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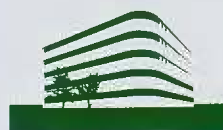

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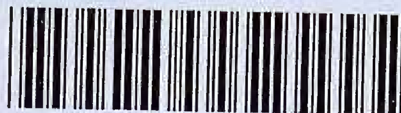

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260 Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis

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Fundación Juan March

Lecture Course
Molecular Biology of the Rhizobium-Legume Symbiosis

Organized by

T. Ruiz-Argüeso

T. Bisseling
P. Boistard
J. A. Downie
D. W. Emerich
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T. Ruiz-Argüeso
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Serie Universitaria

260

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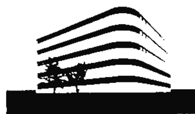

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INDEX

PAGE

PROGRAMME.. 5

INTRODUCTION. T. Ruiz-Argüeso................................ 7

PRESENTATIONS:

RHIZOBIAL GENES INVOLVED IN THE NODULATION OF LEGUMES. J.A. Downie.................................. 13

FUNCTION OF RHIZOBIUM LEGUMINOSARUM bv. VICIAE NODULATION GENES INVOLVED IN THE DETERMINATION OF HOST SPECIFICITY. H.P. Spaink.................... 15

OXIGEN REGULATION OF SYMBIOTIC NITROGEN FIXATION IN RHIZOBIUM. P. Boistard............................ 21

THE USE OF LAMBDA EXPRESSION LIBRARIES AND pLAFR1 LIBRARIES TO ISOLATE GENES FOR THE CITRIC ACID CYCLE ENZYMES FROM BRADYRHIZOBIUM JAPONICUM. D. W. Emerich.. 24

RHIZOBIAL GENES INVOLVED IN COMPETITIVENESS. J. Olivares.. 27

MOLECULAR BIOLOGY OF THE HYDROGEN UPTAKE HYDROGENASE FROM RHIZOBIUM LEGUMINOSARUM. T. Ruiz-Argüeso...................................... 29

ROOT NODULE FORMATION: A SYSTEM TO STUDY PLANT DEVELOPMENT. T. Bisseling........................... 33

EXPRESSION AND REGULATION OF NODULINS AND NODULIN GENES IN PHASEOLUS VULGARIS L. F. Sánchez........... 35

LECTINS, LECTIN GENES, AND RHIZOBIUM. J. Kijne...... 38

POSTERS:

ON THE REGULATION OF NITRITE ACCUMULATION IN LEGUME NODULES. C. Arrese-Igor...................... 43

MOLECULAR CHARACTERIZATION OF THE MAJOR NODULIN-SPECIFIC TRANSCRIPT OF THE PHASEOLUS VULGARIS. F. Campos.. 44

STUDIES ON MERCURY-TOLERANT RHIZOBIA. J. Chaudhuri.. 45

BRADYRHIZOBIUM JAPONICUM Tn 5-MUTANTS AFFECTED IN MEMBRANE-BOUND NITRATE REDUCTASE ACTIVITY. M. Fernández-López................................... 46

Fundación Juan March (Madrid)

PAGE

CHARACTERIZATION OF THE OXIGEN-DIFFUSION BARRIER IN LUPINUS ALBUS CV. MULTOLUPA NODULES. M. Fernández-Pascual.................................. 47

EFFECTS OF HERBICIDES ISOPROTURON AND CLORTOLURON ON RHIZOBIUM-LEGUME SYMBIOSIS. M. Flores............ 48

A STUDY OF THE nodD GENE OF RHIZOBIUM LEGUMINOSARUM bv. PHASEOLI CIAT899. J.L. Folch.................... 49

STUDY OF THE DENITRIFYING ACTIVITY IN SOME STRAINS OF FREE-LIVING RHIZOBIUM MELILOTI. J.I. García Plazaola.. 50

EARLY NODULINS OF VIGNA UNGICULATA INDUCED BY RHIZOBIUM SP. NGR234. A. Krause..................... 51

NITRATE INHIBITOR ON NODULATION CAN BE OVERCOME IN THE PRESENCE OF THE ETHYLENE INHIBITOR AMINOETHOXYVINYLGLYCINE. F. Ligero.................. 52

IMMUNOLOGICAL AND IMMUNOCYTOCHEMICAL DISTINCTION BETWEEN BRADYRHIZOBIUM SP. (LUPINUS) STRAINS IN NODULES OF LUPINUS ALBUS. M.M. Lucas................ 53

A FAMILY OF SYMBIOTIC SIGNALS PRODUCED BY RHIZOBIUM SP. NGR-234. N.P.J. Price........................... 54

SEQUENCING AND ORGANIZATION OF A GENE CLUSTER REQUIRED FOR THE SYNTHESIS OF AN ACTIVE HYDROGENASE IN RHIZOBIUM LEGUMINOSARUM. L. Rey.................. 55

RHIZOBIUM TRIFOLII GENES INVOLVED IN SURFACE POLYSACCHARIDES AND NODULE DEVELOPMENT. B. Sánchez.. 57

IDENTIFICATION AND GENETIC ANALYSIS OF AN FNR-LIKE GENE IN RHIZOBIUM LEGUMINOSARUM bv. VICIAE INVOLVED IN REGULATION OF NITROGEN FIXATION GENES. A. Schlüter.. 59

SALINITY EFFECT ON NITROGENASE ACTIVITY IN LEGUME NODULES. R. Serraj.................................. 60

LOCALIZATION OF GENES IMPLIED IN SYNTHESIS OF EXTRACELLUAR POLISACARIDES. M. Ternero.............. 61

LIST OF INVITED SPEAKERS.................................... 63

LIST OF PARTICIPANTS.. 67

Fundación Juan March (Madrid)

PROGRAMME

1. Monday 27:

First Session:	**Nodule formation and development**
J.A. Downie	- Rhizobial genes involved in the nodulation of legumes.
H.P. Spaink	- Function of Rhizobium leguminosarum bv. viciae nodulation genes involved in the determination of host specificity.
N.P.J. Price	- Short oral presentation.
Second Session:	**Nodule functioning**
P. Boistard	- Oxygen regulation of symbiotic nitrogen fixation in Rhizobium.
M. Fernández-Pascual	- Short oral presentation.
D.W. Emerich	- The use of Lambda expression libraries and pLAFR1 libraries to isolate genes for the citric acid cycle enzymes from Bradyrhizobium japonicum.
C. Arrese-Igor	- Short oral presentation.

2. Tuesday 28:

Third Session:	**Nodule complementary functions**
J. Olivares	- Rhizobial genes involved in competitiveness.
F. Ligero	- Short oral presentation.
T. Ruiz-Argüeso	- Molecular biology of the hydrogen uptake hydrogenase from Rhizobium leguminosarum.
A. Schlüter	- Short oral presentation.
Fourth Session:	**Symbiosis-related plant genes**
T. Bisseling	- Root nodule formation: a system to study plant developtment.
A. Krause	- Short oral presentation.
F. Sánchez	- Expression and regulation of nodulins and nodulin genes in Phaseolus vulgaris L.
J. Kijne	- Lectins, lectin genes, and Rhizobium.

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INTRODUCTION

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INTRODUCTION

Rhizobium and Bradyrhizobium bacteria are unique among microbes in their ability to induce nitrogen-fixing nodules on legumes. This symbiotic interaction has an strategic importance, particularly in the context of food production, since it allows the legume plants to thrive in nitrogen-impoverished soils . In addition to their agricultural interest, the Rhizobium/Bradyrhizobium-legume symbiosis provides a good experimental system to study the molecular basis of several relevant problems in plant sciences, such as the specificity of recognition between plants and microbes , or the elucidation of plant development organogenesis. Furthermore, the analysis of the symbiosis at the molecular level has produced a large data base leading to related advances in bacterial genetics, regulation of gene expression, metallo-protein chemistry, plant diseases or bacterial ecology.

The process of nodule formation and nitrogen fixation requires the participation of genes from both the plant host and the bacterial microsymbiont. These include genes involved in specific recognition between the eukaryote and the prokaryote partners, in invasion of plant cells and in nodule development and functioning. Still other genes are involved in ancyllary functions such as hydrogen recycling, although they are not required for the symbiosis itself. A differential expression of these genes takes place in the bacteria and in the nodule plant cells in response to extracellular small molecules that are exchanged between both symbionts. Several of these interactive molecular signals are starting to be identified now, and the regulatory changes that are needed to elicit the nitrogen fixation mechanisms are begining to be understood.

It was the spirit of this Lecture Course held in Fundación Juan March to provide a forum where postdoctoral or advanced postgraduate students and young scientists will have the oportunity to interact in a direct manner with reseachers working on different molecular aspects of the Rhizobium-legume symbiosis and get adquainted with the latest developments in the field. With this aim in mind, four sessions were programmed along the two days of the course. The first session was dedicated to review the - molecular genetics of legume nodulation. Special emphasis was placed in recognition, molecular signaling and host specificity. The second session dealed with nodule functioning, and was centered on molecular aspects of oxygen regulation of nitrogen fixation and carbon metabolism in nodules. In the third session, two possibilities of increasing nitrogen fixation by manipulating genes involved in competitiveness of Rhizobium strains for nodulation and

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in hydrogen recycling in nodules were analysed in detail. The fourth session was dedicated to review plant genes related to the symbiosis and the functions of their products. The review lectures were followed by extended time of formal and informal discussions. The participants also contributed by presenting their research lines and recent data in posters (17) that were exposed all along the Course. In addition of the lectures of the invited speakers, short oral presentations were given by six participants selected after evaluation of the poster abstracts.

Finally, I thank the Fundación Juan March for generously supporting the Lecture Course, and specially aknowledge Andrés González for his warm help during the organization and development of the Course.

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FIRST SESSION

J. A. DOWNIE
H. P. SPAINK

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RHIZOBIAL GENES INVOLVED IN THE NODULATION OF LEGUMES

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Highly specific recognition occurs between leguminous plants and the rhizobia that nodulate them. The specificity in this interaction is mediated in part by a regulon of nodulation genes in the bacteria. These *nod* genes are involved in the formation of signals that are recognised by legume roots establishing the appropriate communication between plant and bacterium to establish the initial stages in the symbiotic interaction.

