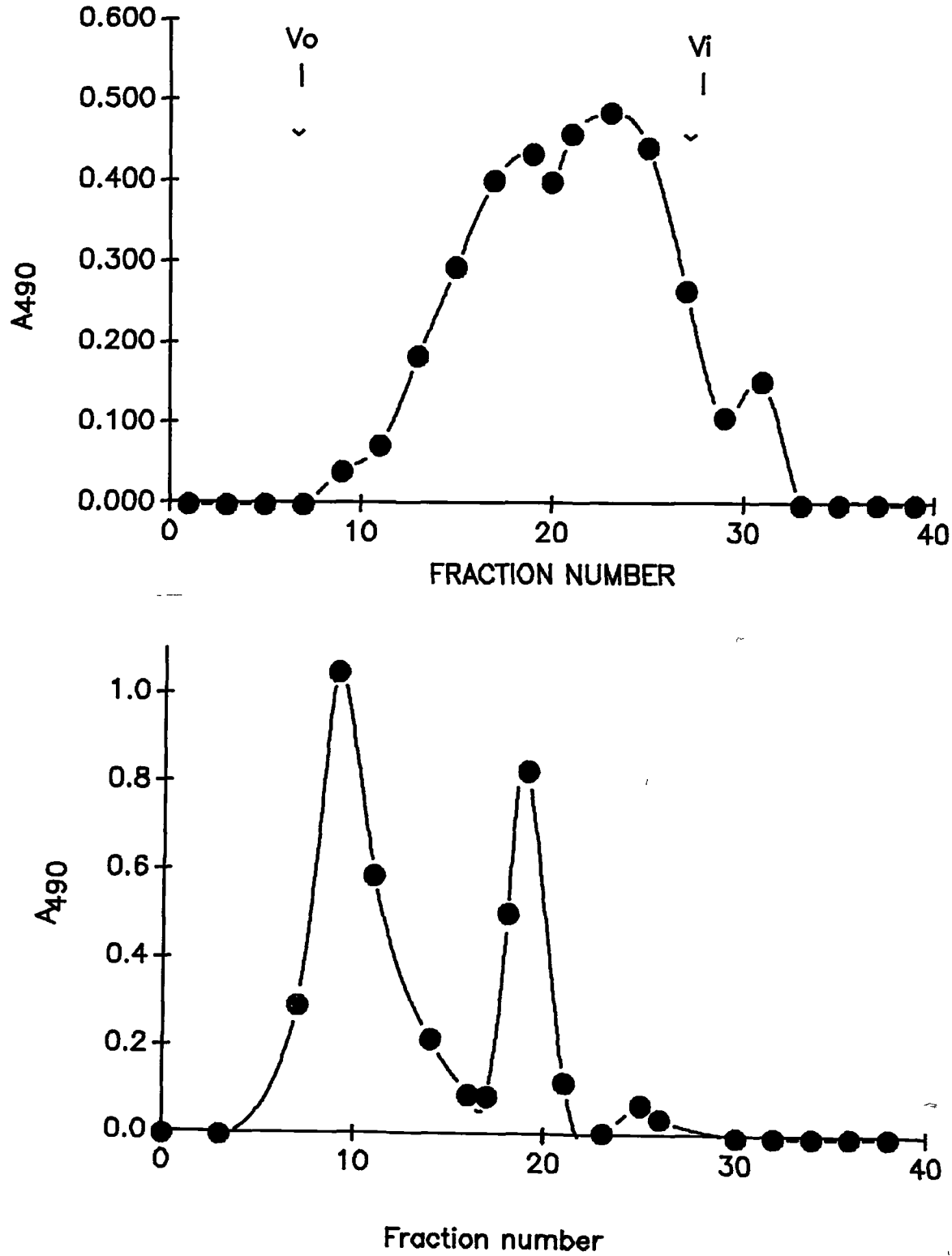


Figure 27 A BioGel A5m elution profile of soluble exopolysaccharide from the parent strain *R leguminosarum* biovar *viciae* VF39Sm^{r} (A) and the mutant strain *R.leguminosarum* biovar *viciae* JB45(A) The column was run as described in section M29 The marks on the left and right indicate the void volume and the bed volume respectively

strain's EPS eluted in almost in the entire range of the column indicating that this EPS is heterogenous in size, Figure 27(a) This result is in agreement with the results reported for *Rhizobium leguminosarum* biovar *viciae* 128C53, (Carlson and Lee, 1982), and therefore may represent a general property of the EPS of *Rhizobium leguminosarum* biovar *viciae*

The elution profile of the mutant strain, JB45, was different to that of the wild-type strain Two peaks were resolved, a major high molecular weight peak eluted in the void volume whereas a minor peak with a molecular weight of $\simeq 10^4$ daltons eluted near the included volume, Figure 27(b) The identity of the latter peak is not known, however, no KDO, the biochemical marker for LPS, could be detected in this peak

Chemical Composition of EPSs

Acidic soluble EPS from the wild-type and mutant strain were prepared as outlined in M24 and M25

Gas-liquid chromotography / Mass spectrometery analysis of the alditol derivatives of the hydrolysed polysaccharides of the mutant and wild-type strains revealed that both strains' EPS was composed of the same molar ratio of galactose, glucose and galacturonic acid (R Carlson, personal communication)

Such analysis while revealing that the mutant and wild-type strain possess the same glycosyl residues, does not reveal whether the O-acyl substitution patterns of these polysaccharides is the same Therefore the chemical shifts of the hydrogen atoms of the O-acyl substituents were compared using proton magnetic resonance

EPS from both strains was isolated according to M 25 Following isolation the native EPSs were found to form solutions which were too viscous to yield NMR spectra, (data not shown)

The viscosity of the samples was reduced by depolymerization of the polymers with ultrasonic irradiation as described in M31

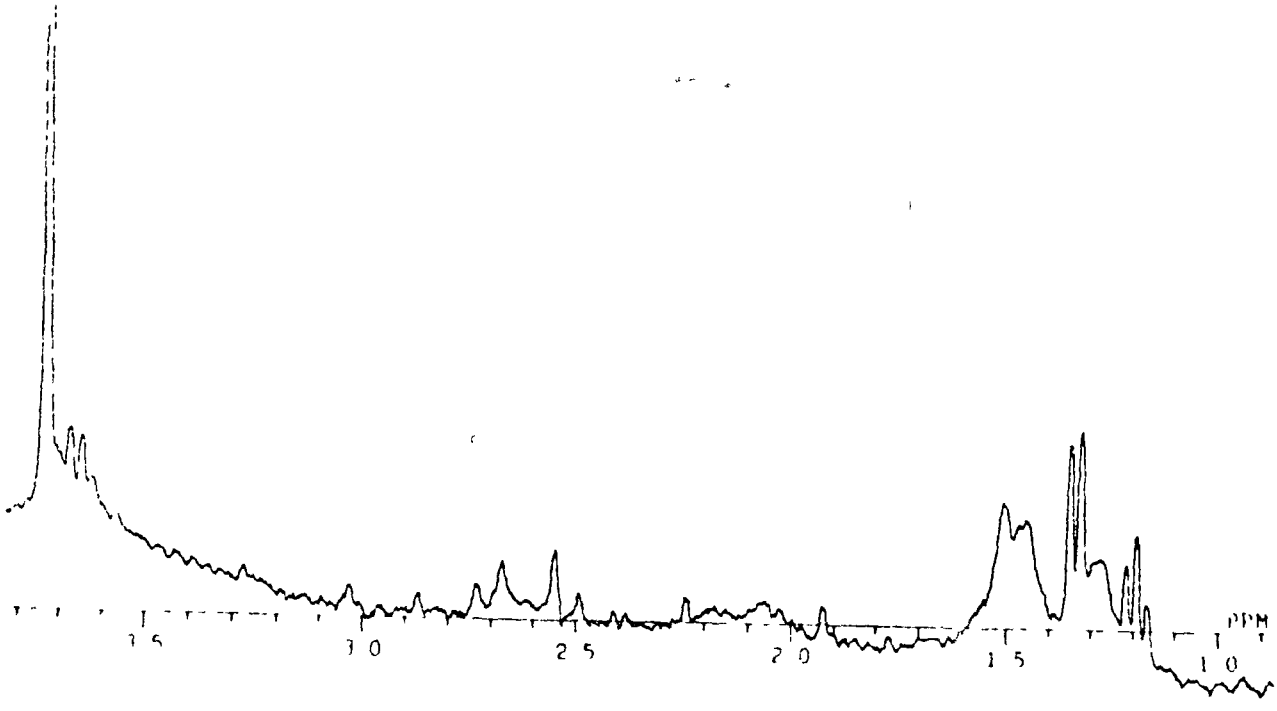


Figure 28(a) Proton NMR spectrum of the high molecular weight fraction from the mutant strain JB45 This fraction was depolymerized with ultrasonic irradiation for 20 min as described in section M31

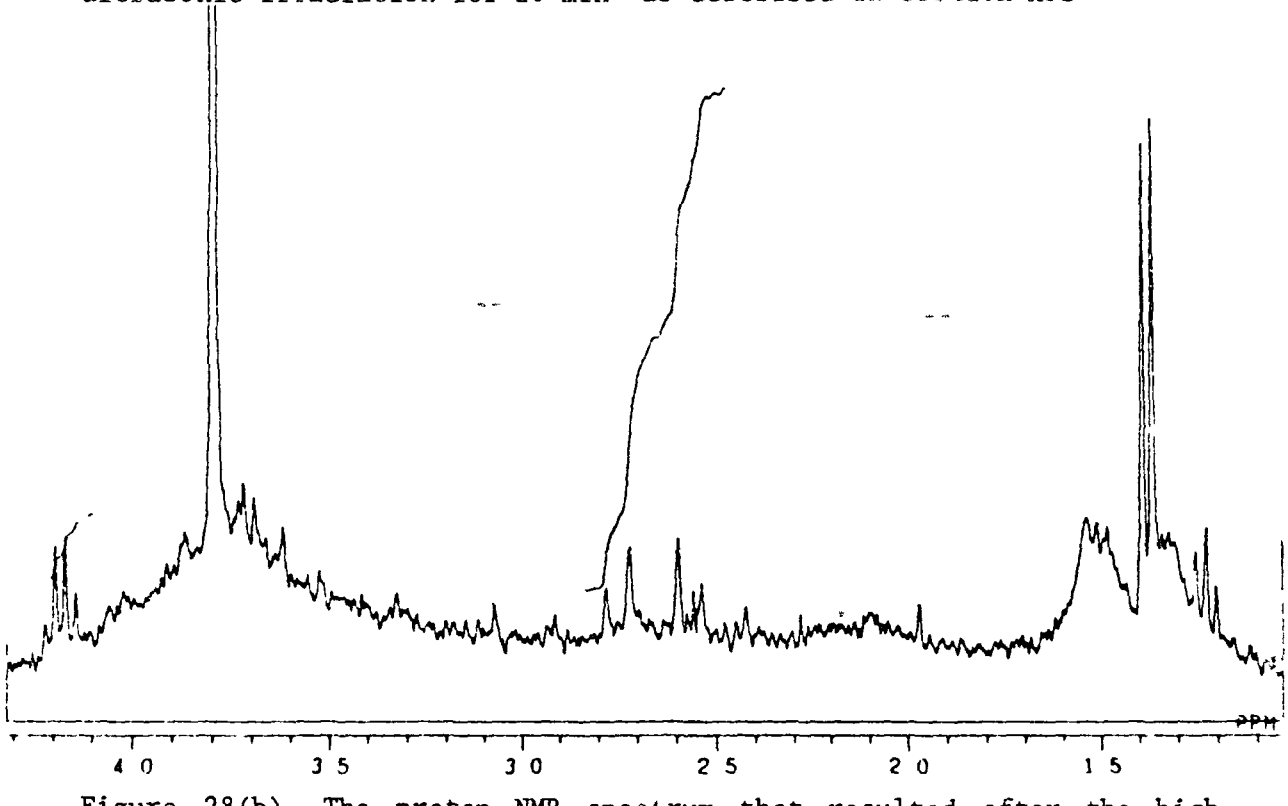


Figure 28(b) The proton NMR spectrum that resulted after the high molecular weight fraction from JB45 was depolymerized for 40 min as described in section M31

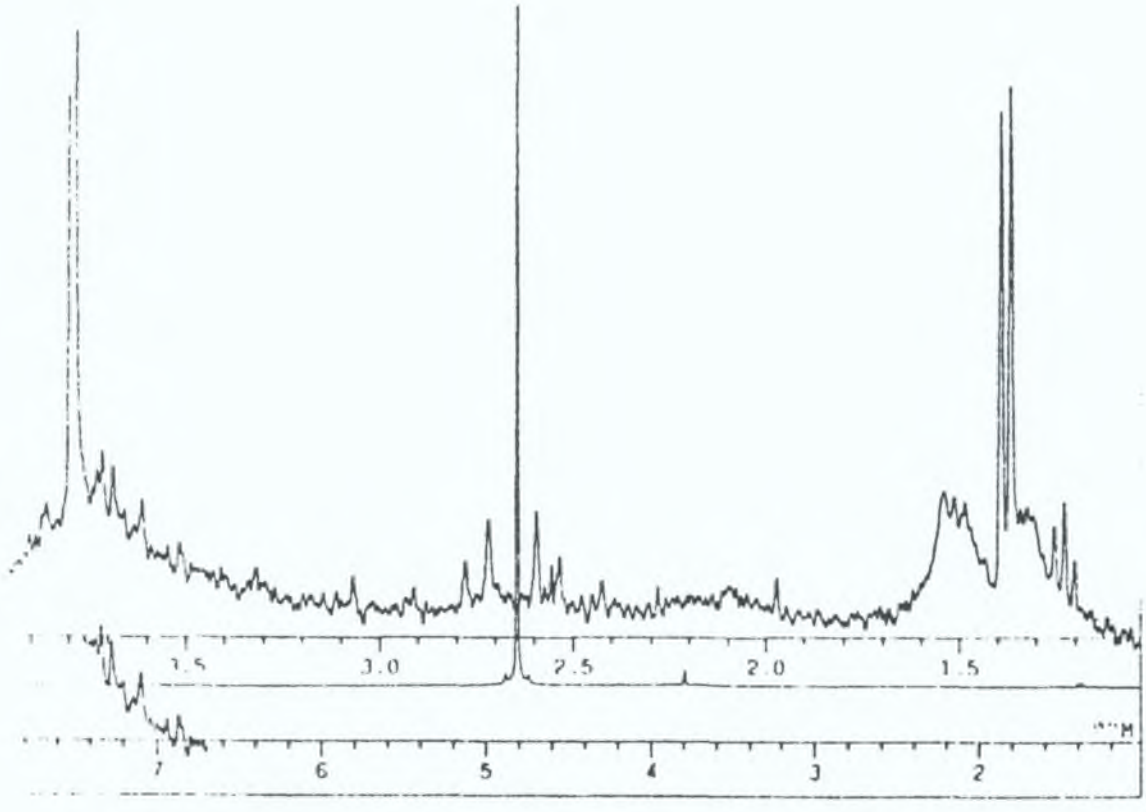


Figure 28 (c). The proton NMR spectrum which resulted after the high molecular weight fraction from the mutant strain JB45 was irradiated with ultrasound for 60 min.