Many different *nod* genes have been identified in several strains of rhizobia. This work on *nod* gene identification has encompassed a wide variety of rhizobia including the biovars *viciae trifolii* and *phaseoli* of *Rhizobium leguminosarum*, as well as *R. meliloti*, *R. fredii*, *R. loti*, *Bradrhizobium japonicum* and other *Bradyrhizobium* spp and *Azorhizobium caulinodans*. Although there are clear differences with regard to range, arrangement and numbers of *nod* genes among these strains, some clear common features have been identified. It is evident that in all of these rhizobia most (but not all) of the *nod* genes are under the control of transcriptional activators encoded by *nodD* genes. In most strains these appear to be multiple copies of *nodD* genes although in the *R. l.* bv. *viciae* and bv. *trifoli* strains studied only single copies of *nodD* have been found. It appears that the NodD proteins bind to flavones, isoflavones (or even chalcones in some cases) secreted from legume roots and this interaction leads to activation of *nod* gene transcription. There is specificity in the NodD-flavone interaction and thus only certain strains of rhizobia will react to certain legumes. The presence of multiple copies of *nodD* presumably increases the range of flavone-like molecules that can be recognised by an individual rhizobial strains.

The NodD proteins bind to highly conserved promoter regions (that extend over about 50 nucleotides) upstream of the *nod* operons they control. Using these promoter regions as DNA hybridisation probes several *nod* operons have been identified and different rhizobia have different numbers of these transcriptional units. In one strain of *R. l.* bv. *viciae* all of the *nod* genes downsteam of these promoter regions have been sequenced and 12 *nod* genes in four operons under NodD control have been identified. In *R. meliloti* six operons have been found and eighteen *nod* genes have been identified thus far. Although there are obvious *nod* gene homologues in common between these two strains there are also *nod* genes unique to each strain.

Many of the inducible *nod* gene products are involved in the biosynthesis of glycolipid signal molecules similar to the acylated sulphated tetraglucosamine molecule (Nod Rm-1) characterised by Lerouge et al (Nature 344, 781-784) from *R. meliloti*. The *nodABC* gene products are necessary for the synthesis of the signal and NodM appears to be involved in the formation of the additional glucosamine precursors required to make the glycolipid. Other *nod* genes products such as NodF and NodE are homologous to proteins (acyl carrier proteins and condensing enzyme) involved in fatty acid biosynthesis and are likely to play a role in determining the type of acyl group on the glycolipid. Although NodF and NodE are highly conserved among some rhizobia, there is genetic evidence to show that they play a key role in determining specificity - presumably by determining the type of acyl substitution. NodG is also similar to an enzyme (β ketoacyl reductase) of fatty acid synthesis and could also determine the type of acyl substituent (*R. meliloti* has *nodG* but *R. leguminosarum* does not).

The *R. meliloti nodHPQ* genes are also key determinants of host specificity and they do this by making a precursor for attachment of a sulphate group and mediating its transfer to the sugar backbone of the glycolipid. Significanlty, these genes are absent from *R. leguminosarum* which makes a glycolipid signal that is not sulphated.

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It is now clear that all of the different rhizobia have a common group of *nod* genes (at least *nodABC*) that are required for a "core" signal that can be subsequently modified by other *nod* gene products to make a group of related but distinct signals that can be specifically recognised by different legumes.

In addition to these glycolipid signals there appear to be other *nod* gene products whose precise role is not known but appear unlikely to be involved in the synthesis or modification of the glycolipid. One of these is the *R. l.* bv. *viciae* NodO protein which is a Ca^{2+}-binding secreted protein that has the potential to interact directly with plant membranes. There are other *nod* genes from *R. meliloti* and *B. japonicum* whose functions have yet to be established; it is possible that these gene products make ancillary signals or stimulate infection such that they improve the overall efficiency of nodulation in certain legumes. However since mutation of the *nodABC* genes in any rhizobial strain totally blocks nodulation and formation of the glycolipid signals, it is evident that this plays the central role in nodule organogenesis.

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Function of Rhizobium leguminosarum bv. viciae nodulation genes involved in the determination of host specificity

Herman P. Spaink and Ben J.J. Lugtenberg
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Introduction

The host-specific interaction between leguminous plants and bacteria of the genera Rhizobium and Bradyrhizobium results in the formation of nitrogen-fixing root nodules. At least two steps of molecular signalling between plant and bacteria appear to be involved in the determination of host-specific nodulation. In the first step flavonoids excreted by the plant induce the transcription of bacterial nodulation genes (called nod or nol genes) (reviewed in references 1 and 2). The host-specificity of this induction process involves the bacterial NodD protein which presumably directly interacts with the flavonoids (3,4). In the second step, the bacterium, by means of the nod genes, produces one or more signals (5,6,7,8) which activate plant genes (9) and trigger root responses like root hair curling (10) and nodule meristem induction (6,8,11). In Figure 1 a model is shown which compiles the present knowledge of the R.leguminosarum nod genes, the presumed localization of their translational products, and their function. In this presentation we give more details about the biochemical function of the Nod proteins which are involved in the determination of host specificity.

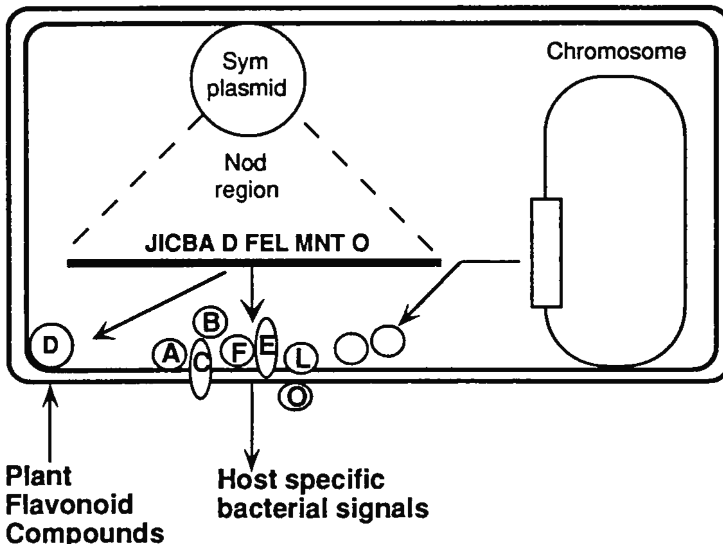

Figure 1. The role of the nod genes of R.leguminosarum bv viciae in the interaction with the host plant.

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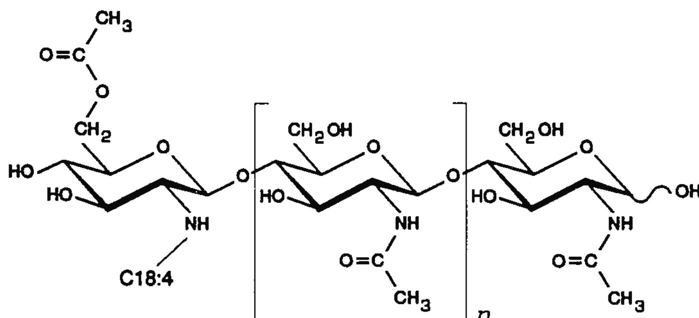
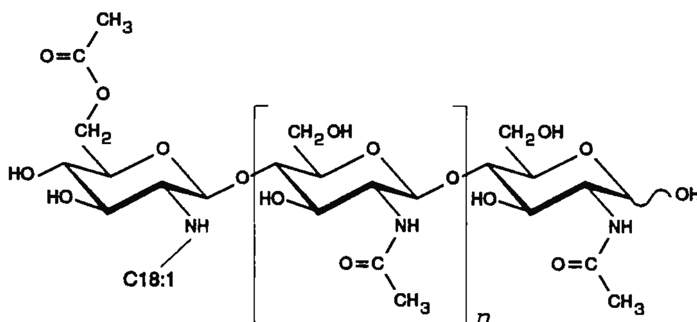
A. *nodABC FEL* - dependent metabolites



n	nomenclature	mass
2	NodRlv-IV (Ac,C18:4)	1088 Da
3	NodRlv-V (Ac,C18:4)	1291 Da

B. *nodABC L* - dependent metabolites



n	nomenclature	mass
2	NodRlv-IV (Ac,C18:1)	1094 Da
3	NodRlv-V (Ac,C18:1)	1297 Da

Figure 2. Nod signals produced by wild type R.leguminosarum bv. viciae strain RBL5560. This figure is based on the data presented by Spaink et al. (8). In the nomenclature used, the Roman numeral following the species indication (Rlv) refers to the number of glucosamine units, while the terms in parenthesis indicate O-acetylation, fatty acyl carbon number and degree of unsaturation, respectively. Also indicated is the predicted nominal molecular mass of the Nod metabolites.

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The NodD-flavonoid interaction

Based on indirect evidence it has been proposed that the NodD protein directly binds to the inducing flavonoids (Figure 1). Several groups have also shown that cytoplasmic NodD protein, purified from NodD overproducing strains, is capable of specific binding to the promoter regions of the flavonoid-inducible nod genes. However, two lines of evidence indicate that the process of NodD protein activation takes place at the cytoplasmic membrane: (i) The inducing flavonoids accumulate in the cytoplasmic membrane (12) and (ii) the NodD protein was localized in the cytoplasmic membrane of wild type Rhizobium cells (13). Considering these results it is well imaginable that the development of an in vitro transcription activation system with NodD protein will require the presence of membranes.

The NodE protein determines host specificity by its involvement the production of a novel fatty acid moiety on lipo-oligosaccharide signal molecules

Using radioactive acetate as a precursor, it was shown that the regulatory nodD gene as well as the nodABC and nodFEL operons of R.leguminosarum biovar viciae are involved in the production of at least five metabolites which are excreted into the growth medium (7,8). Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used as a test system for the purification of the excreted radiolabelled Nod metabolites from a Rhizobium strain containing a wild type Sym plasmid, Sym plasmids with nod gene mutations, or cloned nod genes (8). The purified Nod metabolites were identified using mass spectrometry and NMR analysis (Figure 2). The results indicated all to be lipo-oligosaccharides similar to the signal molecule NodRm-1 of R.meliloti discovered by Lerouge et al. (5). However, several structural differences of the R.leguminosarum biovar viciae Nod metabolites with the NodRm-1 molecule were observed. (i) The Nod metabolites of R.leguminosarum are devoid of sulphate and (ii) contain an additional O-acetyl moiety (iii) their N-acyl group is different and (iv) the number of N-acetylglucosamine units of the sugar backbone of some of the Nod metabolites is different. Furthermore, the two NodFE-related Nod metabolites had a characteristic UV spectrum (as demonstrated by the HPLC diode array analysis) with an absorbtion maximum at 303 nm.