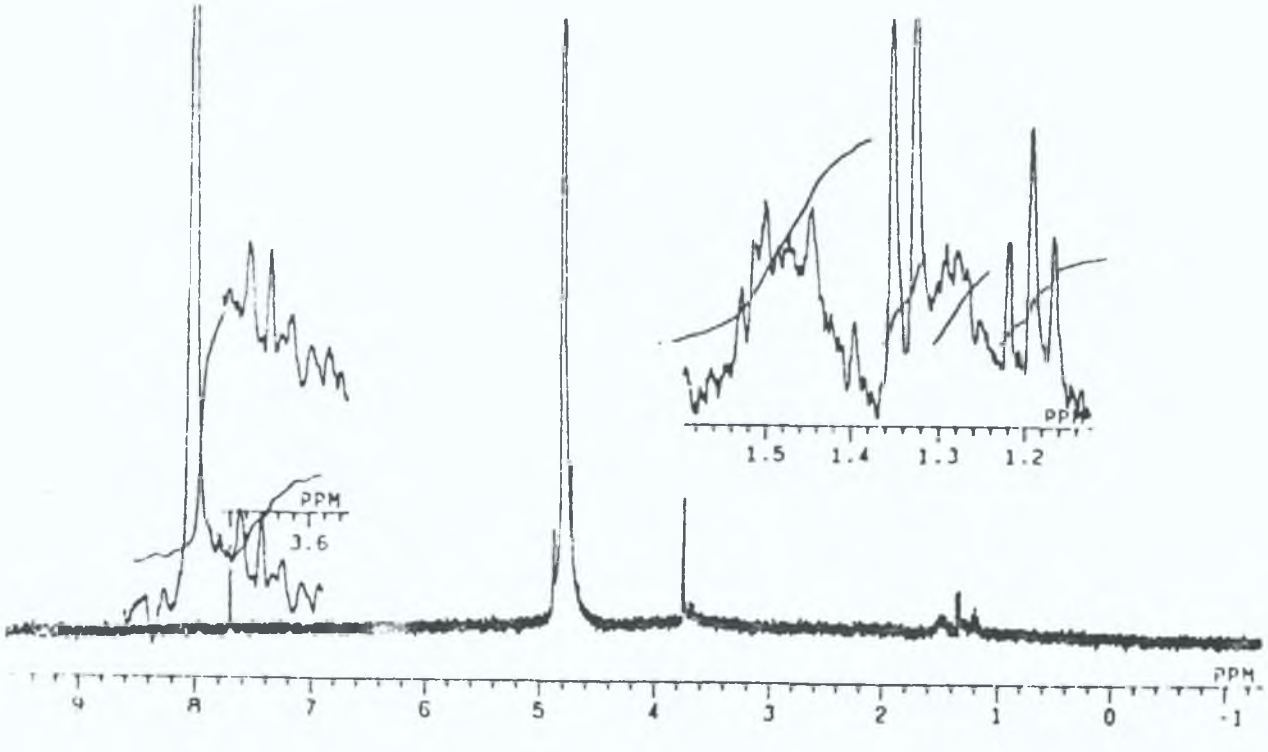


Figure 29(a). Proton magnetic resonance spectrum of a high molecular weight fraction from the wild-type strain VF39Sm^{r} following 20 min. ultrasonic irradiation

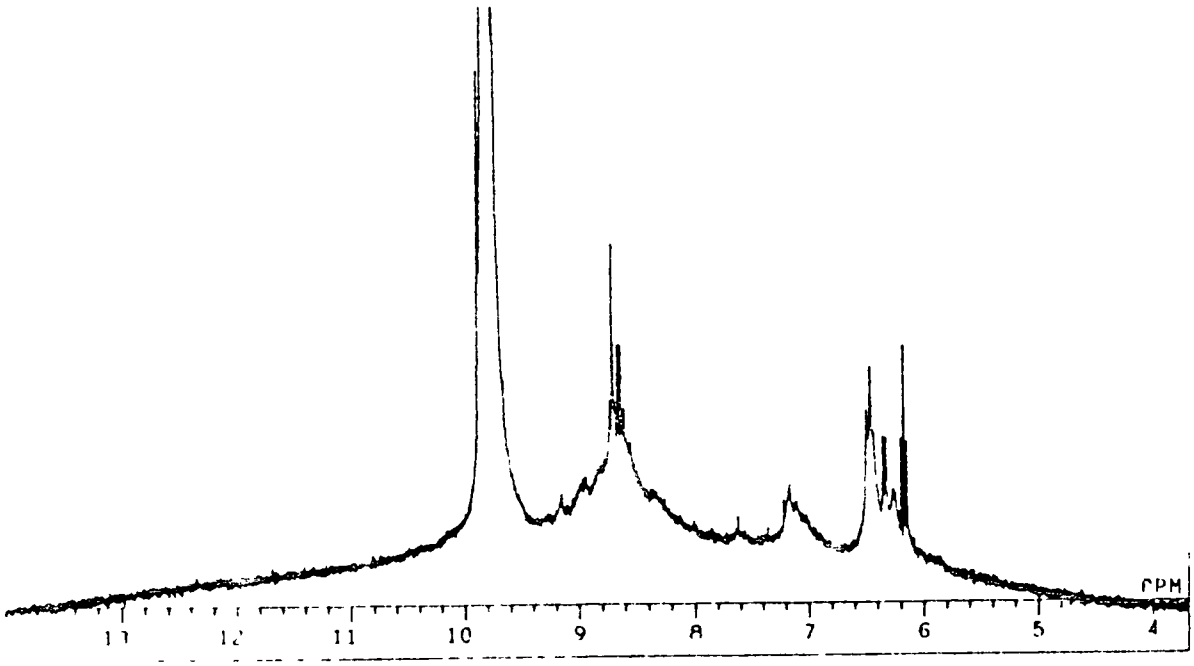


Figure 29(b) Proton NMR spectrum of a high molecular weight fraction from the wild-type strain VF39Sm^r following 40 min ultrasonic irradiation

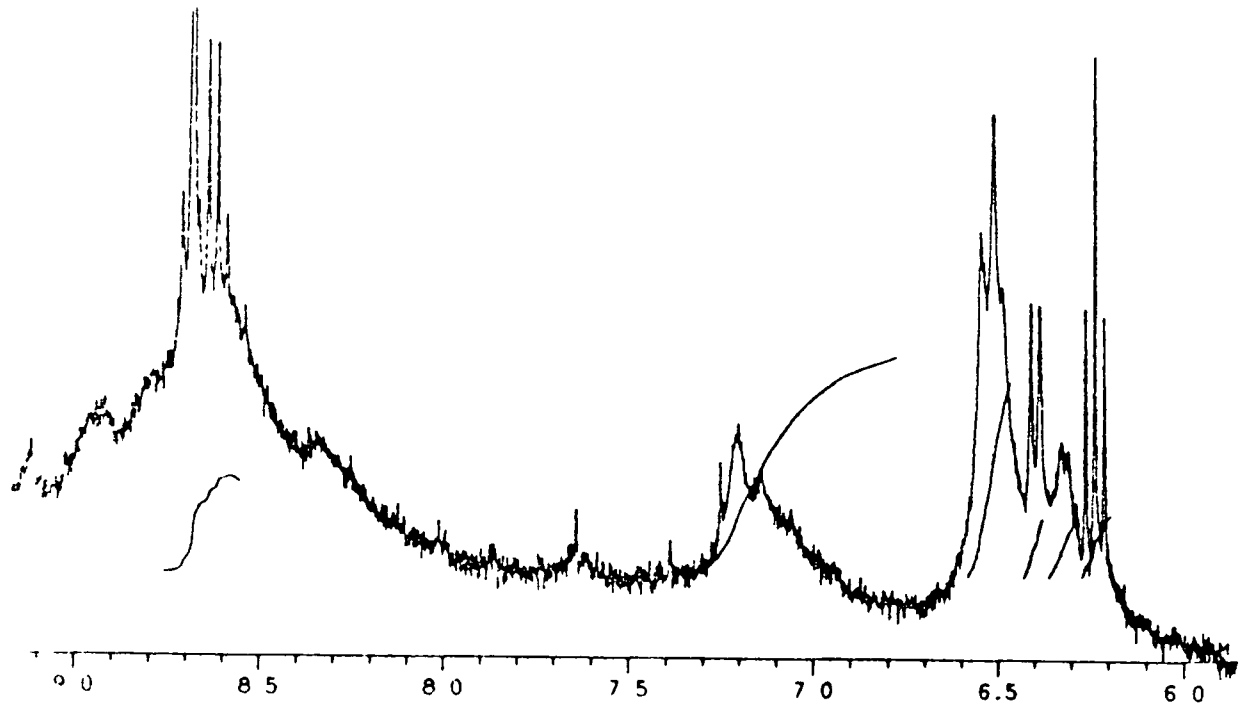


Figure 29(c) Proton magnetic resonance spectrum of a high molecular weight fraction from the wild-type strain VF39Sm^r after 60 min ultrasonic irradiation

The ^{1}HNMR spectra of the wild-type strain's EPS after sonication for 20, 40, and 60 mins are shown in Figure 28 (a), (b), and (c) respectively The ^{1}HNMR spectra of the EPS isolated from the mutant strain following irradiation for 20, 40, and 60 mins are shown are in Figure 29 (a), (b), and (c) respectively It is clear from Figures 28 and 29 that the viscosity of the polymers was reduced and so it was possible to obtain NMR spectra However the line width of the ^{1}HNMR signals was broadened possibly because of residual viscosity and it was not possible to assign the signals to particular O-acyl substituents, specifically to the methyl and methylene hydrogen atoms of the acyl substituents which resonate in between chemical shifts of 2 9 and 1 3 Nevertheless from examination of these spectra it can be seen that the spectrum from the wild-type and the mutant strains are identical

To verify that all the signals present in these spectra were due to hydrogen atoms present in the EPS samples and that were not due to residual cetylpyridinium chloride (which was used in the isolation procedure), the ^{1}HNMR spectrum of CPC was obtained and is shown in Figure 30(a) A sample of CPC was also used to "spike", a sample of the wild-type depolymerized EPS and the ^{1}HNMR spectrum was obtained, Figure 30(b) It can be seen with reference to Figure 30 that all of the signals in the EPS spectra were in fact due to EPS hydrogens

Therefore all the discernible, structural features of the acidic EPS from *Rhizobium leguminosarum* biovar *viciae* VF39Sm^{r}, and its LPS mutant strain JB45 were identical

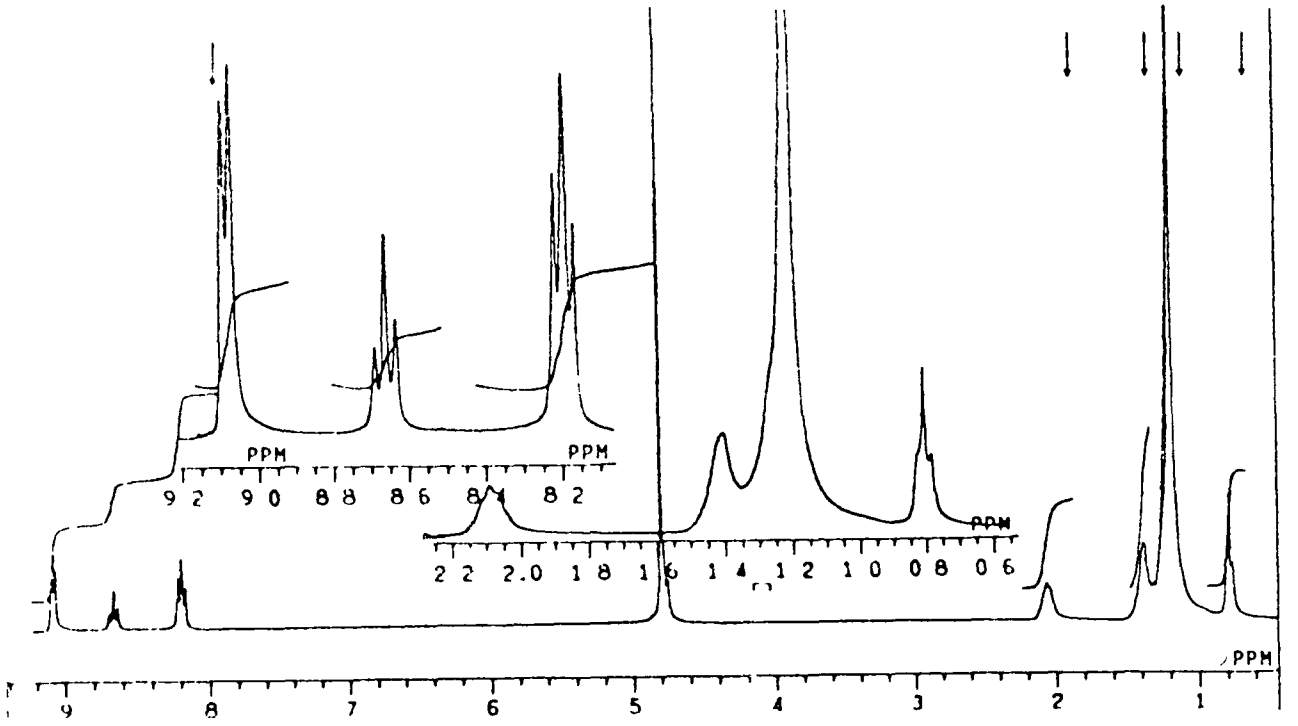


Figure 30(a) The proton NMR spectrum of cetylpyridinium chloride, the compound used in the EPS isolation procedure

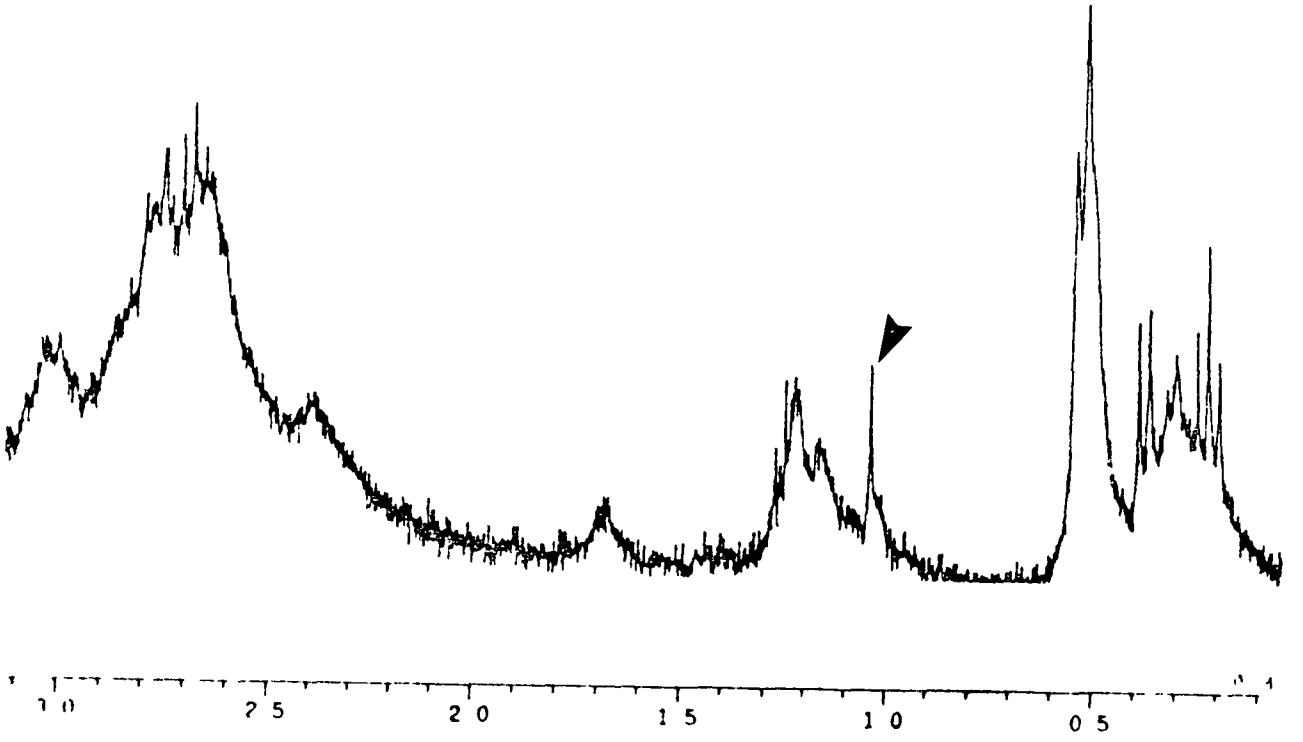


Figure 30(b) The proton nuclear magnetic spectrum of a high molecular weight fraction from the wild-type strain VF39Sm^{r} which had been depolymerized by treatment for 60 min with ultrasonic irradiation and "spiked" with cetylpyridinium chloride (CPC) The arrow indicates a chemical shift due to CPC