The results of the structural analysis of Nod metabolites of the nod mutant show that of the R.leguminosarum nod genes only the nodABC genes are required to produce a basic lipo-oligosaccharide structure. The nodFEL genes are required to produce Nod metabolites with a different N-acyl chain and an additional O-acetyl substituent. Based on the similarities between all Nod metabolites known, a nomenclature (Fig. 2) is further on used which is also being used now for the R.meliloti signal compounds (J. Dénarié and J.C.Promé, personal communication).

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SECOND SESSION

P. BOISTARD
D. W. EMERICH

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OXYGEN REGULATION OF SYMBIOTIC NITROGEN FIXATION IN *RHIZOBIUM*

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Together with ammonia assimilation and / or nitrate reduction, symbiotic nitrogen fixation constitutes one of the major routes of entry of nitrogen into the metabolic pathways of *Leguminosae* plants which have developed symbiotic relationships with the nitrogen-fixing prokaryotes *Rhizobium, Bradyrhizobium* and *Azorhizobium*. These symbiotic associations result in the development of root (or exceptionally stem) nodules. In infected cells, differentiated bacteria, called bacteroids, synthetize the nitrogenase enzyme, which reduces molecular nitrogen into ammonia. Nodule formation is under the genetic control of both the plant and the bacteria (Long, 1989).

Oxygen concentration is a key regulatory factor in the establishment of a nitrogen-fixing nodule. Recent results have enabled us to understand to what extent the oxygen concentration regulates the expression or activity of the bacterial genes whose products are needed for symbiotic nitrogen fixation.

1. Nodule anatomy and leghemoglobin control oxygen supply to the bacteroids:

The histology of a mature nodule distinguishes a central tissue including invaded cells, delimited laterally by peripheral tissues such as the vascular bundles and the nodule endodermis. The outer cortex surrounds the nodule. The endodermis and the inner cortex constitute the major barriers to oxygen diffusion (Witty *et al.*, 1986).

Leghemoglobin (Lb) is a hemoprotein found in the cytoplasm of infected cells (VandenBosh and Newcomb, 1988), where it facilitates the diffusion of oxygen to respiring bacteroids (Appleby, 1984). The ferrous Lb exhibits a very high affinity for oxygen. The biological function of Lb could be to allow a fast supply of oxygen for the bacteroids respiratory chains and to maintain a low oxygen concentration around the bacteroids, preventing oxygen damage for the enzyme nitrogenase which is irreversibly inactivated by oxygen. Therefore both the nodule architecture and leghemoglobin participate in making the symbiotic environment microaerobic.

2. Oxygen concentration regulates nitrogen fixation genes expression and activity in *Rhizobium meliloti*:

R. meliloti fixes nitrogen only in symbiotic conditions. The most characterized nitrogen fixation genes in *R. meliloti* are clustered in two groups on the symbiotic megaplasmid pSym.

The first *nif-fix* gene cluster comprises the *nifHDK* structural genes for the polypeptide subunits of the nitrogenase complex, *nifE, N* and *B* required for the synthesis and processing of the iron molybdenum cofactor (FeMoCo) and *fixABCX* which are thought to code for factors involved in nitrogenase specific electron transport (Long, 1989). Symbiotic expression of these genes is activated by the regulatory protein NifA (Szeto *et al.*, 1984). Both *nifA* expression and NifA activity is regulated by oxygen (Ditta *et al.*, 1987; Fisher *et al.*, 1988) . NifA oxygen sensitivity is probably related to a NifA domain which carries cysteine residues, which could be involved in redox activity or metal binding or both .

A second *fix* gene cluster has been identified in *R. meliloti*. This cluster comprises four operons: *fixLJ, fixK, fixNOQP* and *fixGHIS* (David *et al.*, 1988: Batut *et al.*, 1989; Daveran, 1988; Kahn *et al.*, 1989). *fix LJ* and *fixK* are regulatory genes (see below). *fixGHIS* and *fixNOQP* gene products are thought to be membrane located . FixG has homologies with bacterial ferredoxins. (Kahn *et al.*, 1988). FixP has homologies with c-cytochromes. Since *fixN* expression is induced under free-living microaerobic conditions and symbiotic conditions (David *et al.*, 1988), this raises the possibility that *fixNOQP*

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encodes components of a respiratory chain which allows efficient respiration under microaerobic conditions (Daveran, 1988). It is known that the differentiation from free-living bacteria to bacteroids leads to a variation in cytochrome pattern (Appleby, 1984).

In contrast to the first *nif-fix* gene cluster, the *fixN* operon expression does not require the transcriptional activator NifA (David *et al.*, 1988), but instead requires the product of *fixK*. FixK positively regulates *fixN* expression and negatively regulates its own expression and *nifA* expression. FixK bears extensive homology with two *E. coli* transcriptional activators Crp and Fnr which regulate catabolic genes and genes coding for anaerobic electron transport chains, respectively (Batut *et al.,* 1989). Although FixK and Fnr are closely related proteins, FixK lacks the Fnr N-terminal domain, which is likely involved in Fnr oxygen sensitivity (Trageser and Unden, 1989). This suggests that FixK is not involved in oxygen sensitivity in *R. meliloti.*

In the free-living diazotroph *K. pneumoniae*, nitrogen fixation genes are activated by the *nifA* gene product. *nifA* expression is regulated principally by the nitrogen status of the cell and depends of the NtrB/NtrC regulatory proteins (Gussin *et al.*, 1986). In contrast, symbiotic nitrogen fixation is unaffected in *R. meliloti ntrC* mutants (Szeto *et al*, 1987). Instead in *R. meliloti*, it has been shown that the expression of *nifA* and *fixK* is activated *ex planta* in microaerobiosis (Ditta *et al.*, 1987; Batut *et al.* 1989). Consequently, oxygen concentration appears to be the major physiological trigger of symbiotic nitrogen fixation gene expression in *R. meliloti.*

3. FixL is an oxygen sensor protein which regulates *R. meliloti nifA* and *fixK* genes expression by the transcriptional activator FixJ:

In *R. meliloti*, symbiotic and microaerobic expression of *nifA* and *fixK* is activated by a pair of regulatory proteins, FixL and FixJ (David *et al.*, 1988; Batut *et al*, 1989), which belong to a family of two component regulatory systems (Stock *et al*, 1989). In agreement with the proposed regulatory mechanism of these systems, it was predicted that FixL transduces environmental signal(s) to the FixJ transcriptional activator by reversible phosphorylation of FixJ, which modulates its activity.

FixJ has been shown to be a transcriptional activator of *nifA* and *fixK* (Hertig *et al.,* 1989). FixL is an oxygen sensor which modulates FixJ activity in response to the oxygen level (de Philip *et al.*, 1990). More recently, it was shown that FixL is a hemoprotein which catalyzes its own phosphorylation and is a FixJ kinase (Gilles-Gonzalez *et al.,* 1991). A model proposes that FixL senses oxygen through its heme moiety and transduces this signal by controlling the phosphorylation of FixJ.

Nitrogen fixation genes of *R. meliloti* are thus activated by a cascade regulatory pathway in response to oxygen concentration. Nodule morphogenesis, which limits oxygen concentation in the invaded zone, can be viewed as generating a signal which allows the building up of the nitrogen fixing apparatus of the bacteroids to be integrated with the developmental programme of the nodule.

4. Oxygen and symbiotic nitrogen fixation in *R. leguminosarum*, *Bradyrhizobium japonicum* and *Azorhizobium caulinodans*

In other *Rhizobium* species than *R. meliloti* and in species of the genera *Bradyrhizobium* and *Azorhizobium* , available evidences suggest that the regulatory mechanisms which connect nitrogen fixation gene expression to oxygen concentration might be diverse.

Two results suggest that at least some genetic determinants are conserved between *R. meliloti* and *R. leguminosarum*. Microaerobic induction of the hydrogen-uptake genes *hupV* and *hupVI* of *R. leguminosarum* requires the presence of functional *fixLJ* and *fixK* at least in *R. meliloti* (Palacios *et al*, 1990). Elsewhere, in *R. meliloti*, the *R. leguminosarum* gene *fnrN* is capable of promoting microaerobic and symbiotic induction of the *fixN* gene independently of *fixLJ* (Colonna-Romano *et al.*, 1990). FnrN is very similar to Fnr and carries the Fnr N-terminal domain essential for the oxygen regulated activity of Fnr. FnrN could be an oxygen sensor protein in *R. leguminosarum.*

Fundación Juan March (Madrid)

In *B. japonicum*, expression of nitrogen fixation genes occurs during symbiosis in root nodules or in free-living cells under microaerobiosis. Three regulatory elements are involved in oxygen control at different levels. 1). The positive regulatory protein NifA . NifA activity is regulated by oxygen and under microaerobiosis, NifA positively autoregulates its own promoter and binds to the *nifH* promoter in the presence of metal ions. 2). *fixLJ* like genes are present in *B. japonicum*. They are not involved in *nifA* regulation, but in the regulation of other *fix* genes probably involved in anaerobiosis respiration (Anthamathen and Hennecke, 1991). 3). Two functional genes (*rpoN1, rpoN2*) encode for NtrA. *rpoN1* expression is oxygen regulated via a mechanism involving *fixLJ*. The two last points represent clear differences with *R. meliloti* (Fisher *et al.*, 1991).

Interestingly *A. caulinodans nif* gene expression is controlled both by nitrogen availability as well as by oxygen (Ratet *et al.*, 1989)). *fixLJ* genes were caracterized in *A. caulinodans* (Kaminsky and Elmerich, 1991)), and are structurally very similar to *R. meliloti fixLJ* genes. *A. caulinodans fixLJ* genes are essential for *nifA* expression. *fixLJ* mutants are essentially defective in nitrogen fixation *ex planta* and *in planta*.

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The Use of Lambda Expression Libraries and pLAFR1 Libraries to Isolate Genes for the Citric Acid Cycle Enzymes from **Bradyrhizobium japonicum.**

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Several lines of evidence support the relationship between symbiotig nitrogen fixation and organic acid metabolism: (i) organic acid metabolism stimulates ex planta nitrogen fixation better than all compounds tested, (ii) organic acids are actively transported by the peri-bacteroid membranes and (iii) mutants defective in organic acid uptake or metabolism produce nodules with reduced nitrogen fixation activity.