Synthesis of β-(1⟶2)-Glucan

Hot water-ethanol extraction of *Rhizobium meliloti* SU47 cells has been reported to release β-(1⟶2)-Glucan (Zevenhuizen and vanNeerven, 1983) Strains VF39Sm^{r}and JB45 were subjected to this extraction as described in section M30 For comparison, *Rhizobium meliloti* 2011 was included in this analysis as *Rhizobium meliloti* SU47 (the strain from which *Rhizobium meliloti* 2011 is derived) is known to produce a neutral β-(1⟶2)-Glucan (Zevenhuizen and vanNeerven, 1983)

The presence of charged groups on the β-(1⟶2)-Glucan molecule was studied by chromatography on a QAE-Sephadex A-50 anion exchange column using a gradient (5mM to 1M) of ammonium bicarbonate as described in section M30

The elution profile of the *Rhizobium meliloti* β-(1⟶2)-Glucan is shown in figure 31 A major peak of phenol/sulphuric acid material was eluted with millimolar Na_4HCO_3, which indicated that this fraction did not adsorb to the column, and that it corresponded to a fraction that possessed no non-sugar substituents This result is in agreement with those reported previously, (Zevenhuizen and vanNeervan, 1983, Leigh and Lee, 1988)

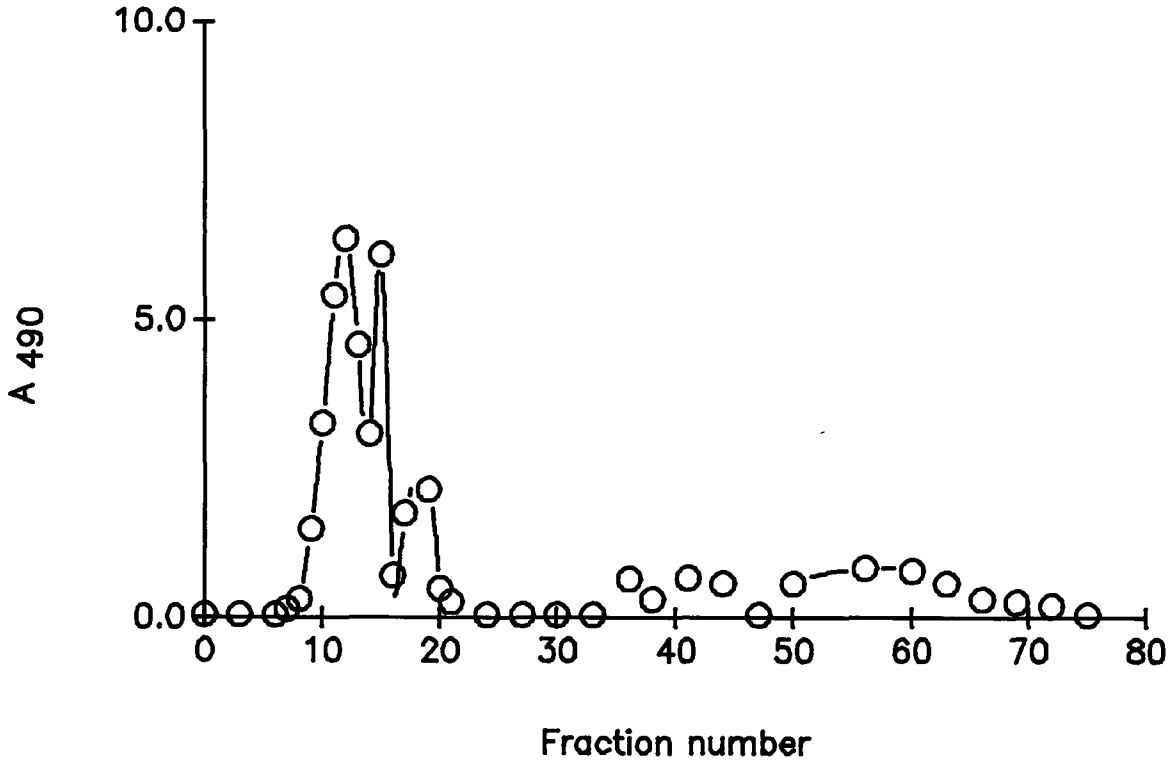
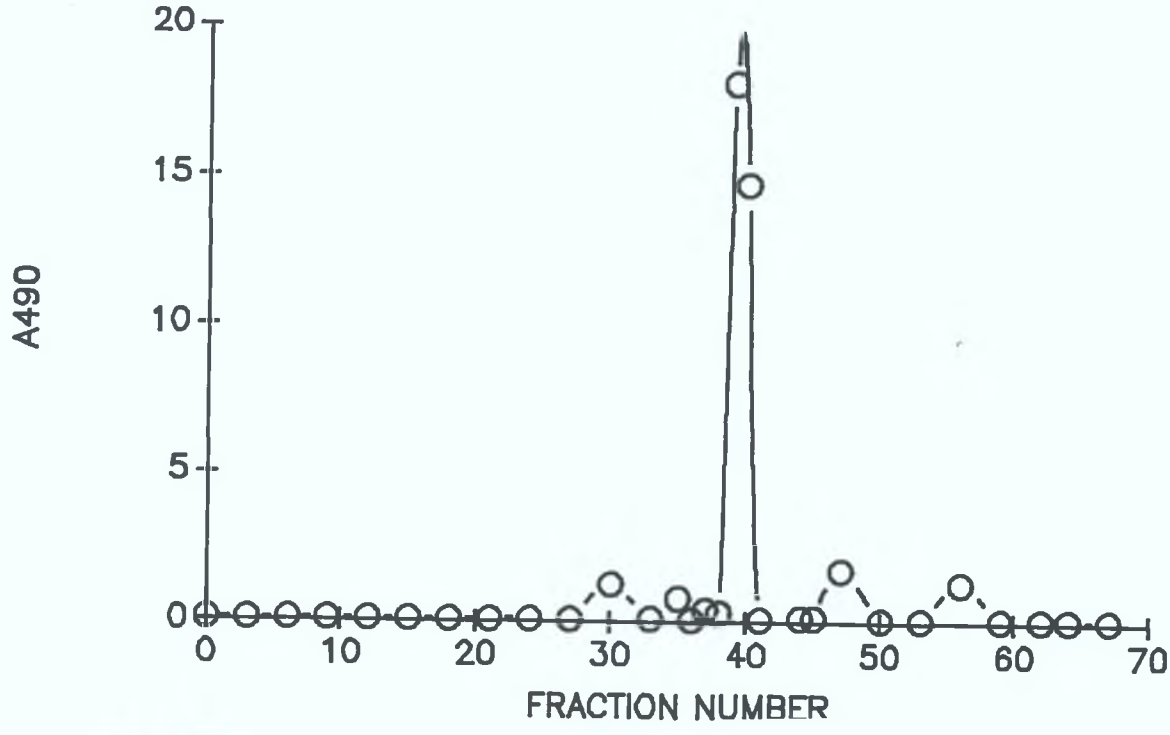


Figure 31 Elution profile on Sephadex QAE A50 anion exchange chromatography of ethanol-water extracts from *R meliloti* cells, using a Na_4HCO_3 gradient The column was run as described in section M30

Anion exchange chromotography of ethanol extracts of *Rhizobium leguminosarum* biovar *viciae* VF39Sm^{r} gave a single peak of phenol/sulphuric reactive material, Figure 32(a) This fraction did adsorb to the column and was only eluted by the sodium bicarbonate gradient, which indicates that this fraction is acidic

In contrast the ethanol-water extracts of the mutant strain JB45, yielded only a slight peak of phenol/sulphuric acid reactive material which eluted in the millimolar range of the gradient Figure 32(b) This indicates that the mutant strain does not produce any acidic β-(1⟶2)-Glucan

Such acidic β-(1⟶2)-Glucans have been reported for several *Rhizobium* species (Batley et al , 1987, Hisamatsu and Yamada, 1987, Miller, 1987) However this is the first report of acidic β-(1⟶2)-Glucan in *Rhizobium leguminosarum* biovar *viciae*



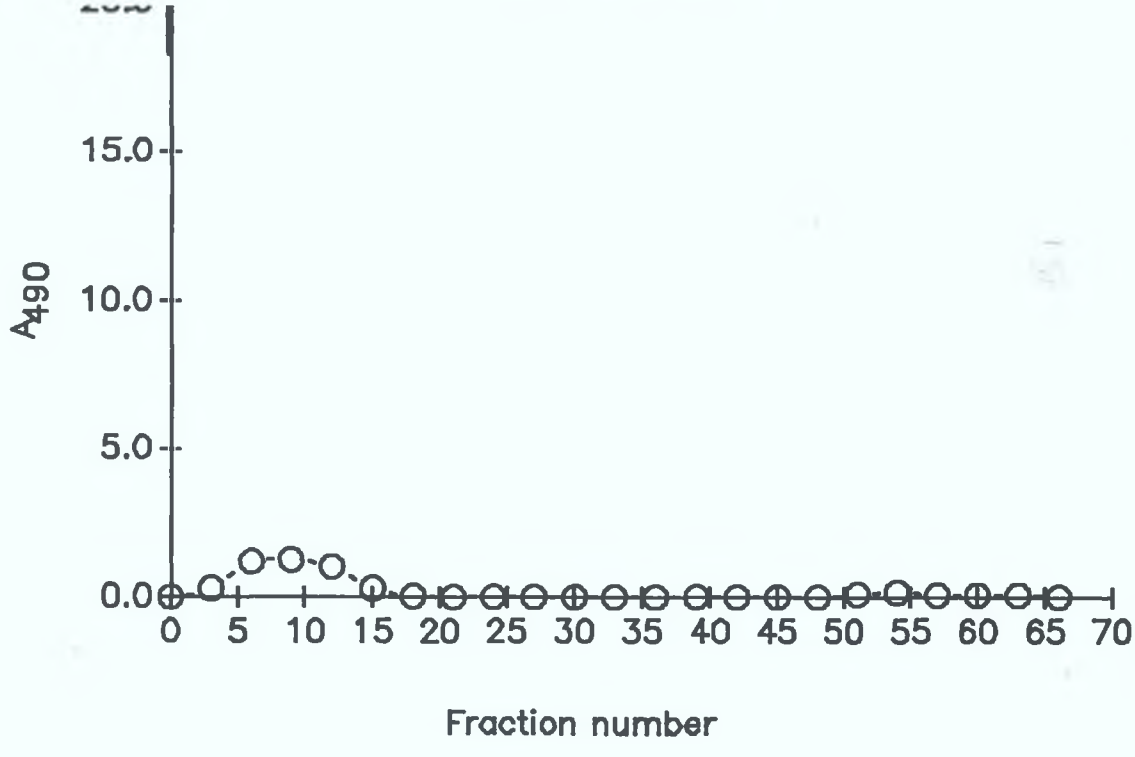


Figure 31 (A). The elution profile on Sephadex QAE A50 anion exchange chromotography of ethanol-water extracts of the wild-type strain *R.leguminosarum* biovar *viciae* VF39Sm^{r} cells. The column was run as described in section M30.

Figure 31(b). The elution profile on Sephadex QAE A50 anion exchange chromotography. of ethanol-water extracts of the mutant strain *R.leguminosarum* biovar *viciae* JB45 cells. The column was run as described in section M30.

Elaboration of Capsules

Capsular polysaccharide was examined by electron microscopy using the glutaraldehyde\ruthenium red\uranyl acetate (GRU) procedure described in M32 *Rhizobium* species do not elaborate a capsule when grown in liquid medium (J Vasse, personal communication), therefore *Rhizobium leguminosarum* biovar *viciae* strains VF39Sm and JB45 were grown on solid medium in order to induce capsule formation

The wild-type strain was shown to produce its capsule by polar secretion, Figure 11, which is a similar mechanism to that reported by Sherwood et al ,(1983) Approximately 14% of a population of *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r cells elaborated capsules, Figure 33

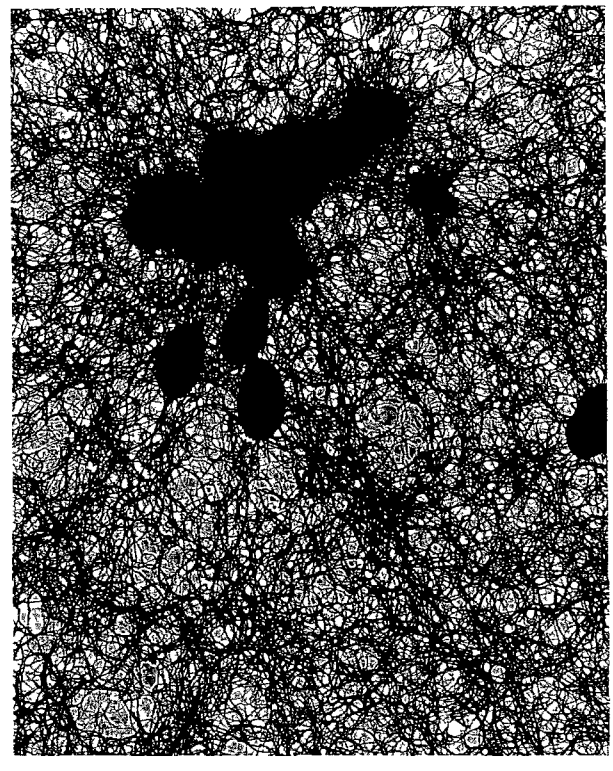


Figure 33 A group of encapsulated wild-type VF39Sm^{r}cells contrasted by use of the Glutaraldehyde-Ruthenium red-Uranyl acetate (GRU) procedure The polysaccharide web and capsules are positively stained by this stain, X 5410

The mutant strain also produced capsules but only 2% of the population of cells examined possessed a capsule, Figure 34 Figure 34 also shows that ruthenium red stained the exopolysaccharide "web" of the mutant strain Since ruthenium red is a hexacationic dye it will only stain acidic polysaccharides, because the "web" is clearly visible in electronmicrographs of the mutant cells this confirms that the EPS of the mutant is in fact acidic

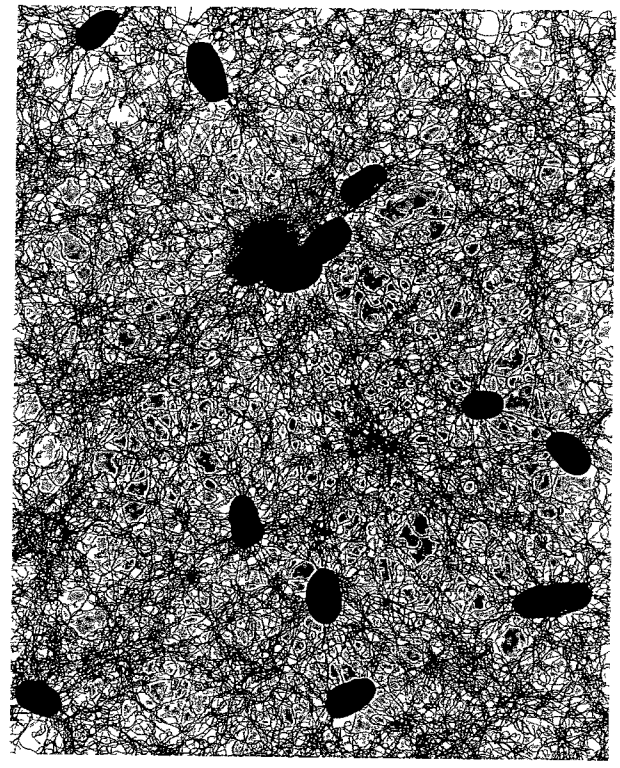


Figure 34 A group of mutant JB45 cells stained by the G/R/U procedure (section M32) The arrow indicates an encapsulated cell The polysaccharide web and capsule are positively stained by this stain, X 4328

Flagella and Microfibrils

It was observed while studying the capsular EPS by the GRU procedure that vortexing five day old plate-grown cultures resulted in a marked disruption of the polysaccharide web enabling other surface characteristics of the mutant to be observed

Figure 35 shows the presence of morphologically normal flagella on mutant cells in spite of the fact that this mutant is non-motile

Figure 36 shows the presence of "sticky microfibrils", which are most probably cellulose fibrils which are thought to play a role in the attachment of *Rhizobium* cells to the plant cell surface (Smit et al , 1988)