Our primary objective was to isolate and sequence the gene for malate dehydrogenase from **Bradyrhizobium japonicum**. The initial attempt to generate mutants lacking malate dehydrogenase and to complement them from a pLAFR1 library. Presumptive mutants defective in malate utilization were obtained after ampicillin-selection for spontaneous mutations, or ultra-violet mutagenesis, and after Tn5 mutagenesis. The majority of these isolates regained the ability to grow on malata after subculturing. A curcory investigation of these mutants indicated some were apparently compensating for the apparent mutation in malate dehydrogenase by expressing malic enzyme, phosphoenolpyruvate synthetase and phosphoenolpyruvate carboxylase to form oxaloacetate independent of malate dehydrogenase.

Rather than attempt to sort out the myriad effects we had witnessed, we decided to first isolate the gene by other methods and then construct defined mutations. A recombinant B, japonicum genomic DNA library was constructed in the fusion protein vector Lambda gt11. Polyclonal antibodies to purified B. japonicum malate dehydrogenase were generated and characterized for their use as probes for screening recombinant expression libraries. The carboxy-terminal portion of the malate dehydrogenase gene was isolated from the Lambda gt11 library and was used as a probe to screen the cosmid libraries of **B.japonicum**. A cosmid clone was solated which hybridized to the probe and was shown to contain a 26 kbp insert. Using Southern blot analysis of restrction digests of this insert, a 3.5 kbp SmaI fragment was isolated which hybridised to the probe. The DNA sequence of the gene was determined from both strands and confirmation that the structural gene for malate dehydrogenase had been isolated was obtained by the complete agreement between the deduced amino terminus of the translated sequence and that determined from sequencing the purified protein. Examination of the DNA sequence downstream of the malate dehydrogenase gene revealed coding regions which when translated exhibited homology to the subunits of succinyl-CoA synthetase and the dehydrogenase subunit of alpha-ketoglutarate dehydrogenase. These structural genes are all preceded by typical -24/-12 promoters. In addition, the malate dehydrogenase gene is preceded by a promoter of the **hemA** type. Two sets of inverted repeats were located upstream of the malate dehydrogenase gene but there was no region which bore resemblance to the NifA upstream activator sequence.

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THIRD SESSION

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Rhizobial genes involved in competitiveness.

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The manipulation of symbiotic nitrogen fixation to increase the productivity of legumes is not easy. Among all the posibilities we can succesfully act on the competitiveness and the efficiency of the process apart from the obtention of strains resistant to phytochemicals and agresive environmental conditions.

The inoculation with superior strains of root nodule bacteria to improve legume productivity in agricultural fields often fails because of the inability of the superior inoculation strains to nodulate in soils with a large population of indigenous rhizobia. Therefore competitiveness is one of the main limitant factors to improve the symbiotic nitrogen fixation by the *Rhizobium*-legume association.

Several characteristics of the bacteria have been related to the competitiveness, such as speed of nodulation, motility, different level of attachment, extracellular polysaccharide production and effectiveness. However, none of them have showin a direct correlation with competitiveness.

Some metabolic properties of the bacteria could be involved in this character. For instance, the synthesis and catabolism of rhizopines that enable the bacteria to be in better conditions to nodulate than those lacking these abilities.

In spite of the interest of this characteristic not too much information at the level of molecular biology has been reported.

Different genes have been reported to be involved more or less directly in the competitiveness. Some of them, as GSN genes, of genotype specific nodulation, that are those bacterial genes that allow nodulation of specific plant phenotypes within a given legume species, are of restricted applica-

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tion. We can show three examples. One belongs to *R. leguminosarum* bv. *viciae* strain TOM which nodulates Afghanistan pea that is not nodulated by european bacterial strains. *nodX* gene has been found as responsible of this particular ability. *nolA* gene from *B. japonicum* strain USDA 110, determines the nodulation of restricting genotypes of soya. Another GSN like-locus has been reported in *R. fredii* strain USDA 257, that allows the nodulation of only unimproved cultivars of soya. Tn*5* mutants of this bacterial strain nodulated cultivars released by plant breeders.

Other genes as *tfx* and *nfe* genes are more directly involved in competitiveness. The first genes determine the biosynthesis of trifolitoxin, a bacteriocin of *R. leguminosarum* bv. *trifolii* strain T24. It was found that this strain was highly competitive for nodulation when coinoculated with a bacteriocin-sensitive strain. Other examples could be presented.

nfe genes, of nodule formation efficiency, are present in *R. meliloti* strain GR4. These genes provide this strain a high competitive character. These genes, that can be transferred to other strains, are expressed under microaerobic conditions since they are NifA dependent. These genes could be of great interest from the applied point of view.

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MOLECULAR BIOLOGY OF THE HYDROGEN UPTAKE HYDROGENASE FROM *Rhizobium leguminosarum*

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Some strains of *Rhizobium* and *Bradyrhizobium* induce in legume nodules the synthesis of a H_2-uptake (Hup) enzyme system that recycles the H_2 generated by nitrogenase during the nitrogen fixation process. In the *B. japonicum* symbiosis, this *hup* system has been shown to save energy and provide other biological advantages as well (1). The first component of the *hup* system is a membrane-bound, [NiFe] hydrogenase which contains two polypeptide subunits of about 35 and 65 kD (1). The genetic determinants (*hup* genes) for the structural subunits of the hydrogenase of *B. japonicum* lie in a cluster of *hup*-specific DNA spanning a region of about 16 kb of the chromosome (2). Hydrogenase positive strains of *R. leguminosarum* contain DNA homologous to *B. japonicum hup* genes. Based on this homology, a recombinant cosmid (pAL618) containing the entire set of *hup* genes required for H_2-uptake in pea nodules was isolated from a pLAFR1 gene library of *R. leguminosarum* strain 128C53 (3).

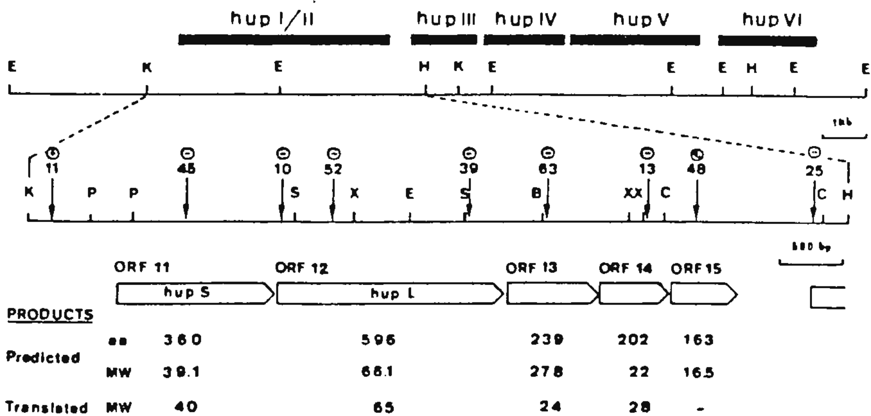

Fig.1: Gene organization of the *Rhizobium leguminosarum hup* operon containing the hydrogenase structural genes. The horizontal black bars show the location of the transcriptional units in pAL618 as defined by site-directed transposon mutagenesis and complementation analysis. The positions of the *Tn5* insertions in the mutants were determined by sequencing and are indicated by vertical arrows. The encircled +/- over the arrows indicate the Hup phenotype associated with each mutant. The open arrowed boxes indicate the position and orientation of the ORFs identified in the sequence. The table below the ORFs shows the number of aa and the molecular weight for the predicted products and the molecular weight of the peptides detected in *E.coli* cells from translation of the ORFs with a T7 promoter/ RNA-polymerase system.

Fundación Juan March (Madrid)

The organization of R. leguminosarum hup gene cluster in the 20 kb DNA insert of pAL618 was investigated by site-directed transposon mutagenesis and complementation analysis (4). The hup genes were found to span over 15 kb and to be organized in six transcriptional units designated hupI to hupVI (Fig 1) (4). By constructing hup-lacZ fusions, we have shown that none of the hup transcriptional units is induced in aerobically grown free-living cells of R. leguminosarum, but transcriptional units hupV and hupVI are activated in vegetative cells in response to microaerobic conditions (5).

In order to identify the hup genes in the R. leguminosarum hup cluster, we have sequenced a 15 kb fragment of the pAL618 DNA insert. Five genes were found in the DNA corresponding to the transcriptional unit hupI/II (Fig 1). Transposon Tn5 insertions within these genes were associated with total or partial reduction of the hydrogen uptake activity observed in the wild-type strain. The five genes belong to a single operon which is transcribed from an NtrA-dependent promoter. The two first genes of this operon code for the small (hupS) and large (hupL) subunits of the R. leguminosarum hydrogenase (6). Sequence homology data suggested that the other three genes (ORF13, ORF14 , ORF15) code for proteins of a membrane protein complex involved in transferring electrons from hydrogenase to ubiquinone in the electron transport chain.

Ten other genes were identified in the DNA corresponding to transcriptional units hupIII, hupIV, hupV and hupVI (see abstract by L. Rey et al in this meeting). The exact functions of these genes are not known yet. However, all of them seem to be required for the synthesis of an active hydrogenase. The gene products from operons hupV and hupVI had homology to components of the hyp operon of Escherichia coli (7), which is required for the synthesis of the three hydrogenase isoenzymes of this bacteria, and may be involved in incorporation of metals (Ni, Se) or metal clusters (Fe-S) into the hydrogenase.

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Fundación Juan March (Madrid)

FOURTH SESSION

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ROOT NODULE FORMATION: A SYSTEM TO STUDY PLANT DEVELOPMENT

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INTRODUCTION

Legume root nodule formation is induced by bacteria of the genera ***Rhizobium***, *Bradyrhizobium* or *Azorhizobium*. A particular bacterium species is able to interact with one or a limited number of legume species. Rhizobia attach to the roots of their host and cause a characteristic curling of the host's root hairs. The rhizobia then invade the plant by way of a newly formed tube called the infection thread. Meanwhile, cells in the root cortex start to divide and form the nodule primordium. Infection threads enter individual primordium cells, and bacteria are released from the infection thread into the cytoplasm of the target cell. Bacteria then differentiate into their endosymbiotic forms named bacteroids, and begin to fix nitrogen by the action of the enzyme nitrogenase, a process that is assisted by the morphology and physiology of the root nodule. Ammonia is transported from the bacteroids to the plant cytoplasm, where it is assimilated and then transported to other parts of the plant.
Our research program especially concentrates on the developmental aspects of root nodule formation and the molecular interactions that guide this development.