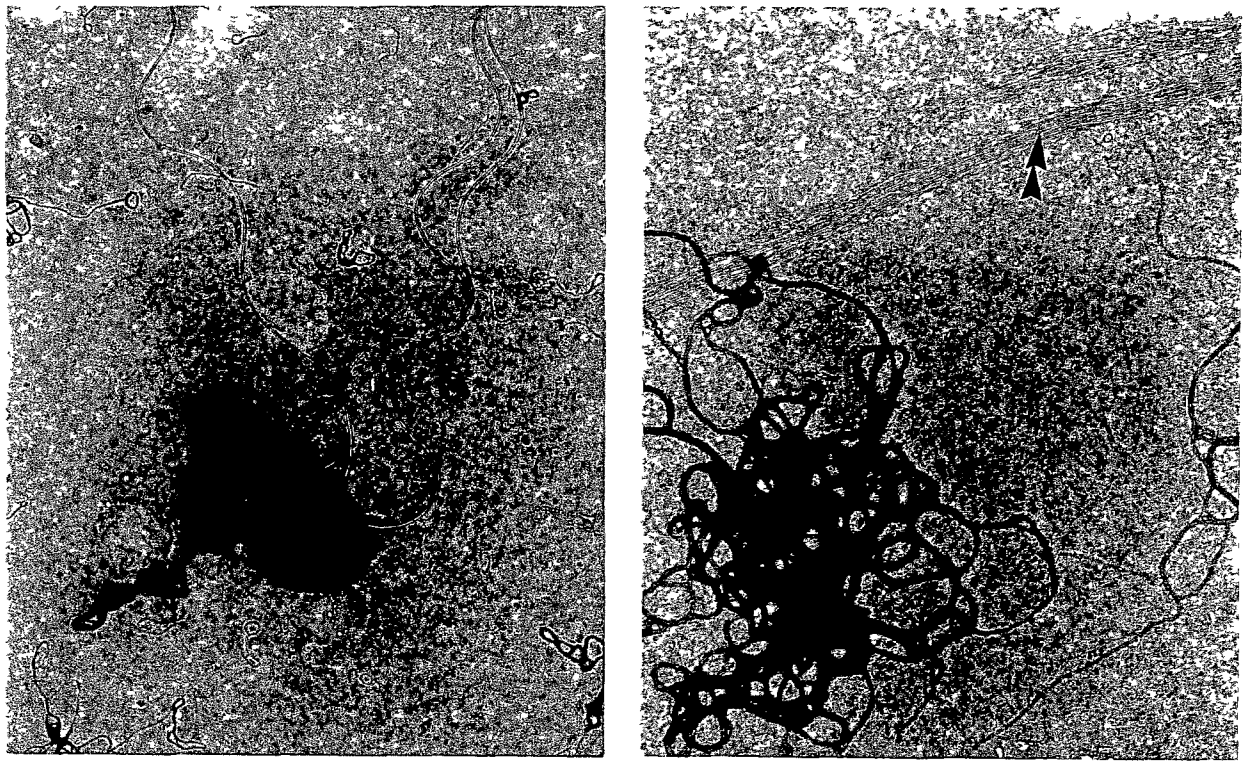


Figure 36 Flagella negatively stained by use of the GRU procedure, X 16232.

Figure 37 Negatively stained sticky microfibrils ("cellulose") double arrow, could be distinguished from positively stained EPS by the G/R/U procedure, X 54100

Outer Membrane Proteins

Lipopolysaccharide molecules are known to interact with proteins in the outer membrane of gram-negative bacteria Rough mutants of enteric bacteria are known to contain lower levels of protein in their outermembranes, (Ames et al , 1974, Koplow and Goldfine, 1974),therefore it was of interest to examine the outer membrane proteins of the mutant isolated during this study

Outer membranes from cells grown in complex medium were prepared according to the method of deMaagd and Lugtenberg,(1986), as described in sectionM33 Outer membrane protein profiles were obtained by PAGE in 10% gels followed by staining with coomassie blue as described in section M21

Comparison of the protein profiles of the wild-type *Rhizobium leguminosarum* biovar *viciae* VF39Sm with that of its surface mutant JB45, showed that all the protein bands present in VF39Sm, were also present in strain JB45, (data not shown) However some of the lower molecular weight bands in the mutant strain were less intense that the corresponding bands in the wild-type strain

Outer membranes were isolated from cells grown in modified VMM which contained either 1μM $FeCl_3$ (low iron), or 10μM $FeCl_3$ (high iron) The outer membranes were isolated from cells grown in such minimal medium by the method outlined in section M34 SDS- PAGE was performed in 10% gels and stained with coomassie blue as described in M21

When the outer membrane protein profiles of the mutant strain, JB45, were compared to its parent strain at least four extra bands ranging in size between 61K and 85K were evident, Figure 17 The wild-type strain could be induced to produce proteins of this size range by growth under iron-limiting conditions, Figure 37 The mutant strain was unable to grow in medium containing only 1μM $FeCl_3$

Rhizobium species become iron stressed when grown in liquid medium where the concentration of free iron is <1μM $FeCl_3$ and

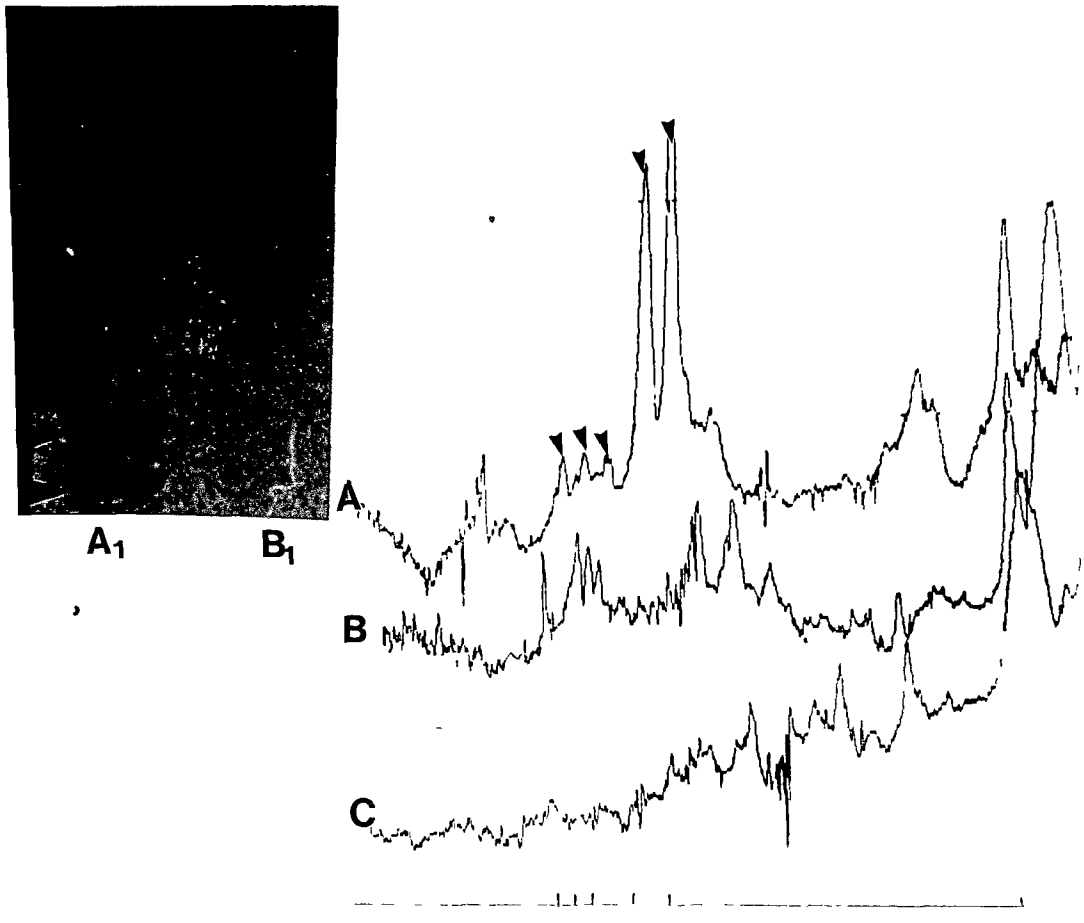


Figure 37 Polyacrylamide gel electrophoresis of solublized outer membranes isolated from cells grown in minimal medium

Densitometer scans of

A The wild-type strain grown in iron limiting conditions (1μM $FeCl_3$)

B The mutant strain JB45 grown in medium containing 10μM $FeCl_3$

C The wild-type strain grown in medium containing 10μM $FeCl_3$

A_1 The lane from which the densitometer scan A was obtained

B_1 The lane from which the densitometer scan B was obtained

Arrows indicate the protein bands that were induced in the wild-type strain in response to iron limitation

Rhizobium leguminosarum biovar *viciae* VF39Sm responds to iron stress by synthesising a siderophore (G Reigh personal communication)

In response to iron stress bacteria express proteins termed iron regulated outer membrane proteins, IRMPs In enteric bacteria growth on low iron medium induces the appearance of the siderophore enterochelin as well as specific outer membrane proteins Three IRMPs of molecular weight 82K, 79K, and 77K are induced in *Salmonella typhimurum* (Bennet and Rothfield, 1976), and proteins of molecular weight of 83K, 81K, and 74K are expressed as major proteins in *E coli* in response to iron limitation, (Ichichara and Mizushima, 1977) In this study *Rhizobium leguminosarum* biovar *viciae* was shown to induce a triplet of protein bands with molecular weights of 86K, 82K, and 72K as well as two other proteins having molecular weights of 67K and 61K, Figure 17 Therefore the 86K, 82K and 72K appear to be conserved between enteric bacteria and the soil bacterium *Rhizobium leguminosarum* biovar *viciae*

The surface mutant *Rhizobium leguminosarum* biovar *viciae* JB45 expresses the three proteins constitutively

Two other bands having molecular weights of 67K and 61K are derepressed by iron-limitation in the wild-type strain *Rhizobium leguminosarum* biovar *viciae* VF39Sm In the mutant strain JB45, only the 61K protein is expressed and it was expressed at a lower intensity than that of the wild-type 61K protein, Figure 17 The 67K IRMP could not be detected in the mutant strain JB45, Figure 17

Kvach et al , (1977) have shown that the ability of *Salmonella typhimurium* LPS core mutants to acquire iron is proportional to the severity of the core defect The *Rhizobium leguminosarum* biovar *viciae* LPS mutant JB45 exhibits a similar inability to transport iron

DISSCUSSION II

The surface mutant isolated during this study lacks at least the O-antigenic part of its LPS molecule It has been suggested that this part of the LPS molecule may interact with a plant receptor and that this interaction is essential for the formation of an effective symbiosis (Bohool and Smidt, 1974, Kato et al , 1979, Halverson and Stacey, 1986, Wolpert and Albersheim, 1976) deMaagd et al , (1990), dispute this claim on the basis that their is a very large variety of structure found in the LPS of *Rhizobium leguminosarum* biovar *viciae* strains (Carlson et al , 1983, Carlson et al , 1978) and because of the isolation of a *Rhizobium leguminosarum* biovar *viciae* mutant with an altered (but not lacking) an O-antigen which gave rise to a Fix^+ phenotype (deMaagd et al , 1989)

deMaagd et al , (1989), also isolated mutants of *Rhizobium leguminosarum* biovar *viciae* 248 which lacked the O-antigen of the LPS molecule Although slight changes in membrane protein profiles were observed with these mutants, neither this nor the effect of the change in the surface charge (due to the loss of hydrophilic sugar residues) on symbiotic function can be ignored

Brink et al , (1988), noted that replacement of an O-antigen of an LPS mutant strain of *Rhizobium leguminosarum* biovar *trifolii* ANU843 with that of another strain or even by an unnatural O-antigen results in the restoration of the wild-type nodulation phenotype

The above evidence argues that LPS serves as an important structural entity rather than a specific signal for the interaction with the host-plant

The mutant JB45 lacks the immunodominant O-antigenic oligosaccharide, it may also lack sugars on the LPS II molecule as the LPS II band from JB45 migrates slightly faster than the corresponding band from the wild-type strain Furthermore JB45 exhibits pleiotrophic effects not reported for the LPS mutants by deMaagd *et al* , (1989) and Priefer, (1989) This suggests that the defect in LPS structure is more severe than that of the LPS mutants of *Rhizobium leguminosarum* biovar *viciae* isolated by the Dutch and German groups

Table 4 Comparison of the surface features of *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r and JB45

	VF39Sm^r	JB45
LPS	LPS I LPS II	LPS I
Reaction with Antiserum directed against VF39Sm^r LPS	+	-
EPS (quantity)	100%	13%
(size distribution)	heterogenous	2 defined peaks
(O-Acyl substitution)	+	+
(sugar composition)	identical for EPS of both strains	
Acidic β-glucan	+	-
Motility	+	-
Flagella	+	+
Growth of 1μM $FeCl_3$	+	-
Expression on IRMPs on 10μM $FeCl_3$	-	+
Autoagglutination in high [Ca^{2+}]	-	+
Production of siderophore *	+	++++
Sensitivity to Lysozyme	+	++++

*Geraldine Reigh personal communication
++++ significantly more than that of the wild-type

Rhizobium meliloti mutants with multiple carbohydrate defects have been isolated *ExoB* synthesises only the low molecular weight EPS which unlike that of the wild-type strain is not succinylated, has an altered LPS structure and fails to produce any β-(1-2)-glucan *ExoC* synthesises no EPS, has an altered LPS structure and does not produce any β-(1-2)-glucan The surface defects of the mutant *Rhizobium leguminosarum* biovar *viciae* JB45 are summarized in Table 7

Although the mutant strain *Rhizobium leguminosarum* biovar *viciae* JB45 produces less EPS than the wild-type strain it is chemically identical to that produced by the wild-type strain unlike the *exoB* mutant of *Rhizobium meliloti* The EPS produced by the wild-type strain of *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r is heterogeneous in size unlike that produced by *Rhizobium meliloti* which forms two defined peaks The smaller of these peaks represents the unpolymerized repeating octasaccharide and the larger peak is composed of the polymerized heteropolysaccharide *Rhizobium leguminosarum* biovar *viciae*

JB45 produces two defined peaks, the larger of which represents a high molecular weight fraction chemically identical to that of the wild-type strain The lower molecular weight peak was not identified but no KDO (the biochemical marker for LPS) could be detected in this peak

The wild-type strain *Rhizobium leguminosarum* biovar *viciae* VF39Sm^r produces an acidic β-glucan which the mutant strain JB45 did not A possible explanation for the simultaneous defects in both LPS and β-glucan synthesis is that the Tn5-Mob insertion occurred in a gene which codes for a function involved in an early non-specific step in the synthesis of both oligosaccharides (see Figure 10) Alternatively, an alteration in a regulatory function that affects more than one pathway may be involved

The mutant *Rhizobium leguminosarum* biovar *viciae* JB45 produced morphologically normal flagella although it was non-motile This result is consistent with the results of deMaagd *et al* , (1989), who also showed that loss of motility does not result in loss of symbiotic ability Loss of motility as a result of LPS mutation has been described for deep rough mutants of *Salmonella typhimurium* (Ames *et al* ,1974)

Slight changes in intensity of some of the outer membrane proteins were observed in the mutant strain JB45 compared to those of the wild-type strain when the strains were grown on complex medium These changes were minor compared to those observed in deep rough mutants of *Salmonella typhimurium* (Ames *et al* , 1974)

Significant changes in the outer membrane protein profiles of the mutant strain JB45 compared to the wild-type strain were observed when the strains were grown on minimal medium Some of the outer membrane regulated proteins were expressed constitutively in the mutant strain Strong LPS-LPS interactions are essential in the correct formation of the outer membrane of Gram-negative bacteria if this membrane is to serve as a barrier against diffusion (Nikado and Vaara, 1985) The interaction between the carbohydrate moieties of the LPS molecule may produce a surface layer that contributes to the stability of the outer membrane Loss of the O-antigen in strain JB45 probably results in the disorganization of the outer-leaflet The carbohydrate chain of the LPS molecule is also thought to interact with proteins in the outer membrane (Ames *et al* , 1974) Therefore any decrease in the integrity of the outer membrane would result in the loss of efficiency of proteins which function therein The constitutive expression of IRMPs and the failure of JB45 to grow on low iron medium represent manifestations of this Similar results have been reported for *Salmonella typhimurium*, in that the ability of *Salmonella typhimurium* LPS core mutants to acquire iron decreases as the severity of the core defect increases (Kvach *et al* , 1977)