Plant nodulin genes

The plant proteins that are specifically formed during the formation and functioning of a root nodule are called nodulins. The nodulin genes have been operationally defined as early and late nodulin genes according to the timing of their expression during nodule development.
The late nodulin genes comprise a large group of genes that are expressed around the onset of nitrogen fixation. Late nodulins aid in the function of a root nodule by creating the physiological conditions required within the nodule for nitrogen fixation, ammonium assimilation, and transport. Among the identified late nodulins are the leghemoglobins, uricase, and subunits of sucrose synthase and glutamine synthetase. In terms of development, late nodulins are truly late, because the full nodule structure with all its defining characteristics has developed before late nodulin gene expression become detectable. Relatively few nodulin genes have been identified that are expressed in the developing root nodule well before the onset of nitrogen fixation, these are the early nodulin genes.
We have isolated 2 early nodulin cDNA clones encoding proteins involved in the infection process, pPsENOD5 and pPsENOD2 (4, 5). Both proteins are proline rich. ENOD12 is composed of two repeating pentapeptides containing two pro residues each, whereas ENOD5 might be an arabinogalactan like protein. The ENOD2 early nodulin (6) has a similar structure as ENOD12, but this gene is specifically expressed in the nodule parenchyma. In addition to these proline rich early nodulins we isolated cDNA clones of two early nodulin ENOD3 and ENOD14 (5), that are not proline rich. The ENOD3 and ENOD14 early nodulins are about 60% homologous. This are small peptides of about 6 kDa containing 4 cysteine residues, with a spatial distribution indicating that it are metal binding proteins.

Rhizobium nod-genes

The rhizobial genes required for nodule formation and nodulin gene expression include the nodulation (*nod*) genes, several groups of genes concerned with the structure of the

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outer surface of the bacterium (the *exo*, *lps*, and *ndv* genes), and a number of less well defined genes. Formal proof for the involvement in the induction of the expression of early nodulin genes has only been demonstrated for the *nod* genes. Upon transfer of the *nod* gene region, the recipient non-nodulating *Agrobacterium* gained the ability to nodulate. The *nod* gene products, therefore, are the most likely candidates to be signals eliciting nodule development.

The *nod* genes fall in three classes, common, host-specific, and *nod*D. The common *nod* genes include, among others, the *nod*ABC genes that are found in all rhizobial species. The common *nod* genes of different species are structurally very similar and functionally interchangeable. Mutations in the *nod*ABC genes abolish completely the ability to nodulate, which underlines their pivotal role in nodule development.

The host-specific *nod* genes determine the specificity of nodulation on a particular host. Upon mutation of such a *nod* gene, nodulation is delayed or reduced, or the host range is altered.

The third type of *nod* genes is *nod*D, of which is the only *nod* gene that is constitutively expressed in both the free-living and symbiotic states of *Rhizobium*. In combination with flavonoids excreted by plant roots, the NodD protein probably acts as a transcriptional activator for all other *nod* genes.

Upon induction of the *nod*-genes rhizobia secrete Nod factors which are lipo-oligosaccharides (1). These Nod factors are able to induce root hair deformation (1), cortical cell division (2) and they are also involved in the infection process since they induce the expression of the early nodulin genes ENOD5 and ENOD12.

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EXPRESSION AND REGULATION OF NODULINS AND NODULIN GENES IN *Phaseolus vulgaris* L.

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INTRODUCTION

Legume nodule formation has been divided into three major stages: "preinfection", "infection and nodule formation" and "nodule function" (1). Nodulins are plant proteins that accumulate specifically or preferentially in nodules. Early nodulin genes are detected during the "infection and nodule formation" stage (2). Late nodulin gene expression starts at about the onset of nitrogen fixation (3). Recently, some nodulins have been found to be expressed in other parts of the plant distinct from the nodule (4,5).

Late nodulins comprise the best-known nodule-regulated proteins (3,6). They include enzymes involved in nitrogen assimilation, in carbon metabolism, and in amine and ureide biogenesis (7). Proteins located in the peribacteroid membrane (PMB) (6) and the leghemoglobins (Lbs) (8) also belong to this group. Several enzymes involved in these metabolic processes have been identified as nodulins (eg. uricase II); others are common to several organs but show enhanced activity in nodules (glutamine sinthetase).

GLUTAMINE SYNTHETASE REGULATION IN *Phaseolus vulgaris* ROOT-NODULES.

Glutamine synthetase (GS) and glutamate synthase (GOGAT) activity increse along with nitrogenase activity during nodule formation. Both enzymes are responsible for assimilating the ammonia derived from nitrogen fixation (9). We will consider only recent data of Glutamine Synthetase in this report.

Glutamine synthetase in *Phaseolus vulgaris* root-nodules, is expressed as two different isoforms denominated GSn-1 and GSn-2 (10). GSn-1 is expressed during nodule development concomitantly with nitrogenase activity (11). The nodule GSn-1 is composed by a polypeptide called gamma and the GSn-2 isoform is composed by the beta polipeptide, which is expressed also in roots and leaves (11). The GS gamma polypeptide is expressed before nitrogenase activity (12) but its maximal induction requires normal nitrogen fixation as reported for other nodulins (13). Expression of the

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gamma (gln-gamma) and beta (gln-beta) GS genes in Lotus corniculatus transgenic plants, demostrated that gln-gamma is expressed in the infected zone of the mature nodules where ammonia assimilation takes place, and gln-beta is expressed in the nodule parenchyma (cortex) (14). These results strongly suggest that GSn-1 (gamma) is the reponsible for ammonia assimilation in bean nodules. Furthermore, in bean plants grown in argon (Ar), where nitrogen fixation is reduced, the expression of the gamma polypeptide is inhibited, suggesting that ammonia regulates its expression (15). On the other hand, in nodulated bean plants grown in an enriched CO2 atmosphere (1000 ppm), nitrogenase activity increased two to three-fold; a significant reduction of the GS-gamma, but not of the GS-beta polypeptide was also observed (Ortega, J.L., et al, Submitted to Plant Physiology). Taking these different results together, it seems that both high and low nitrogenase activities can be correlated with a reduction fo GS-gamma. These data suggest that the carbon/nitrogen balace within the nodule, and not ammonia availability per se, may be the main nodulating factor of GS expression.

NODULIN REGULATION IN COMMON BEAN NODULES INDUCED BY BACTERIAL MUTANTS.

Nodulin expression was evaluated in nodules of common bean induced by R. phaseoli mutants and an Agrobacterium transconjugant. Elicited nodules were either "empty" arrested, slow-developed or ineffective. The expression levels of ENOD2, uricase-II, leghemoglobin and nodulin-30 (Npv-30) genes were compared in nodules induced by wild type and mutant strains at initial (12 d) and terminal (21 d) developmental stages.

Uricase-II mRNA (considered to be a late nodulin) was detected in "empty" nodules produced by three different mutants, suggesting the existence of novel regulatory conditions for this nodulin during early stages of nodulation. Accumulation of ENOD2 and uricase-II transcripts were observed in slow-developed but not in arrested nodules. Npv-30 and Lb are only expressed in nodules containing infected cells; however, their relative levels differ depending on the nodule-inducing mutant. Ineffective strains produced nodules with similar initial development and nodulin gene expression, but decreased amounts of late nodulin transcripts at the terminal stage (16). The possible signals involved in the regulation of these genes will be discussed.

NODULIN GENE FAMILIES IN BEAN NODULES.

We have previously reported that in common bean-nodules a group of very abundant-nodule transcripts encode for a group of 30-kD (Npv-30) products (17). Sequence analysis of two cDNAs and

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a genomic clone revealed high homology with a nodulin gene family from soybean. The gene products of this family have two domains that are arranged in paired Cys-X7-Cys motifs, resembling zinc-finger sequences; all products also exhibit a conserved region that encodes for a putative signal peptide. These nodulin families might have a common function, most likely as metal ion carriers in infected cells (Campos, F., et al. 1991 Submitted to Plant Mol. Biol.).

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LECTINS, LECTIN GENES, AND RHIZOBIUM

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A. INTRODUCTION

Lectins are sugar-binding (glyco)proteins other than enzymes or antibodies (Barondes 1988). Sugar-binding is specific and reversible. E.g., wheat germ agglutinin (WGA: wheat lectin), binds N-acetylglucosamine and its oligomers, whereas soybean lectin is a galactose/N-acetylgalactosamine-binding protein. Because of their unique properties, lectins are useful tools for (i) separation and characterization of glycoproteins and other glycoconjugates, (ii) histochemical studies of cells and tissues, (iii) typing and fractionation of various types of cells, and (iv) description and induction of changes occurring on cell surfaces during developmental and pathological processes (Sharon and Lis 1990). Plant and animal lectins can be grouped into families comprising lectins with common structural properties and a significant amino acid sequence identity. Within a family, sugar-binding specificities of its members may vary considerably. Legume lectins constitute the best-characterized family of lectins.

B. LEGUME LECTINS

Leguminous plants (Fabaceae), like pea, bean and soybean, produce a homogeneous group of secretory lectins (Van Driessche 1988). Legume lectins accumulate in protein storage vacuoles in the cotyledons, and may constitute up to 10% of total seed protein. These seed lectins are synthesized during seed development together with the more abundant seed storage proteins. Other plant tissues usually contain very small amounts of lectin, either being present in vacuoles, in cell walls, in intercellular spaces, at the root surface or in the rhizosphere (e.g., Vodkin and Raikhel 1986, Diaz et al. 1990). Most legume tissues contain one lectin or a limited set of near-identical isolectins. For some legumes however, presence in the same tissue of two or more lectins with different sugar-binding specificities has been described. Specific lectin ligands from plant tissues have not been characterized yet.

C. STRUCTURE OF LEGUME LECTINS

Legume lectins consist of two or four subunits, usually made up of single polypeptide chains with a Mw of 25-30 kDa. In Vicieae lectins, the two subunits consist of a heavy beta-chain and a light alpha-chain, due to additional processing. Each lectin

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subunit is dome-shaped, and its structure is primarily determined by two pleated sheets. The single sugar-binding site at the top of each subunit is mainly composed of parts of loops connecting the strands of the pleated sheets (e.g., Derewenda et al. 1989). Differences in amino acid composition of these loops are correlated with differences in sugar-binding specificity between legume lectins. Presence of a calcium (and a manganese) ion in a highly conserved site at the top of each subunit is necessary for obtaining the required structure of the sugar-binding site. In addition to sugar-binding sites, legume lectins contain several sites or pockets for hydrophobic ligands.

Most legume lectins are N-glycosylated. However, glycosylation is not required for the biological activities of glycoprotein lectins (Hoffman and Donaldson 1987).

C. BIOSYNTHESIS OF LEGUME LECTINS

A small family of genes usually encodes legume lectins and lectin-like proteins. Lectin genes do not contain introns, a property shared with genes encoding various storage proteins and enzyme inhibitors (e.g., Fisher and Goldberg 1982, Forde et al. 1985, Hattori and Nakamura 1989). The genetical basis of lectin biosynthesis differs between legumes. In Phaseolus, the seed lectin PHA is composed of five isoforms of the polypeptides PHA-E and PHA-L in different combinations. These polypeptides are encoded by two tandemly linked genes, dlec1 and dlec2, respectively (Hoffman and Donaldson 1985). Data on non-seed bean lectin (genes) are lacking. In Dolichos, leaf/stem-lectin DB58 and seed lectin are encoded by separate, homologous and closely linked genes (Harada et al. 1990), whereas in pea, seed and non-seed lectin are encoded by the same single gene (Gatehouse et al. 1987, Kaminski et al. 1987, De Pater et al. in preparation). In addition to functional genes, a lectin gene family may comprise pseudo-genes (Kaminski et al. 1987).

The molecular basis of differential expression of lectin genes in legume tissues is unclear yet.

Lectins are synthesized as a prolectin on the rough endoplasmic reticulum. After removal of the leader peptide, the protein enters the secretory system of the plant cell. Posttranslational modifications like N-glycosylation or peptide cleavage may take place in the Golgi apparatus or in the protein storage vacuoles, either before or after folding of the peptide chain. A similar biosynthetic pathway has been found for the lectin-like alpha-amylase inhibitor from bean (Chrispeels and Raikhel 1991).

D. LEGUME LECTINS AND RHIZOBIUM

Rhizobium bacteria induce formation of nitrogen-fixing root nodules in roots of leguminous plants. This nodulation is host-plant-specific, e.g., R. leguminosarum biovar viciae nodulates pea, lentil, and vetch, whereas R.meliloti is the symbiotic partner of alfalfa. In addition to other determinants of

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specificity, legume root lectin plays a role in specific interactions of rhizobia with its target root cells. In pea, the pattern of location of pea lectin (Psl) on the root surface entirely corresponds with the susceptibility of root epidermal cells to infection by Rl bv viciae bacteria (Diaz et al. 1986). Psl molecules enhance the accumulation of infective rhizobia on pea root hair tips (Kijne et al. 1988). In addition, Psl is probably involved in development of a rhizobial infection site. Transformation of white clover (hairy) roots with the pea lectin gene confers upon these roots the ability to be infected (and nodulated) by Rl bv viciae (Diaz et al. 1989). In soybean, secreted root lectin increases the proportion of the rhizobial population that is capable of efficient nodulation (Halverson and Stacey 1986). Both rhizobial lipo-oligosaccharides (Lerouge et al. 1990) and extracellular saccharides (Abe et al. 1984) are candidates for representing bioactive lectin ligands. The signal-transduction pathways in which legume lectins and its rhizobial ligands participate are presently unknown.

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POSTER SESSIONS

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ON THE REGULATION OF NITRITE ACCUMULATION IN LEGUME NODULES

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It has long been known that nitrate supply inhibits nitrogen fixation in legume nodules. Among several hypotheses proposed to explain this inhibition, nitrite, the product of nitrate reduction, has been regarded as a good candidate for the causative factor of nitrogenase activity decline. Although recent reports tend to dismiss a major role of nitrite in the short-term, it is still considered a potential inhibitor of nodule metabolism in the medium or long-term.

By using an extraction medium which nearly supressed enzyme activity and a very rapid manipulation of nodules, we found that nitrite did not accumulate following nitrate supply during an 8-days time-course experiment. Also, longer exposures did not cause any nitrite accumulation at significant levels.

However, nitrite rapidly built up in these nodules after detachement. This process is mediated by oxygen concentration within the nodule since, in the presence of pure N_2 gas, nitrite accumulation was 4-times greater and, conversely, it was prevented by exposure to pure O_2. Furthermore, nitrite produced in detached nodules under atmospheric conditions was scavenged by transfering these nodules into 100% oxygen.

These results probably explain the controversy about nitrite formation in nodules following nitrate supply. However, restriction of oxygen availability could lead to nitrite accumulation within nodules. The key role of oxygen in inorganic nitrogen metabolism is currently under study.

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MOLECULAR CHARACTERIZATION OF THE MAJOR NODULIN-SPECIFIC TRANSCRIPT OF THE PHASEOLUS VULGARIS

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During the symbiotic interaction between the soil bacteria **Rhizoboium leguminosarum** bv. **phaseoli** and the roots of the common bean (**Phaseolus vulgaris**), nitrogen-fixing nodules are induced. The identification of about 20 nodule-specific host proteins (Nodulins) from P. **vulgaris** has been described. A group of abundant nodulin transcripts that encode for proteins in the 30 kD range were detected by in vitro translation from common bean module mRNA, this group of nodulins has been named nodulin-30. These nodulins exhibit a peculiar pattern in 2D-PAGE. We haved cloned and characterized the gene for one the nodulin-30 proteins. The nucleotide sequence of a cDNA and corresponding genomic clones has been completed. The deduced amino acid sequence of this nodulin-30 gene shows homology with the major nodulin gene family from soybean (nodulin-22, nodulin-23, nodulin-26B, nodulin-27, nodulin-44). The nodulin-30 contains two sequences resembling "Zinc finger" motives, the aminoterminal one contains a potential signal peptide sequence and a stretch rich in prolines at the carboxiterminus.

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STUDIES ON MERCURY - TOLERANT RHIZOBIA

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In recent years mercury pollution has become a global problem. Agricultural soil receives Hg-Compounds used as fungicides and seed dresser. Soil microflora including the nitrogen fixing organisms often develop resistance to these toxic chemicals. Many free living N -fixing bacteria play an important role in detoxifying mercury and organomercurial pesticides. We collected several Rhizobial strains from several agricultural farms and screened them for their mercury tolerance. The mercury content of the soil samples and the root nodules were also determined. Mercury volatilizing capacity of these strains were compared with that of some free living diazotrophic bacteria. Only a few rhizobial strains showed moderate mercury resistance properties. This is despite the fact that the soil samples had fairly high concentration of mercury compounds.

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BRADYRHIZOBIUM JAPONICUM Tn 5-MUTANTS AFFECTED IN MEMBRANE-BOUND NITRATE REDUCTASE ACTIVITY.

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Native polyacrylamide gel electrophoresis of membrane vesicles from **Bradyrhizobium japonicum** strain PJ17 cells grown microaerobically with nitrate indicated the presence of two bands expressing nitrate reductase (NR) activity. The relative molecular mass (Mr) of the protein corresponding to each band was 200 kDa and 160 kDa.

Mutation of PJ17 cells was done by mating with **E. coli** strain S17.1 containing the plasmid pJQ18 which carries Tn 5. Mutants were selected by their inability to grow with nitrate as the only nitrogen source under microaerobic conditions and they were unaffected in the utilization of either nitrite, ammonium, glutamate and hypoxanthine when used as the sole nitrogen source. Southern hybridation analysis showed that Tn 5 was present in each mutant. They all had a Fix+ phenotype when inoculated on to **Glycine max.** cv. Williams plants.

Cells of each mutant strain grown aerobically with glutamate and further incubated microaerobically in a medium with nitrate expressed membrane vesiclebound NR activity. Levels of activity, however, were lower, except for mutant GRF6, than that of the parental strain PJ17. After incubation, native polyacrylamide gels electrophoresis of membrane vesicles from strain PJ17 and derivative mutant strains showed that the 200 kDa protein was absent in ten out of the twelve mutants analyzed, and that the 160 kDa protein was missing in the other two mutants. Since any of the mutants was able to use nitrate for growth, both the 200 kDa and the 160 kDa proteins are required for nitrate utilization by cells of **B. japonicum** strain PJ17 cultured under microaerobic conditions.

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CHARACTERIZATION OF THE OXYGEN-DIFFUSION BARRIER IN LUPINUS ALBUS CV. MULTOLUPA NODULES.

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Nowdays it is generally accepted that nitrate-induced decline in nitrogenase activity in legume nodules is due to an increase in the resistance of a variable oxygen diffusion barrier, with the consequent decrease in ATP avalaibility needed for nitrogenase functioning. The aim of this work was to corroborate this hypothesis in lupin nodules.

Lupinus albus L. cv. Multolupa seeds were inoculated with two strains of Bradyrhizobium sp. (Lupinus) differing in their competitiveness characteristics. Plants were grown under controlled conditions and 10 and 20 mM KNO_3 was added 2, 4 and 6 days before harvesting. Characterization of the resistance to oxygen diffusion was performed according to Minchin et al. (1989). Oxygen and Hydrogen concentrations inside the nodules were measured employing microelectrodes designed as in Witty et al. (1987). Histochemical and immunocytochemical studies of nodule carbohydrated components were also carried out.

Results suggest that, similarly to other plants, nitrate increases the resistance to oxygen diffusion in lupin plants. However lupins appear to be more resistant since statistically significative values are obtained only with a 2-fold nitrate concentration and higher exposure times than those needed in other legumes (Minchin el al. 1989).

The increase in resistance seems to be related to nitrate-induced alterations of the nodule cortex. A characteristic feature of lupin nodule cortex is the existence of two or three layers of thick-walled cells. The cell walls are specially thick where surrounding intercellular spaces. Nitrate causes the occlusion of these intercellular spaces with a electron dense material, whose carbohydrated constitution was firstly evidenced histochemically, and then immunocytochemically identified as the glycoprotein decribed by VandenBosch et al. (1989).

The regulatory capacity of the barrier to high external oxygen concentrations seems to be also affected by nitrate application, as evidenced by studies with microelectrodes.