A decrease in the integrity of the outer membrane may explain why JB45 produces less EPS than the wild-type strain The EPS is exported through the outer membrane of the wild-type strain *Rhizobium leguminosarum* biovar viciae VF39Sm^{r} by an ordered polar mechanism Any disorganization in the outer membrane would decrease the efficiency of such a mechanism and results in less EPS being exported in the mutant JB45

Further evidence that the outer membrane of JB45 is disorganized comes from the observation that it is much more sensitive to lysozyme than the wild-type strain (as judged by the quantities of DNA released compared to the wild-type strain) Deep rough mutants of enteric bacteria are known to become more sensitive to lysozyme, this sensitivity is thought to be a result of transient rupture and resealing of the outer membrane (Nikado and Nakae, 1979)

Bridging of negatively charged phosphate groups appears to be important in LPS-LPS interactions (Nikaido and Vaara, 1985) Therefore it is conceivable that mutant JB45 is defecient in this respect Isolated LPS are known to contain cations both inorganic (Na^+, K^+, Mg^{2+},Ca^{2+}) and organic (the polyamines putrescine, spermine, and spermidine as well as ethanolamine), (Galanos and Luderitz, 1975) If such positively charged groups interact with negatively charged groups on outer membrane proteins via bridging of charges by polycations then the loss of an O-antigen would result in a net gain of negative charges on the cell surface When grown on high Ca^{2+} medium the cells of JB45 autoagglutinate, this autoagglutination can be abolished by lowering the concentration of Ca^{2+} ions Calcium cations, because of their variable bond angle and bond length, bridge the negative charges on the surface of JB45 causing the cells to agglutinate When the concentration of Ca^{2+} is lowered the cells repel one another and form an even, turbid culture

Therefore the loss of the LPS I results in the constitutive expression of IRMPs, increased sensitivity to lysozyme, a lowering of the amount of EPS exported, autoagglutination in high Ca^{2+} and a loss of motility All these observations are consistent with the outer membrane of the mutant JB45 being disorganized

RESULTS III

Introduction

The development and morphogenesis of root nodules has been most extensively studied in pea (Newcomb, 1981) and alfalfa (Truchet *et al* , 1980) Normal nodules have been the most frequent subjects for study, however in the recent past nodules induced by bacterial mutants have been subjected to analogous analysis (Truchet *et al* , 1980, Long *et al* , 1982, Kondorosi *et al* , 1984, Noel *et al* , 1985, Hynes *et al* , 1986, Finan *et al* , 1985, Muller *et al* , 1988, deMaagd *et al* , 1989) A full analysis of the nodules induced by the surface mutant *Rhizobium leguminosarum* biovar *viciae* JB45 on *Vicia hirsuta* is described in this chapter The vetches *V hirsuta*, *V lathyroides*, and *V sativa* were chosen because of their small size which made them amenable to technical manipulation The development of *V hirsuta* nodules showed morphologies characteristic of indeterminate nodules and proved to be an excellent model for the study of the development of root nodules

Visual Inspection and Effectiveness of Nodulation

Surface sterilization, germination and inoculation of seeds was carried out as described in M35 and M336 respectively Growth conditions were those outlined in M36

Nine days after inoculation of *V hirsuta* seedlings with the wild-type strain VF39Sm^r, nodules were clearly visible Root deformations were first visualized 14 days after inoculation of *V hirsuta* seedlings with the mutant strain JB45. Twenty two days after inoculation, the wild-type induced nodules showed the characteristic cylindrical shape of indeterminate type nodules and were red in colour The majority of the twenty two day old mutant-induced deformations were similar in size to those observed on day 14. However, occasionally small, white, round-shaped, nodule-like structures were observed

Nitrogenase activity in the nodules was determined using the acetylene reduction assay as described in M3 11 The wild-type induced nodules showed nitrogenase activity as judged by the production of ethylene from acetylene No ethylene was detected on chromatographs of samples taken from the head space of the mutant-induced swellings incubated in the presence of acetylene The mutant was therefore assigned a Fix$^-$ phenotype

Bright Field Microscopy of Cleared Material

To determine if the root deformations induced on *V.hirsuta* seedlings were in fact genuine nodules, these structures were cleared as described in M38. Clearing allowed visualization of the inner histology of these structures, Figure 38. The round shaped nodule primordium Figure 38.was easy to distinguish from cone shaped lateral root meristems, Figure 39. For comparison a cleared nodule of a twenty two day old wild-type induced nodule is shown in Figure 40.

Hybrid structures intermediate between roots and nodules have been reported to occur in alfalfa seedlings inoculated with mutant *Rhizobium meliloti* strains (Dudley *et al.*, 1987; Truchet *et al.*, 1989). No such hybrids were observed on *V.hirsuta* seedlings inoculated with the mutant strain JB45.

Since the mutant strain was capable of inducing a nodule meristem it was assigned a nodule induction positive phenotype (Noi^+).

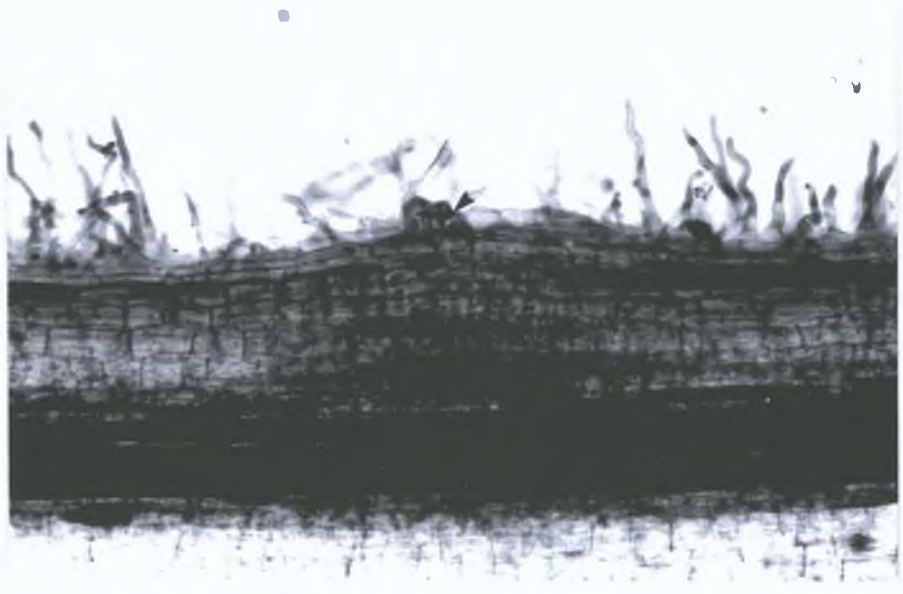


Figure 38. Whole *V.hirsuta* plant which had been inoculated with JB45 werefixed with gluteraldehyde, cleared with sodium hypochlorite and stained with methylene bluetwenty two days after inoculation. The round shaped nodule primordium in the inner cortex of the root was visible. In close proximity to this meristem a curled root containing an infection thread (double arrow) was visible. X 150.

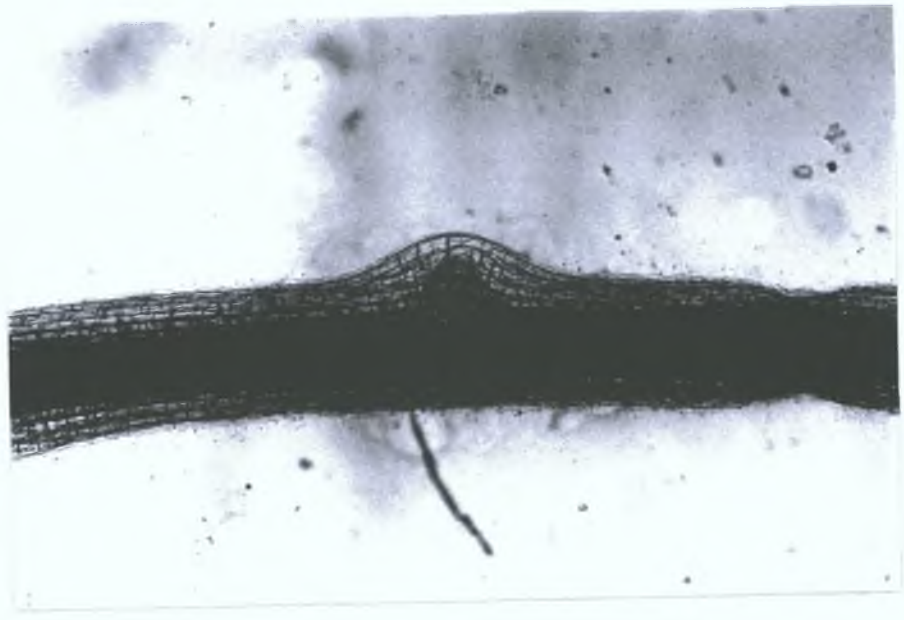


Figure 39. A lateral root primordium with its distinctive cone shape in a V.*hirsuta* root, X 40. The specimen was prepared for microscopy as described in section M38.

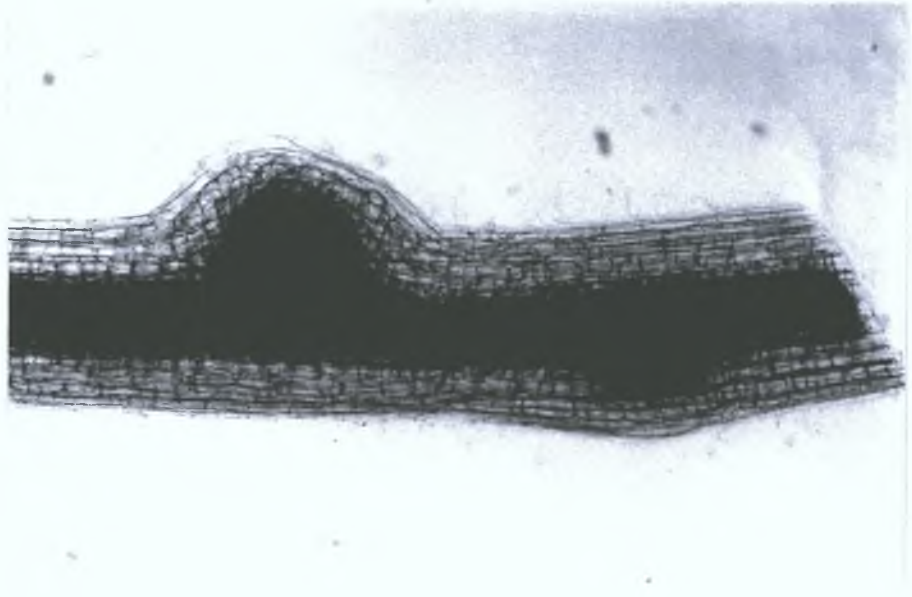


Figure 40. Twenty two day-old mutant induced nodules on *V.hirsuta* roots following clearing to visualize the inner histology of the nodule, X 40.

Infection Phenotypes of *V hirsuta*,*V lathyroides* and *V sativa* Seedlings
Recent studies on alfalfa/*Rhizobium meliloti* interactions have demonstrated that root cortical divisions may occur without root hair curling (Hirsch *et al* , 1984), and some *Rhizobium meliloti* exopolysaccharide mutants are capable of eliciting the formation of nodule-like structures in the absence of markedly curled root hairs or infection threads (Finan *et al* , 1985) Therefore it is important to screen nodules formed by Nod^+ Fix^- bacterial mutants to determine if they cause root hair curling and give rise to infection threads

Infection phenotypes of the wild-type strain were examined on *V hirsuta*, *V lathyroides* and *V sativa* The root systems were examined by bright field microscopy after staining with methylene blue to facilitate observation of infection threads as described in M37 All analyses were carried out within the first 10 days after inoculation of the seedlings

Normal root hairs are straight Exposure of a legume to its homologous host or culture filtrates of its host causes the root hairs to deform Deformations of 360° are refered to as shepherd's crooks Shepherd's crooks usually contain a bright refactile spot from which the infection thread emanates (Vasse and Truchet, 1984)
On *V hirsuta* seedlings inoculated with the wild-type strain $VF39Sm^r$, the root hair curling phenotype (Hac^+) was found in short root hairs or at the basal part of root hairs, Figure 41 As a result, the Hac^+ phenotype was difficult to observe It was found that this phenotype could be most easily viewed at the root hair/cover slip interface The infection thread was seen as a short blue thread emanating from the hyline spot, Figure 42
The Hac^+ phenotype was much easier to detect on *V lathyroides* root hairs after inoculation with the wild-type strain $VF39Sm^r$ The Hac^+ generally occurred in the middle of long root hairs Figure 43, which facilitated viewing of the infection threads, Figure 43

The infection phenotype of the mutant strain JB45 on *V.hirsuta* seedlings is shown in Figure 44. The mutant-deformed root hairs like the wild-type strain, was to be found in short root hairs or at the basal part of long root hairs, Figure 44. On *V.hirsuta* seedlings inoculated with JB45, no difference in the timing or the intensity of infection was observed when compared to the wild-type strain.
On *V.lathyroides* the mutant strain gave rise to the Hac^+ phenotype on long, middle and occasionally short root hairs and the infection phenotype was identical to that of the wild-type, Figure 45.
On *V.sativa* the infection phenotype was also identical to that of the wild-type strain (data not shown).

Therefore the nodules induced by JB45 on *V.hirsuta*, *V.lathyroides* and *V.sativa* arose from classical early events, such as root hair deformation and the formation of shepherd's crooks in the root hairs and by the formation of infection threads.

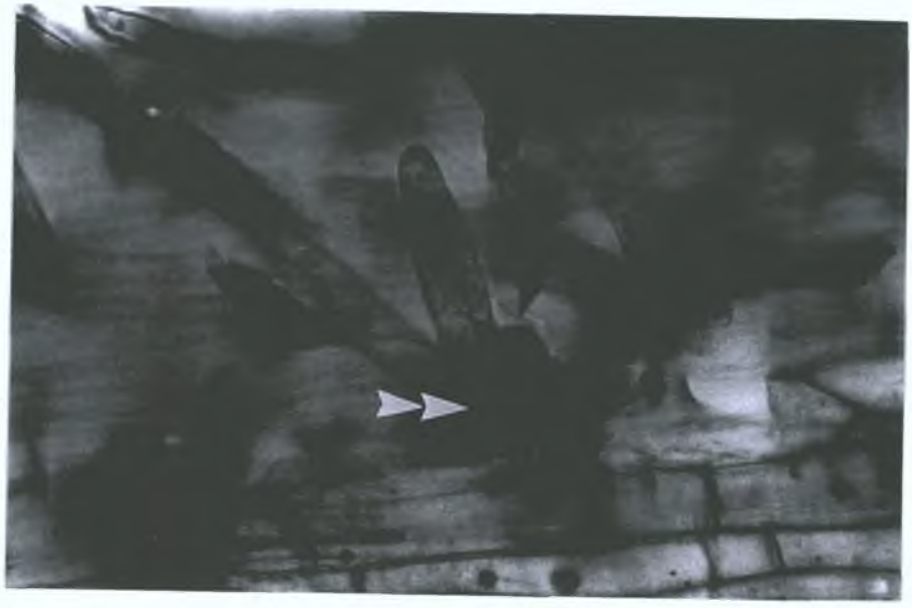


Figure 41. Root hair curling induced by the wild-type strain $VF39Sm^r$ on *V.hirsuta* seedlings showing a shepherd's crook (single arrow), at the basal part of a root hair. The infection thread (double arrow) emanates from the haline spot (triple arrow), X 200.

Figure 42. Hac^{+} phenotype induced on *V.hirsuta* seedlings by the wild-type strain $VF39Sm^{r}$ viewed at the root hair/cover slip interface, X 160.

Figure 43. Hac^{+} phenotype induced by the wild-type strain $VF39Sm^{r}$ on *V.lathyroides* seedlings. Shepherd's crooks are indicated by a single arrow, X 750.

Figure 44. Hac^{+} phenotype induced by the mutant strain on *V.hirsuta* seedlings viewed at the root hair/cover slip interface. The infection thread is indicated by the double arrow, X 200.

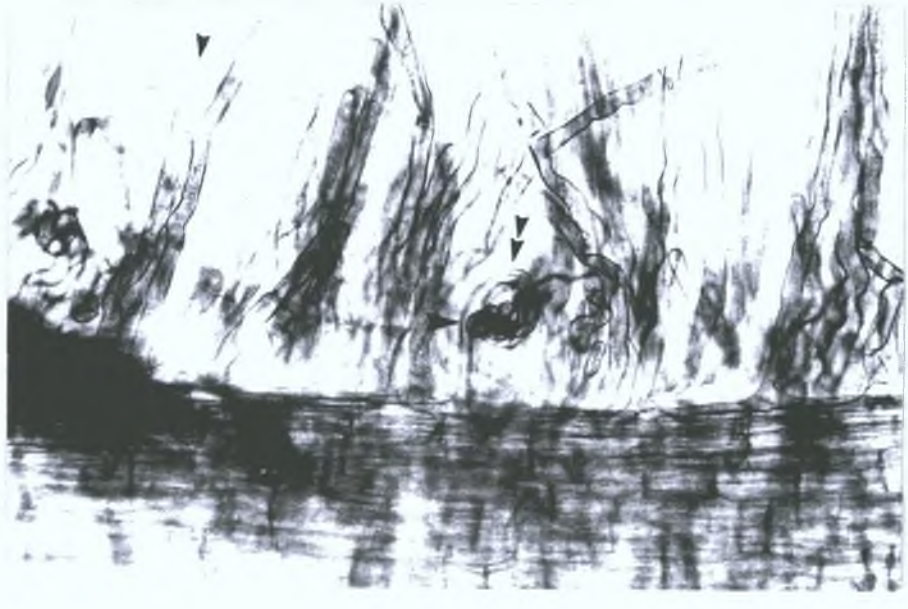


Figure 45. Root hair curling induced by the mutant strain JB45 on *V.lathyroides*.The Hac^{+} phenotype was visible in the middle of long root hairs (single arrow) and the infection thread could be seen within the curled root hair (double arrow), X 100.

Early Nodule Development

Nodules that had been fixed and embedded two weeks after inoculation of *V hirsuta* seedlings, as described in M39, were used to study early nodule development For light microscopy semi-thin sections were stained with fuschin basic and methylene blue, as described in M41 When infection threads were to be located the semi-thin sections were stained only with fuschin basic

Light microscopy of semi-thin sections through nodules induced by the mutant strain JB45, were seen to be composed of an aborting meristem Figure 46a Cells in the central zone did not stain with methylene blue (which stains cytoplasm) indicating that these cells were highly vacuolated and devoid of bacteroids In all examined cases the infection threads were seen to be restricted to the nodule periphery Figure 46b shows an infection thread in the outer cortical cells of the nodule

Thin sections for electron microscopy were stained with the lead citrate-uranyl acetate procedure described in M42 Electron microscopy of thin sections through two week old mutant-induced nodules confirmed that these nodules were devoid of bacteria, Figure 47 Examination of infection threads located in the cortical cells of the nodules revealed the presence of normal infection threads, Figure 48a However, the bacteria within these infection threads were electron dense which was indicative of senescent bacteria Figure 48b shows the presence of proplasts near the plant cell wall The electron dense areas within these proplasts are composed of phytoferritin Phytoferritin only accumulates in the proplasts of nodule cells induced by Fix$^-$ mutant bacteria (F deBilly personal communication)

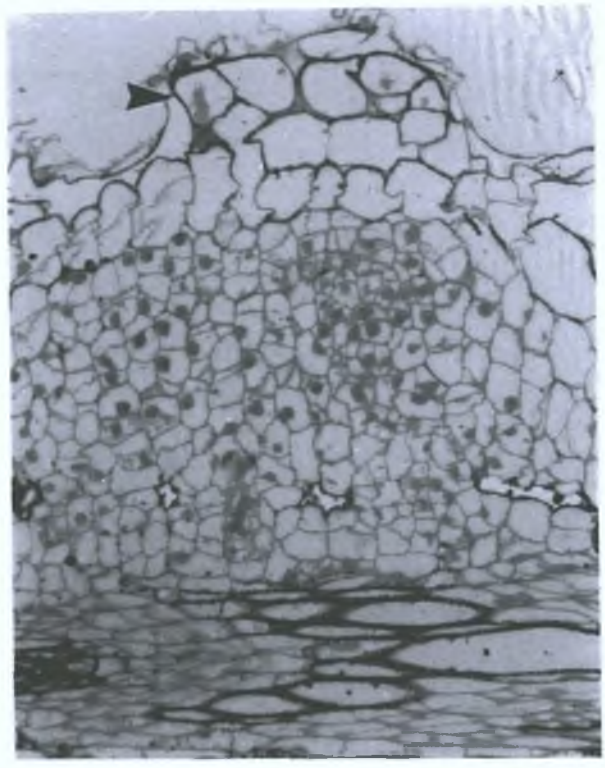


Figure 46a. Semi-thin section through a nodule induced by the mutant strain JB45, showing that the nodule was composed of an aborting meristem. The cells in the central tissue did not stain with methylene blue indicating that they were vacuolated and devoid of bacteria. The plants vascular bundles can be seen at the bottom of the plate. The infection thread is seen in the outer cortical cells (arrow), X 140.

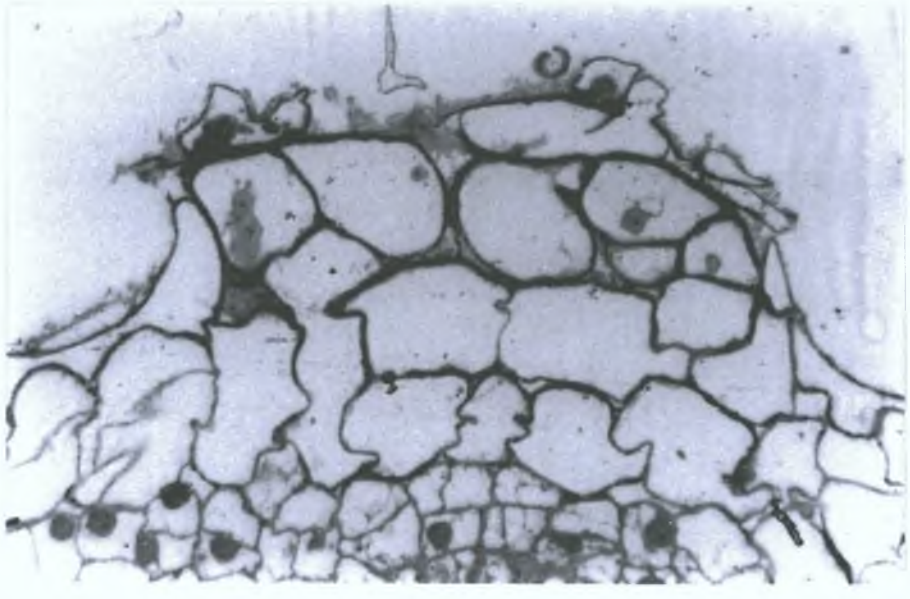
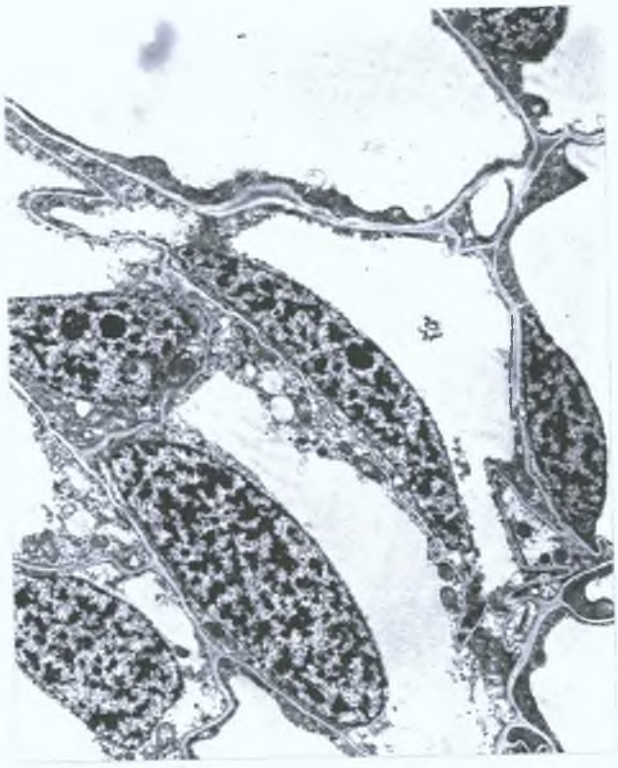


Figure 46b. The same section as described in the legend to Figure 46a except that the infection thread is shown at a higher magnification, X 327.



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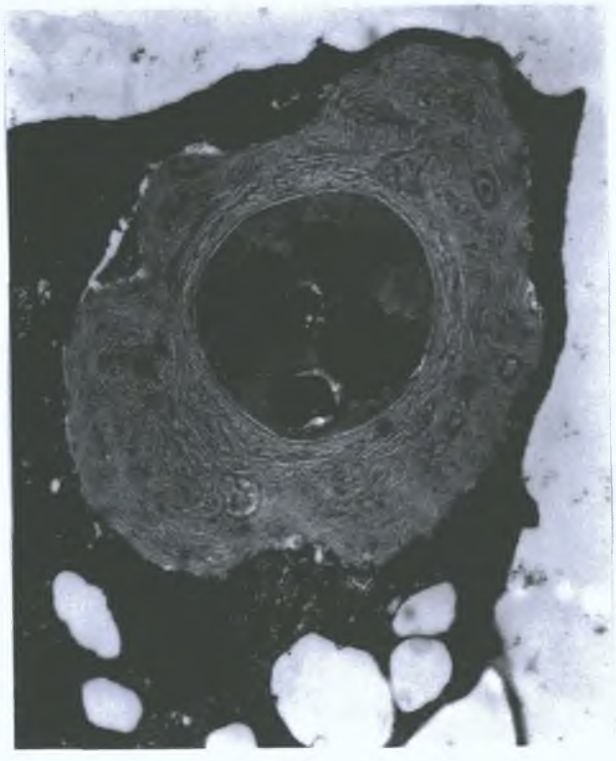


48a

48b

(see overleaf for legends to figures)

Figure 47. Transmission electron microscopy of nodular cells of the central tissue on a nodule induced by JB45 on *V.hirsuta* seedlings. The bacterium-free cytoplasm is reduced to a thin layer against the cell wall (CW), X 4520.

Figure 48a. Transmission electron microscopy of a thin-section through a two-week old mutant induced nodule which shows a normal infection thread. The bacteria within the thread are electron dense hence senescent, X 10820.

Figure 48b. Transmission electron microscopy of a thin section through a two-week old mutant induced nodule showing an infection thread with a normal morphology although the bacteria within the thread are senescing, X 3246.

Figure 49. Transmission electron microscopy of two adjacent nodular cells of the central tissue of a two-week old mutant induced nodule. The cytoplasm is restricted to a small area near the cell wall (CW), and proplasts containing electron dense phytoferritin (arrow) can be seen. Lipid droplets can also be seen (double arrow). X 16230.

Nodule Differentiation

Twenty two days after inoculation of *V.hirsuta* with the wild-type strain the nodules induced showed normal intermediate type differentiation, Figure 51. In the distal part of the nodule normal meristematic cells were evident, and some of these cells were still undergoing division, Figure 51.

Below the meristamic zone was the invasion zone (zone II), where numerous invasions could be seen, Figure, 50. Bacteria were released by a process of endocytosis as shown in Figure 52. The differentiated bacteroids with the characteristic shapes of nitrogen fixing bacteroids, Figure 53, were located in the nitrogen fixing zone (zone III), Figure 50. In the proximal part of the nodule the senescent zone (zone IV), the bacteroids appear electron dense and were senescing, Figure 54.

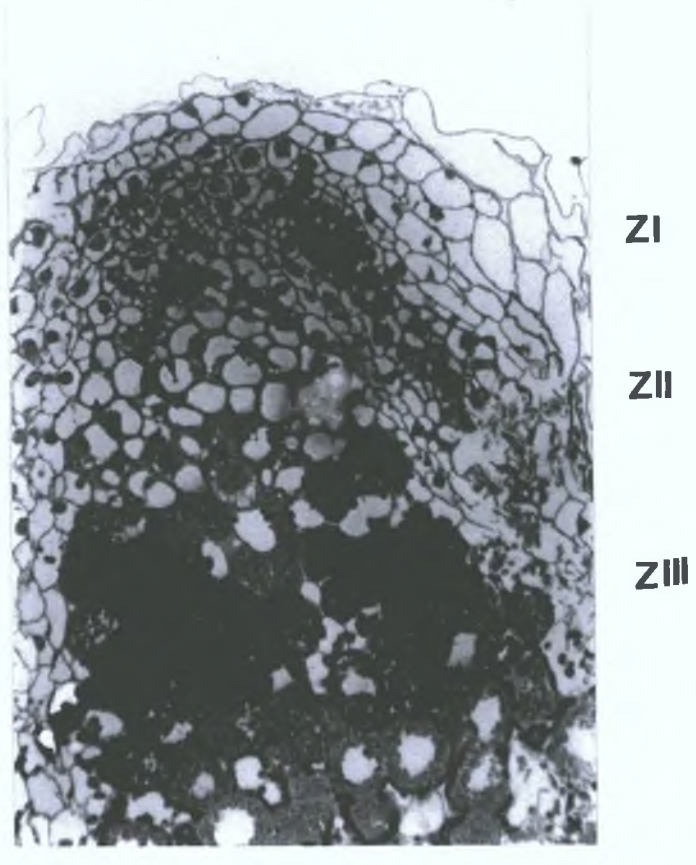


Figure 50. Semi-thin section through a wild-type induced nodule showing zonation typical of indeterminate nodules. ZI, an apical meristematic zone; ZII, an infection zone with numerous infection threads (arrows), ZIII, nitrogen-fixing zone.

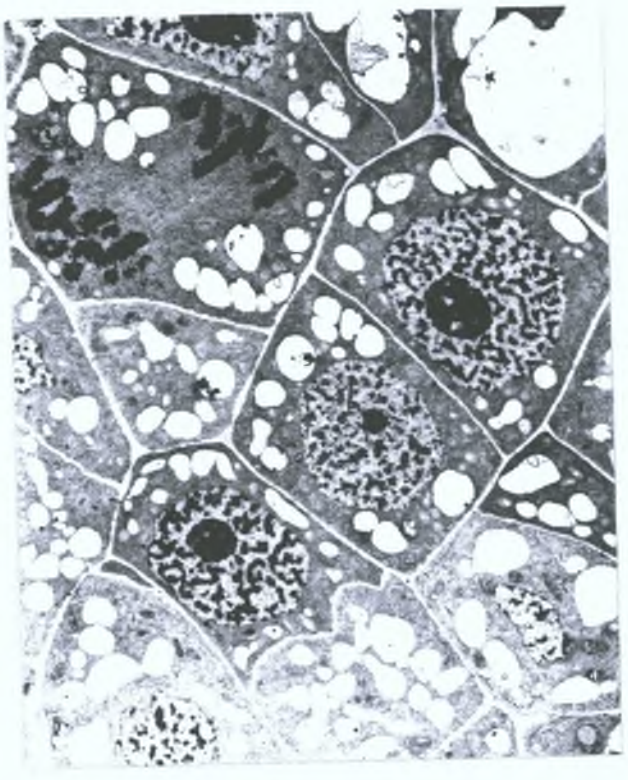


Figure 51. Transmission electron microscopy of cells in Zone I of a Twenty-two-day old wild-type induced nodule. Divisions are still seen to be taking place and one of these cells is in anaphase, X 2164.