Minchin et al. (1989). Planta 180: 46-52
VandenBosch et al. (1989). EMBO J. 8:335-342.
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EFFECTS OF HERBICIDES ISOPROTURON AND CLORTOLURON ON *Rhizobium*-LEGUME SYMBIOSIS

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It has been demonstrated by several researchs carried out during the last years that most of the herbicides may exert an inhibitory action on growth and biological activity of soil microbial populations.

In previous investigations we have studied the effects of different herbicides on the following parameters of *Rhizobium*-Legume association:

- Growth of rizobia in axenic culture.
- Nodulation ability of rizobia pre-treated with herbicide.
- Nitrogen fixation ability of nodules developed by rizobia pre-treated with herbicide.

The experiments have been performed with different groups of herbicides, in doses similar to recommended field doses. It has been also assayed both commercial mixtures (Trade-marks) and its active ingredients free from excipients. Results shown in this work correspond to a pair of substituted urea herbicides: Clortoluron and Isoproturon on four strains of *Rhizobium meliloti*.

The assayed concentrations, 5 and 10 ppm, do not exert a significant effect on growth of *Rh. meliloti* in axenic culture. However, when rizobia cultivated in the presence of herbicide are washed and then inoculated to alfalfa plants (*Medicago sativa*), a significant decrease in the number of nodulated plants and number of nodules per plant is observed. In the nodules developed, ARA-determined Nitrogen fixation decrease significantly with the pre-treatment of 5 ppm of both herbicides, while 10 ppm completely inhibit this parameter.

From the results obtained, we may conclude that both herbicides affect the symbiosis association by reducing rizobia infectivity on legume as well as nodule Nitrogen fixation ability.

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A STUDY OF THE *nodD* GENE OF *Rhizobium leguminosarum* bv. *phaseoli* CIAT899

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In our laboratory we have isolated a region of the symbiotic plasmid of strain CIAT899 which contains the *nod* genes. We have identified several *nod* genes by hibridization, including the *nodD* gene. Using a *nodD* probe from *R. meliloti*, we have isolated a 0.6 kb fragment which carries *nodD* and this fragment was cloned into plasmid pCR1. This plasmid contains a region of *R. leguminosarum* bv.*trifolii* RS1051 symbiotic plasmid which is not involved in nodulation nor nitrogen fixation. The resulting plasmid pCR3827 (pCR1 + *nodD* from CIAT899) was tested for its ability to extend the host range of *R. leguminosarum* bv. *trifolii* RS1051 to nodulate bean plants. On its normal host (clover) this strain nodulates and fixes nitrogen normally, while in bean plants it is only capable of producing little, white and non-fixing nodules. When the *nodD* gene of strain CIAT899 is forced by recombination into the symbiotic plasmid of strain RS1051, the results obtained are similar, with the difference that the number of nodules increases considerably.

In order to further study this phenomena, we have complemented strain RS1051 :: *nodD* with several clones which carry different sets of *nod* genes of strain CIAT899. On the other hand, we have introduced the 0.6 kb fragment which carries *nodD* to a strain baring a *lacZ* fusion to *nodA* promoter region, so as to look for the ability of this *nodD* gene to induce the *nod* genes and its interaction with flavonoid compounds. Results of the nodulation tests and the *nodA-lacZ* inductions will be presented.

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Study of the denitrifying activity in some strains of free-living *Rhizobium meliloti*

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The ability to denitrify under low oxygen conditions seems to be a characteristic widely distributed between the slow-growing Rhizobia (4), and more scarcely within the fast-growing group, except on *Rhizobium meliloti* where 37 % of the strains that have been tested showed denitrifying activity (1), but with much lower rates than *Bradyrhizobium japonicum* (6). Both bacteroids and free-living bacteria denitrify (10), being the oxygen the main regulatory factor in the system.

It has been suggested that this ability could enhance the survival under anoxic environments and support the anaerobic growth (4,9). It also could be of great importance as a mechanism of nitrite detoxification (3,6), considering the sensitivity of the nitrogenase to this anion (7,8).

Nine strains were tested, showing a great variety in the final products and rates of denitrification; up to date all the *R. meliloti* strains tested were complete denitrifyiers (5,6). Thus, four reduced nitrate to nitrogen, other four carried out the reduction until nitrous oxide and only one, was only able of reducing nitrate to nitrite. For the following experiments the strain 102-F-51 was used because of its good growing conditions and high rates of denitrification.

The desasimilatory NR activity showed to be constitutive and not inducible by the presence of nitrate and / or the low oxygen tensions. However, NiR appeared strongly inducible by the oxygen concentration, but it seems to require a treshold nitrite level to start its activity. This mechanism could be assumed as a system of nitrite detoxification (2), channeling the electrons of the respiratory chains to this anion (3). This hypothesis explaines the transitory increase observed on nitrite concentrations before the appearance of nitrous oxide, when the cells were grown microaerobically. So, the rates of denitrification were five times higher with nitrite than nitrate but in no case this activity supported anaerobic growth.

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EARLY NODULINS OF VIGNA UNGICULATA INDUCED BY RHIZOBIUM SP. NGR234.

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Developmental changes in the synthesis of root-hair proteins were followed in the symbiosis between the promiscuous legume Vigna unguiculata and the broad host-range Rhizobium sp. NGR234. Comparison of the two-dimensional electrophoresis patterns of proteins isolated from root-hairs inoculated with wild-type or hair-deformation minus mutants revealed a series of 12 symbiosis-specific proteins, which can be divided into two classes. Synthesis of three proteins were repressed 4 days after inoculation with Rhizobium (class I), one of which was also a root-hair specific protein. Nine proteins were induced by Rhizobium 1, 2 or 4 days after inoculation (class II), of which three proteins (hadulins 15, 31, and 44 kD) were transiently expressed. Five proteins (including hadulins 15 and 31) were first visible 24 h after inoculation, while only one of these (hadulin 15) was not induced by R. fredii USDA257S1 (Nod^+, Fix^+ on V. unguiculata). Hadulin 15 may therefore represent a strain-specific and legume-specific protein. After 2 days, three additional proteins became apparent, while another (hadulin 44) appeared on day 4. All 12 proteins seem to be involved in root-hair deformation and nodule development.

To clone genes, which encode the symbiosis-specifc proteins identified in Vigna unguiculata, a cDNA-libary was constructed from mRNA extracted out of root-hairs inoculated with Rhizobium sp. NGR234. Root-hairs were isolated at the day 1 and 4 after inoculation. Differential screening of the libary is just in progress.

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NITRATE INHIBITOR ON NODULATION CAN BE OVERCOME IN THE PRESENCE OF THE ETHYLENE INHIBITOR AMINOETHOXYVINYLGLYCINE

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In previous works we reported for the first time both inoculation induced ethylene evolution and a positive correlation between nitrate in growth medium and ethylene evolved from uninoculated and inocculated alfalfa roots. Negative correlation between ethylene evolution and nodulation rates was also found (J. Plant Physiol. 1986, 1987). In the present work we have studied the effect of ethylene biosynthesis inhibition with aminoethoxyvinylglycine (AVG) on nodulation of alfalfa using a combination of 4 NO_3 levels (2.5, 5, 10, and 20 mM) by 4 AVG concentrations (0, 1, 10, and 20 μM). AVG sharply enhanced the nodule formation on alfalfa roots and this effect increased with NO_3 supply. It produced as many as 4.3,2.4,2.3 and 1.5-fold more nodules than controls in plants grown on 20, 10, 5, and 2.5 mM NO_3 respectively, by day 19 after inoculation. AVG induced ethylene biosynthesis inhibition was studied in 10 mM NO_3 fed plants. The concentration of AVG which induces as many as more than 2-fold the number of nodules per plant (10 and 20 μM), also inhibited ethylene biosynthesis by 5 to 10-fold (with respect to the controls) 48 h. after inoculation and inhibitor addition. These results reinforce our hypothesis that NO_3 effect could be mediated through phytohormone ethylene. Furthermore this has led us to think that endogenous ethylene may be involved in autoregulation of nodulation by the plant. AVG increased nodulation also results ina slightly but significant (P=0.05) increase in acetylene reduction activity, though this did not involve a better plant growth.

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IMMUNOLOGICAL AND IMMUNOCYTOCHEMICAL DISTINTION BETWEEN *BRADYRHIZOBIUM* SP. (*LUPINUS*) STRAINS IN NODULES OF *LUPINUS ALBUS*.

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The aim of this work was to know if the immunological techniques enable us to distinguish strains of *Bradyrhizobium* sp. (*Lupinus*) when they coexist in the legume nodules of *Lupinus albus* L. cv Multolupa.

The conventional technique of fixing in glutaraldehyde and embedding in Araldite was applied to 30-40 days-fresh nodules tissue, according to Vivo et al. (1989). ELISA technique showed that no cross-reactions were obtained between the strains ISLU 65 and Hig 5_2, that were used to inoculate the *Lupinus* seeds. It was possible to differentiate ISLU 65 and Hig 5_2 strains in suspensions of free-living cells by the specific reaction between colloidal gold-goat anti-rabbit IgG and IgGs raised against each of the two strains.

Localization of colloidal gold on the DNA fibrilis of the bacteroids was observed regardless of the strain that elicited the IgG, suggesting that both strains share a common epitope. On the bacterial wall the immunogold labelling was very specific 5 ug/ml IgG was enough to obtain a good immunolabelling. Colloidal gold was observed on the inner and outer membrane of the bacterial wall. At the polar region of the bacteroids an accumulation of periplasmic substance was produced increasing the width of the bacteroid wall. The application of polyclonal IgG obtained against free-living bacteria has allowed the immunoidentification of two strains regardless of nodule stage of development. No special distribution of the strains in the nodules was observed when both strains coexist together. Gold particles were not found on subcellular organelles or membranes of plant nature, such as, mitochondria, nucleus, Golgi apparatus, cell wall and peribacteroid membrane.

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A FAMILY OF SYMBIOTIC SIGNALS PRODUCED BY RHIZOBIUM SP. NGR-234.