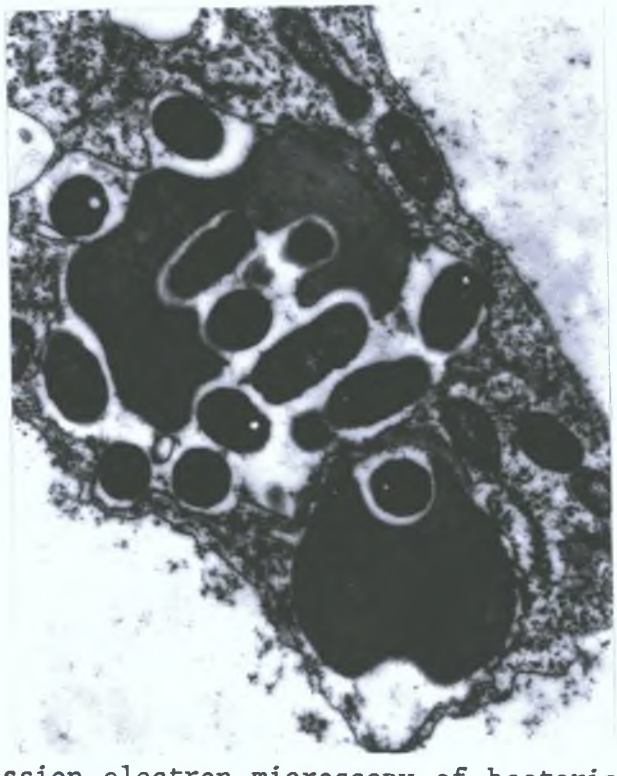


Figure 52. Transmission electron microscopy of bacterial release from an infection thread by endocytosis, X 10820.

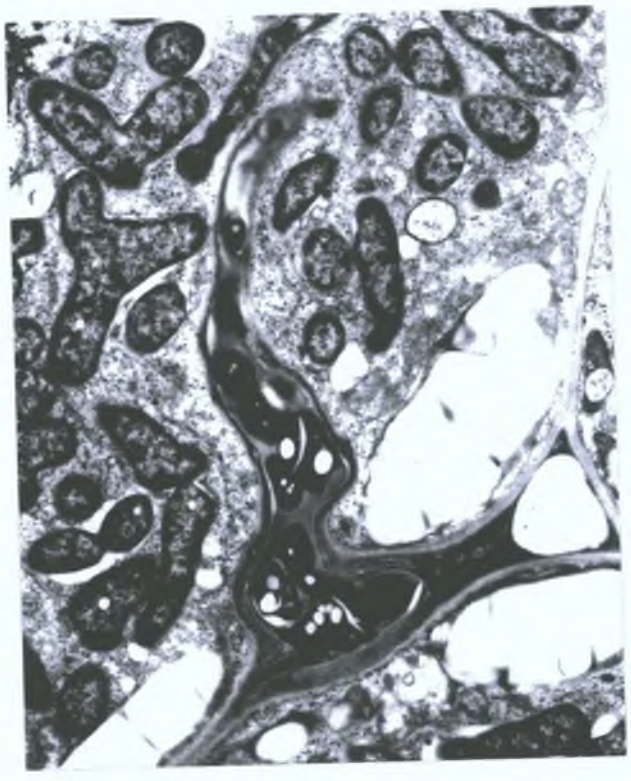


Figure 54. Transmission electron microscopy of cells in Zone III, the nitrogen-fixing zone. The characteristic X and Y shapes of nitrogen fixing bacteroids can be seen alongside an infection thread, X 6492.

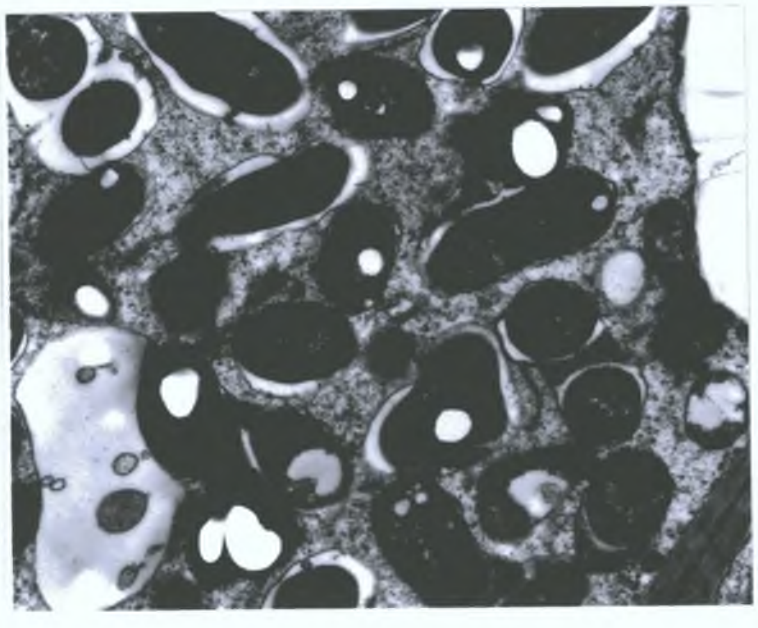


Figure 54. Transmission electron microscopy of bacteroids in the senescent zone. These bacteroids are electron dense compared to those shown in Figure 53, X 10820.

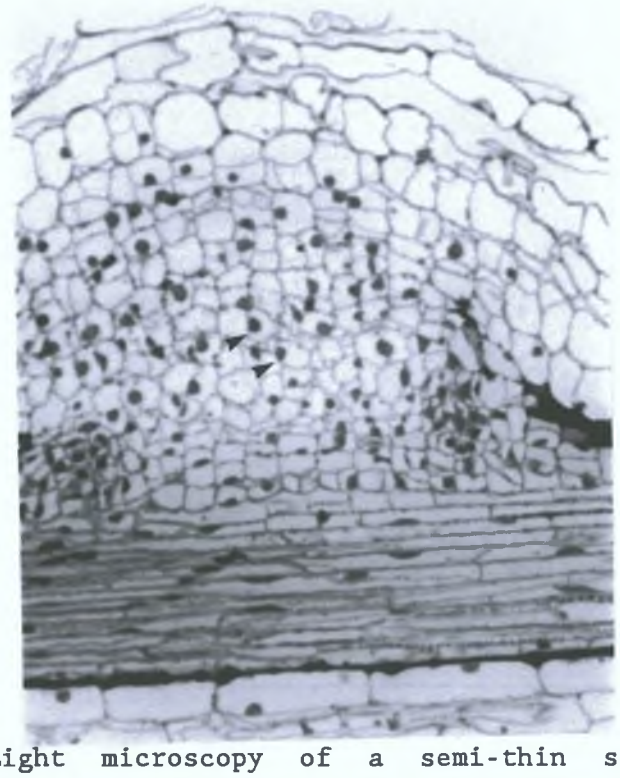


Figure 55. Light microscopy of a semi-thin section through a twenty-two-day old mutant-induced nodule. The cells in the central zone are devoid of bacteria. These nodules were similar in size and histology to those observed two weeks after inoculation of *V.hirsuta* seedlings with the mutant strain. The arrows indicate the presence of large nuclei, X 75.

Twenty two day old mutant-induced nodules were similar in size and shape to those two week old mutant-induced nodules already described. Figure 55 shows a section through a twenty-two-day old mutant-induced nodule which shows the cells in the central zone to be vacuolated and devoid of bacteroids. Starch grains and a large nucleus are clearly visible in these cells. Electron microscopic examination of thin sections through these nodules verified that these nodules were devoid of bacteroids Figure 56.

Occasionally small round nodules were observed at 22 days after inoculation on *V.hirsuta*, *V.lathyroides* and *V.Sativa*. Light microscopy of semi-thin sections through these nodules on *V.hirsuta* showed that the central tissue did stain with methylene blue indicating that these nodules may have been infected. Electron microscopy revealed that these small round nodules fell into three categories.

The first category differed from those nodules previously described in

that infection threads did penetrate the central zone of the nodule, Figure 57, although no bacteria were released into the host cells.

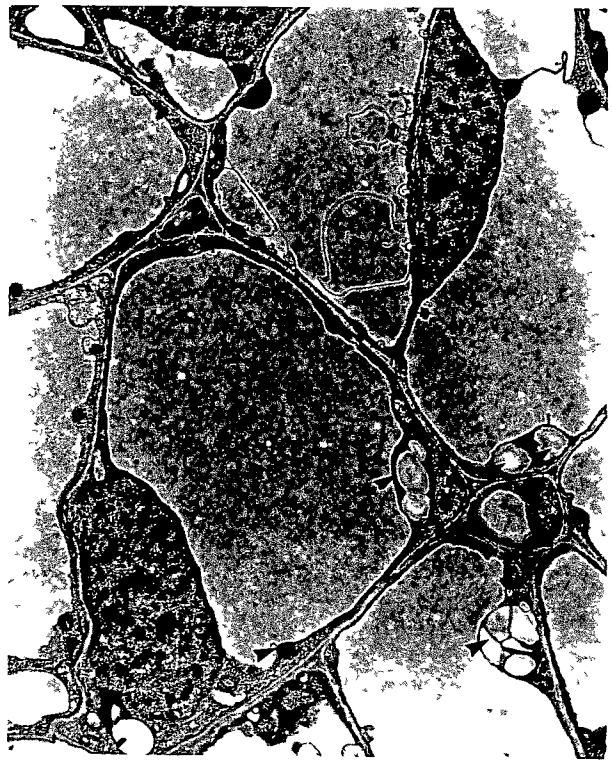


Figure 56. Transmission electron microscopy of a thin-section through the central tissue of a twenty-two-day old mutant induced nodule The nucleus and cytoplasm are confined to a small area alongside the cell wall and numerous amyloplasts are visible (arrows) The cells are devoid of bacteroids, X 2250

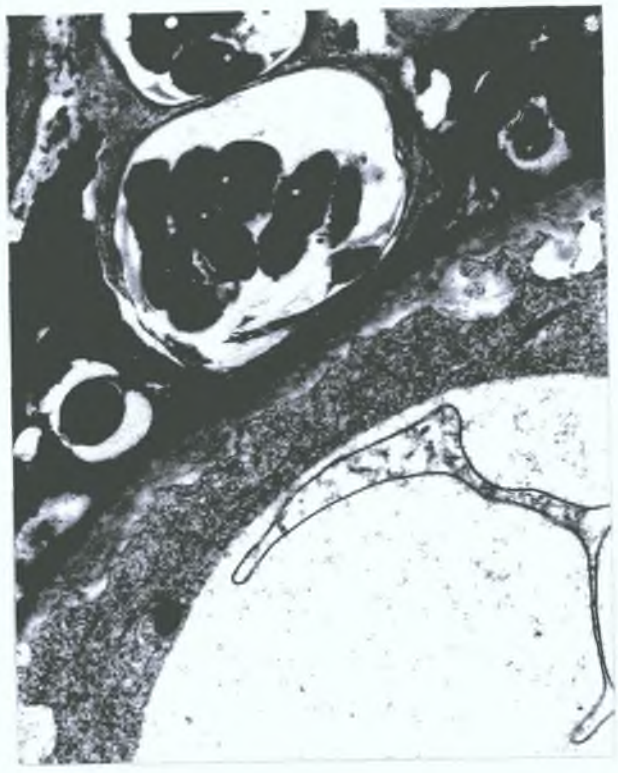


Figure 57. Transmission electron microscopy of an infection thread in the central tissue of a twenty-two-day old mutant-induced nodule, X 10820.

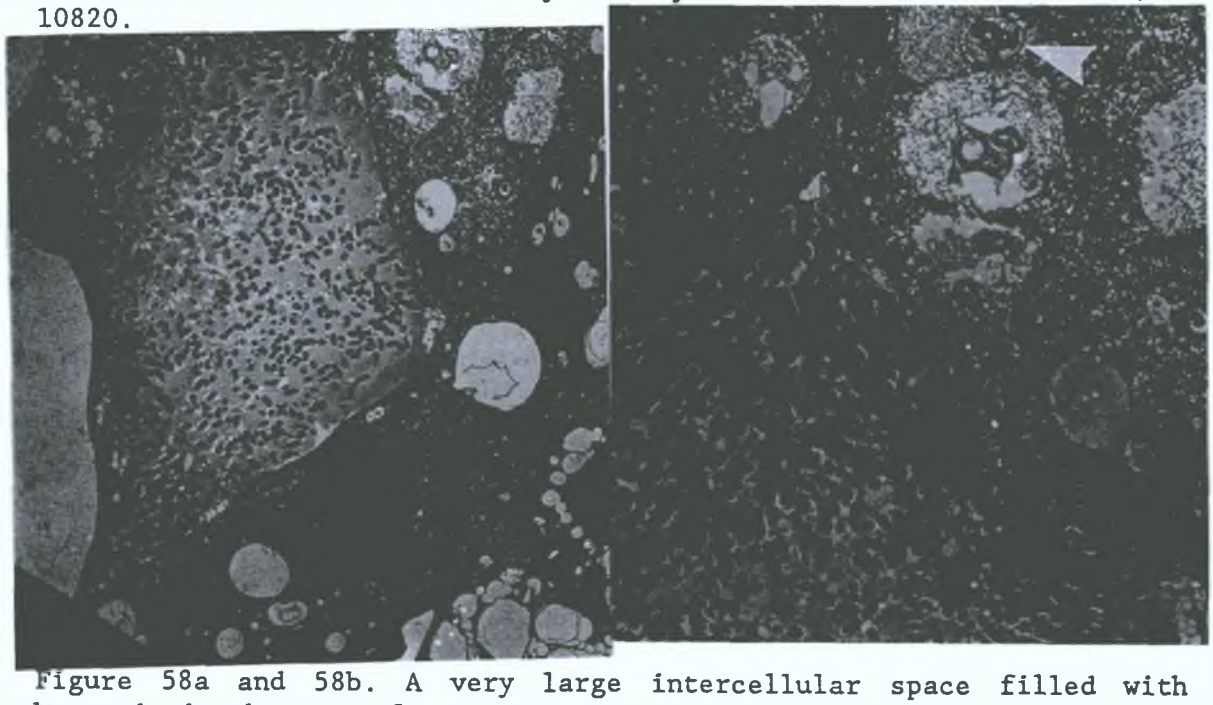


Figure 58a and 58b. A very large intercellular space filled with bacteria in the central part of a three-week old mutant induced nodule. Figure 58a shows that this bacterium filled area is not a cell because of its angular shape, X 1082. Figure 58b shows the presence of an infection thread (arrow) near this large intercellular space, X 2164.

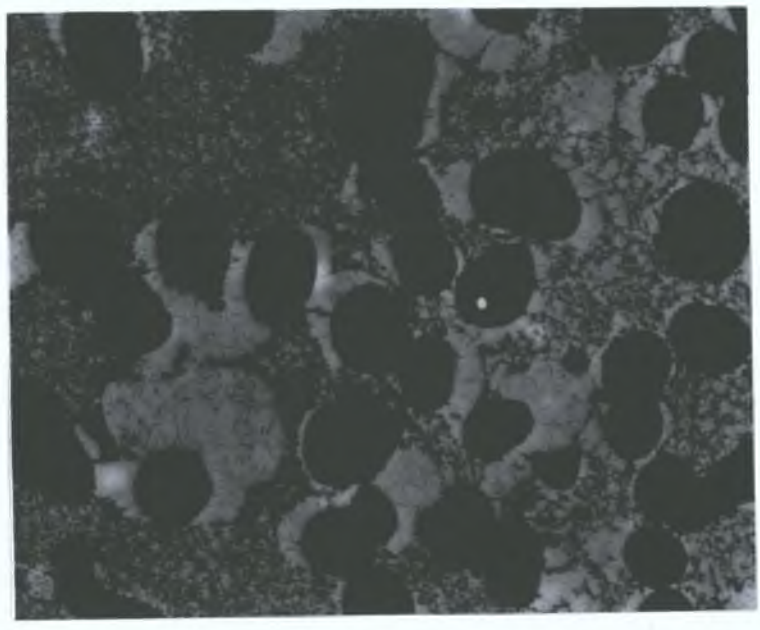


Figure 59. Transmission electron microscopy of bacteria in an intracellular space which was located in the central tissue of a three-week old mutant induced nodule, X 12986.

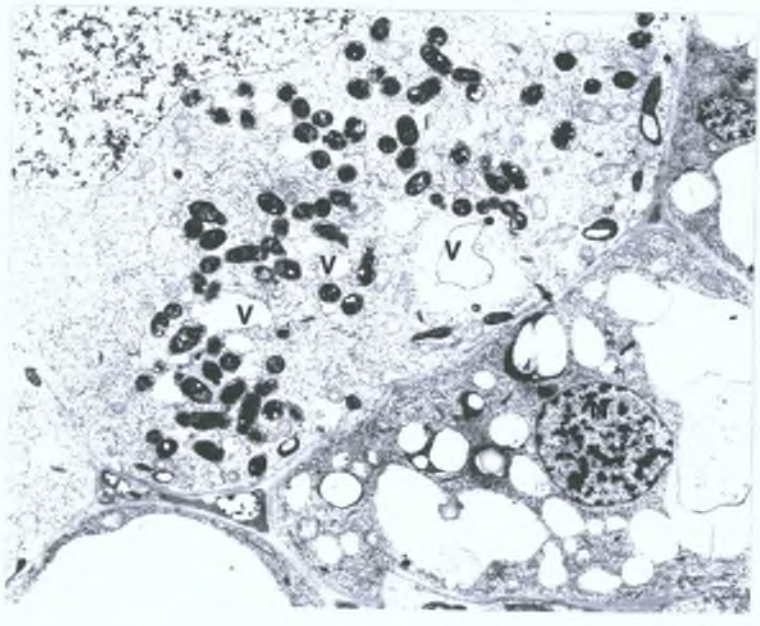


Figure 60. Transmission electron microscopy of cells in the central tissue of a three-week old mutant induced nodule showing that some bacterial release did take place. Very few bacteria were released compared to in the wild-type induced nodules. These plant cells were highly vacuolated (V-Vacuoles; N-Nucleus), X 6492.

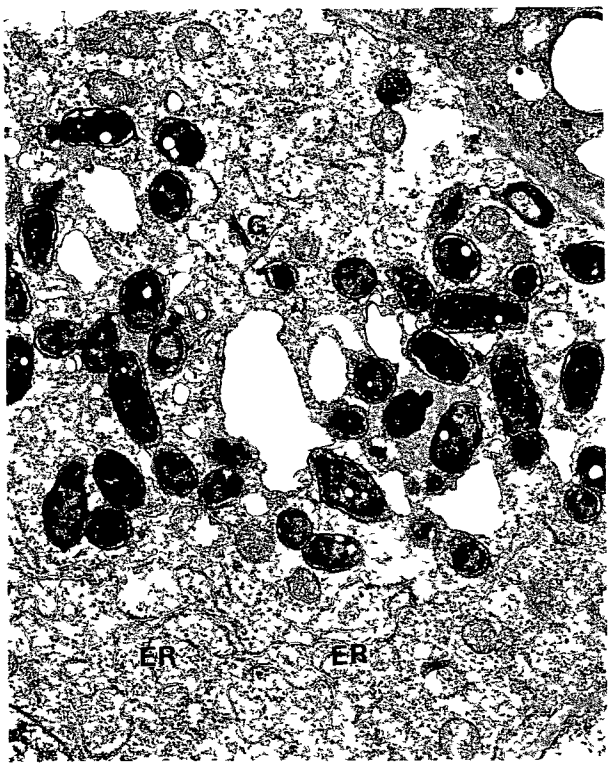
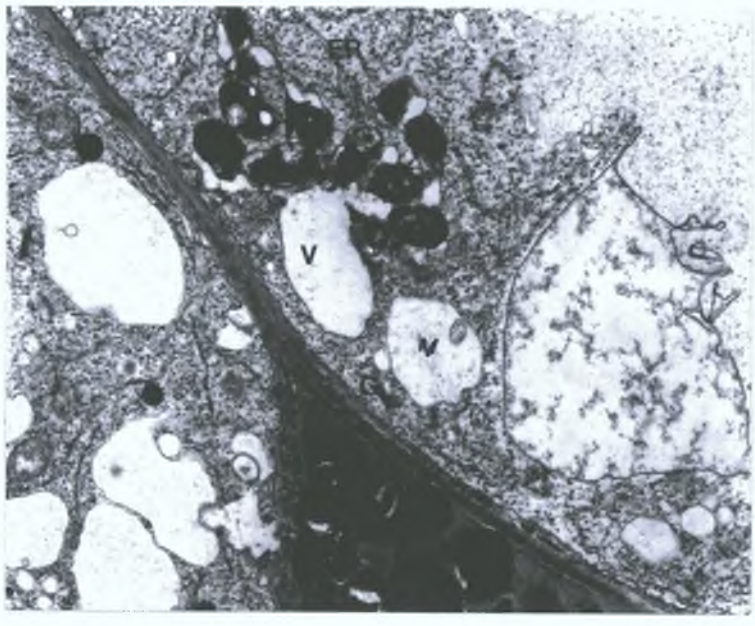


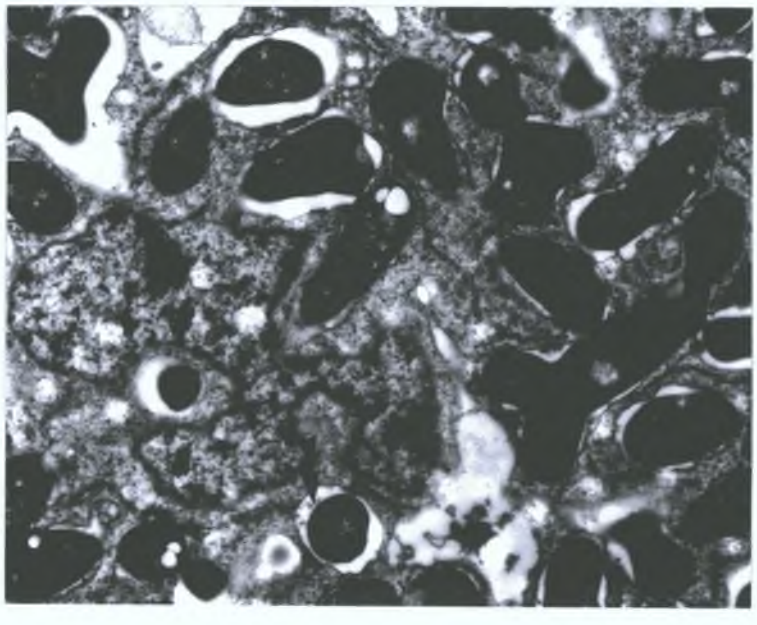
Figure 61 Transmission electron microscopy of the central tissue of a three-week old mutant nodule The bacteroids are electron dense and senescent The plant cells are highly vacuolated and contatin copious quantities of endoplasmic reticulum (ER) and golgi apparatus (G) which are indicative of a senescent plant cell, X 6000

The second category contained very large intracellular spaces filled with senescent bacteria (not bacteroids), Figure 58a,b In these nodules infection threads were not restricted to the nodule periphery and were observed (very rarely) in the central tissue of the nodule Figure, 58b

Figure 59 shows these bacteria to be very electron dense and devoid of peribacteroid membranes.

The third category of nodules contained infection threads in the central zone and bacteria were in fact released However very few invasions were observed compared to the number of infection threads induced by the wild-type strain Very few bacteria were released compared to the numbers released by the wild-type strain, Figures 60 and 61 These bacteroids senesced very quickly and no bacteroid development was observed The plant cells into which the bacteroids were released were highly vacuolated, Figure 61, and had extensive development of endoplasmic reticulum and golgi appratus, which are indicative of a senescing plant cell The peribacteroid membrane surrounding these bacteroids appeared normal, however the bacterial cell envelope did not have a normal morphology The envelope appeared to have a crinkled topography Figure, 52, and was very different from the normal envelopes observed in wild-type induced nodules, Figure 63





(See overleaf for legends to figures)

Figure 62 Transmission electron microscopy of an infected mutant induced nodule twenty-two days after inoculation of *V hirsuta* seedlings The arrow indicated the crınkled topography of the bacterial cell envelope (G-Golgi apparatus, V-Vacuole, ER-Endoplasmic reticulum, CW-Plant cell wall, N-Nucleus), X 13561

Figure 63 Normal bacteroıds twenty-two days afterinoculation of *V hırsuta* seedlings with the wild-type strain The double arrow indicates the smooth even morphology of the bacterıal envelope

DISCUSSION III

The nodules induced on the roots of *V.hirsuta* seedlings by the mutant strain JB45, arose from classical events such as root hair curling and the formation of infection threads. A correlation between the presence of capsules and exopolysaccharide and the degree of infectivity of *Rhizobium leguminosarum* biovar *viciae* has been established (Napoli and Albersheim, 1980). Since mutant JB45 produces less capsules and less EPS than the wild-type strain then it would be expected to infect *V.hirsuta* less efficiently than the wild-type strain. This was found not to be the case. A possible explanation is that *V.hirsuta* seedlings inoculated with JB45 do not regulate the degree of infection by a negative feed-back mechanism because nodule development is arrested. Feed-back regulation of nodule formation has been reported for *Rhizobium meliloti*/alfalfa symbiotic interactions (Caetano-Annolés and Bauer, 1988).

In nodules induced by the mutant strain the infection thread was restricted to the nodule periphery. LPS mutants of *Rhizobium leguminosarum* biovar *phaseoli* also initiate infection threads however these infection threads cease to develop, usually within the infected root hair or within the cortical cells and as a consequence no plant cells within the nodule become infected. These infection threads had abnormal morphology as they were seen to be greatly swollen (Noel *et al.*, 1986). The infection threads induced by these *Rhizobium leguminosarum* biovar *phaseoli* LPS mutants differ from those induced by the LPS mutant strain, JB45, on *V.hirsuta* in several important respects:

1. No cessation of infection thread development within the root hair cells was observed; 2. The infection threads appeared normal in both shape and size and 3. Occasionally a very small number of infection threads penetrated the central tissue of the nodule. An LPS mutant of *Rhizobium meliloti fix*-21, has a symbiotic phenotype on alfalfa plants similar to that which *Rhizobium leguminosarum* biovar *viciae* JB45 exhibits on *V.hirsuta* seedlings, in that it mostly forms abortive infection threads but on a few occasions the infection thread penetrates the central tissue of the nodule and does release bacteria (Hanks *et al.*, 1987; Kieber *et al.*, 1987).

deMaagd *et al* , (1990) and Priefer (1990) reported that LPS mutants of *Rhizobium leguminosarum* biovar *viciae* form small white nodules in which only a few bacteria are released into the host plant cytosol. These nodules are similar in size and histology to those occasionally induced on *V hirsuta* by JB45 The reason why only a few nodules of this type were induced may be related to the fact that JB45 does not produce any acidic β-glucan. β-2-glucan from *Rhizobium leguminosarum* biovar *trifolii* has been implicated in infection thread and nodule formation on clover plants (Higashi and Abe 1980, Abe *et al* , 1982) β-Glucan from *Rhizobium meliloti* has been shown to be required for osmoadapation (Dylan *et al* , 1990a) *Rhizobium leguminosarum* biovar *viciae* JB45 cells were shown to senesce within the infection threads which raises the possibility that an ability to osmoadapt may be required for *Rhizobium* cells to survive within the infection thread

When infection threads did penetrate into the centre of the nodules in some cases bacteria were released These bacteroids had morphologically normal peribacteroid membranes. The bacterial outer membrane contained within the peribacteroid membrane was not normal and it assumed a crinkled type morphology The *Rhizobium leguminosarum* biovar *viciae* LPS mutants isolated by deMaagd *et al* , (1989) did not show this abnormal outer membrane topography

A physical association between the peribacteroid membrane and the bacterial cell surface could account for the concomitant division of the peribacteroid membrane and the intracellular bacteroids (Robertson and Lyttleton, 1984) In pea and in vetch usually only one bacteroid is surrounded by a peribacteroid membrane If an interaction between the outer surface of the bacteria and the bacteroid membrane is responsible for such concomitant division then it would be expected that more than one JB45 cell should be present per bacteroid membrane since the outer membrane of JB45 is disorganised and most probably unable to participate in such a putative interaction. In nodules in which JB45 cells are released into the plant cell cytoplasm no differences in the numbers of bacteroids contained within a single peribacteroid membrane

was observed However the numbers of bacteria that were released were low and it is possible that not enough bacteroids were examined in order to observe this phenomenon Alternatively such an interaction between membranes may be a requirement for bacteroid division and development

Brewin *et al* ,(1986) have suggested that the LPS structure of *Rhizobium leguminosarum* biovar *viciae* changes between the free-living and bacteroid state This new LPS structure may confer on the outer membrane of the bacteroid the ability to expand as the bacteroids increase in size to accomidate 40 times the volume of the free-living cell The *Rhizobium leguminosarum* biovar *viciae* JB45 mutant lacks a portion of its LPS molecule and therefore its LPS molecule may not serve as a substrate for the attachment of this new LPS structure thus giving rise to the Bad$^-$ phenotype observed

A hypothesis that LPS I protects against host defences only after infection thread formation has been initiated has been proposed (Cava *et al* , 1989) These workers propose that the LPS I structure would mask surface features which would elicit the host defence mechanism During the course of this study no host defence mechanisms (such as cell wall thickening) were observed in nodules where the infection threads were restricted to the nodule periphery The rapid senescence of the host plant cells into which bacteria were released could be described as a host defence response, however it is unlikely that this response is initiated in response to the lack of an O-antigen masking other surface characteristics as the bacteria are surrounded by a membrane which is derived from the host's plasmalemma

The cytological studies on nodules induced by *Rhizobium leguminosarum* biovar *viciae* JB45 demonstrate that intact LPS and β-glucan is required, possibly at different stages, in nodule development

Conclusions

The role of surface polysaccharides in the development of the symbiotic interaction between *Rhizobium leguminosarum* biovar *viciae* and its plant host *V.hirsuta* has been addressed by mutational analysis The mutant studied has multiple carbohydrate defects which gave rise to an unusual symbiotic phenotype The results reported in this study can be used to speculate on the exact roles of these surface polysaccharides in the development of root nodules

deMaagd *et al* , (1989), and Priefer, (1989) have demonstrated that *Rhizobium leguminosarum* biovar viciae LPS plays a role in the later stages of nodule development The findings of this work are in agreement with these studies However, the mutant analysed in this work exhibits a different phenotype on *V hirsuta* in that in this mutant the maintenance of continued penetration by infection threads is impaired Since JB45 not only has an altered LPS structure but also fails to produce an acidic glucan, then it can be speculated that this β-glucan may function in the continued penetration of the infection thread This differs in the case of *R meliloti* where it has been suggested that β-Glucan functions at the level of infection thread formation, (Dylan *et al* , 1986) Therefore, it is noteworthy that hot-water extraction of *R leguminosarum* biovar *viciae* cells, yields an acidic fraction, whereas such an extraction of *R meliloti* yields a neutral fraction In the light of these results it would be of interest to characterize nodules induced by *Rhizobium leguminosarum* biovar *viciae* which lack β-Glucan only In *Rhizobium leguminosarum* biovar *phaseoli* LPS mutants, infection thread development is abnormal whereas JB45 induces the formation of normal infection threads LPS mutants of *R meliloti* form effective symbioses with alfalfa plants (Long *et al* , 1989) Therefore the role of LPS in the development of the symbiotic state differs not only between species of *Rhizobium* but also between biovars

The results of this study show that surface polysaccharides are important in the cell-cell interactions of Rhizobium/Legume symbiosis

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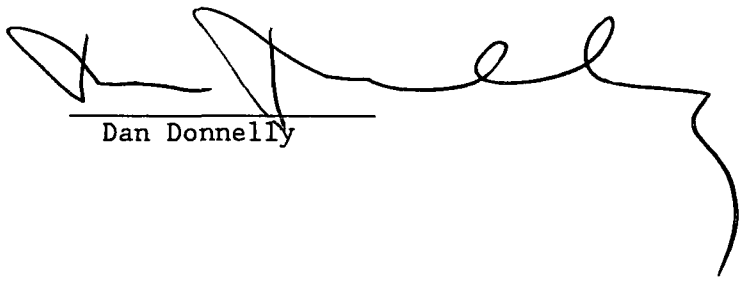
I declare that all the work reported in this thesis was performed by Dan Donnelly



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