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The initial response of leguminous plants to rhizobial infection is a deformation of root hairs, a process which is also induced by sterile rhizobial culture filtrates. This response is host specific and has recently been shown to be due to secreted signal molecules called Nod-factors, the biosynthesis of which is encoded by the rhizobial *nod* genes. Here we report the occurence of two groups of Nod-factors, NodNGR1 and NodNGR2, that are secreted by broad host range *Rhizobium* sp. NGR234. Biosynthesis of both is Sym plasmid determined and induced by the presence of flavones via the transcriptional regulator protein NodD1. Moreover, their production is completely abolished by deletion of the common *nodABC* genes. NodNGR2 causes root hair deformations analogous to early rhizobial infection on *Macroptilium atropurpureum* and on *Vigna unguiculata*, two heterologous host plants of NGR234, at concentrations down to 10^{-11}M. Chemical and biochemical analysis of NodNGR2 showed that it is a family of structurally related compounds all of which contain a common sulphated and acetylated pentasaccharide moiety of ß-1,4 linked glucosamine units. Their structural diversity arises from the nature of the N-linked acyl chain attached to the non-reducing end of the oligosaccharide, and in the positioning of O-acetyl substituents.

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SEQUENCING AND ORGANIZATION OF A GENE CLUSTER REQUIRED FOR THE SYNTHESIS OF AN ACTIVE HYDROGENASE IN RHIZOBIUM LEGUMINOSARUM.

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The nucleotide sequence of the structural genes for the polypeptides of the hydrogenases from different bacteria are now known. However besides the structural polypeptides other specific gene products are also required for the complete biosynthesis of an active hydrogenase. In R. leguminosarum, the DNA inserted in cosmid pAL618 contains the complete set of genes necessary for H_2-oxidation by pea bacteroids (1). These genes are apparently organised in five or six trancriptional units. Unit hupI/II contains the structural genes of the R. leguminosarum hydrogenase (2). We present here the nucleotide sequence and the gene organization of a DNA region comprising transcriptional units hupIII, hupIV, hupV and part of hupVI. The analysis of the sequence revealed the presence of ten ORFs (Fig 1). These ORFs are likely to be coding sequences in the light of the following observations: i) All of them are preceded by plausible ribosome binding sites and fall within high coding-probability regions; ii) Tn5 insertions within many of these ORFs completely supressed the Hup+ phenotype, indicating that their gene products are required for H_2 uptake; iii) Some of the ORFs have been expressed in E. coli cells by the T7 RNA polymerase/promoter system. The molecular weight or the translated polypeptides agreed with the size of the corresponding predicted proteins from the ORFs.

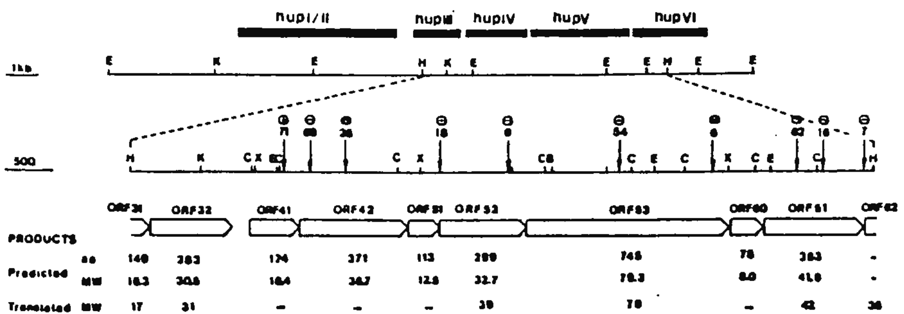

Fig.1: Genetical and physical map of a gene cluster required for the hydrogenase activity in R. leguminosarum. The horizontal black bars show the location of the transcriptional units in pAL618 as defined by site-directed transposon mutagenesis and complementation analysis. The location and orientation of the ORFs are shown by open arrowed boxes. The position of the Tn5 insertions in the mutants were determined by sequencing and are indicated by vertical arrows. The table below the ORFs shows the number of aa and molecular weight for the predicted products and the molecular weight of the peptides detected en E. coli cells from the translataion of the ORFs with a T7 RNA polymerase and promoter system.

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When the predicted amino acid sequences from the ORFs were compared with proteins from data base banks (EMBL, Swissprot), significant similarities were found for some of the ORFs. Most predicted gene products from operon *hup*V and *hup*VI had homology to proteins from the *hyp* operon (3), which is necessary for the activity of the three hydrogenase isoenzymes of *E. coli*, and to gene products from a gene cluster of *Rhodobacter capsulatus* involved in H_2 oxidation (4). The predicted product of the ORF52 is unusual in having the motif CxxCxC in the amino terminus followed by a histidine-rich strecht of 40 amino acids. These motives may be involved in metal binding. ORF52 protein had homology to the *hyp*B gene product wich has a role in nickel metabolism in *E. coli*. The deduced amino acid sequence of ORF53 presents two cystein-rich, zinc finger-like motives that resembles the regulatory motif present in the amino terminus of protein kinases C from *Drosophila* (5) and mammalian and the Cys/Cys motives of different zinc finger proteins (6). The physiological role of this motif, if any, and the function of ORF53 protein are unknown.

In summary, besides the structural operon of the hydrogenase, at least ten other genes are involved in H_2 oxidation in *R. leguminosarum* bacteroids . The exact functions of these genes remain unknown.

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RHIZOBIUM TRIFOLII GENES INVOLVED IN SURFACE POLYSACCHARIDES AND NODULE DEVELOPMENT.

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The *Rhizobium* surface has been sopossed to be involved in adhesion to the root hair surface, determination of host range and nodulation. A firm relation between the *Rhizobium* surface and the development of a nitrogen-fixing root nodule was established by the isolation of mutants that fail to produce extracellular polysaccharide and form a developmentally disturbed nodule. Like other Gram-negative bacteria, *Rhizobium* has an outer membrane, tightly associate with it are the lipopolysaccharides (LPS) and more loosly bound are the extracellular polysaccharides (EPS). The various surface polysaccharides are a complex mixture of different oligomers and polymers, every one of which may be of importance in the symbiosis.

Several genetic loci for *Rhizobium* surface determinants have been identified and the genes are being characterized as detailed as *nif*, *fix* or *nod* genes. Loci affecting acidic EPS have been identified in *R. meliloti*. Strains mutated in seven loci *exoA* through *exoF* fail to produce a particular acidic EPS. The *exoC* mutant also lacks cyclic glucan. A mutation in the locus *exoH* resulted in a strain that produces a slighth modified acidic EPS in which the succinyl modification are absent. The nodules induced by *exoH* mutant have infection threads but bacterial release is rarely observed. In addition to *exo* loci, two *ndv* loci, *ndvA* and *ndvB* have been identified in *R. meliloti*. These genes are homologous to and functionally interchangeable with the chromosomal virulence genes *chv* of *A. tumefaciens*. R. meliloti *ndv* mutants induce nodules with a morphology similar to that of the *exo* mutants. In contrast to *R. melilotl exo* mutants, these mutants are not affected in the syntesis of acidic EPS but they are defective in the biosynthesis of cyclic glucan, just as the *Agrobacterium chv* mutants.

Surface components like LPS have been shown to affect nodule development by isolation of mutants that produce an altered LPS and form nodules with a characteristic aberration, in development. These nodules contain infection threads bacteria however the infecction threads develop abnormal and abort.

R. trifolii RS800 recombinant cosmids pCC11 and pCC71 were isolated on basis of structural and functional homology to the *ndv* genes. 8 Kb *Eco RV* fragment of pCC71 that strongly hybridize with *ndvB* probe was subcloned in pRK293. This plasmid (pCC718) was mutagenized by cloning the Kanamicine catridge into the *Xho*I

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internal site of the *ndvB* region. Using the marker exchange technique we have obtained one *ndvB* mutant, CAR1. This mutant is able to nodulate and fix nitrogen but nodules are different from the nodules that elecit RS800. This was confirmed by microscopy. CAR1 shows no differences in the production of beta 1,2 glucano in relation to wild type. CAR1 grows more slowly than the wild type and exhibit tendency to precipitate in liquid culture, phenotype similar to LPS mutants. CAR1 shows no differences in the motility in relation to RS800. Studies directed to investigate possible alteration in this surface component are being carried out.

In contrast to *R. meliloti* which fluoresce a brigth blue-green colour on plates containing the fluorescent brightner Calcofluor when exposed to UV ligth, *R. trifolii* exhibit a dark phenotype on these media. A different structure and composition of each exopolysaccharide could explain this different behavior. Two strategies were performed to isolate recombinant cosmids containing *exo*-specific sequences from a *R. trifolii* RS800 gene bank: Complementation of calcofluor-dark phenotype R. *meliloti exo* mutants and complementation of its Fix $^-$ phenotype. Recombinant cosmids coming from each procedure were characterized physically and hybridized with R. meliloti *exo* probes to identify the fragment containing *R. trifolii exo* loci. To demostrate genomic localization of *exo* homologues plasmids DNA of *R. trifolii* RS800 were separated by agarosa gel electrphoresis and hybridized with *R. meliloti exo* probes. Our results suggest that *R. trifolii* RS800 harbour chromosomal and plasmidic exo information. We have detected hybridization in a *R. trifolii* plasmid different to symbiotic plasmid.

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Identification and genetic analysis of an *fnr*-like gene in *Rhizobium leguminosarum* bv. *viciae* involved in regulation of nitrogen fixation genes

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Recently an *fnr*-like gene, designated *fnr*N, was found in *Rhizobium leguminosarum* bv. *viciae* (S. Colonna-Romano et al. 1990).
The FnrN protein shows structural homology to *Rhizobium meliloti* FixK (J. Batut et al. 1989) and Fnr, the transcriptional regulator of anaerobic respiration in *Escherichia coli* (S. Spiro and J.R. Guest 1990).
FnrN is able to restore induction of the *fix*N operon and nitrogen fixation ability in an *R.meliloti fix*K mutant. Furthermore *fnr*N complements an *E.coli fnr* mutant for anaerobic respiration with nitrate.
Analysis of the expression of *fnr*N-*lac*Z fusions revealed that the *fnr*N gene like the *R.meliloti fix*K gene is only induced under microaerobic conditions. In the *fnr*N promoter region two sequence motifs were found that show significant homology to the consensus sequence of Fnr-binding-sites (also called anaerobox). Deletion of the distal anaerobox showed that it is absolutely required for *fnr*N expression.
The *fnr*N gene is not expressed in an *R.leguminosarum fnr*N mutant. This result indicates that *fnr*N expression is positively autoregulated. In *R.meliloti* the FnrN protein can be replaced by *R.meliloti* FixK in activating *fnr*N expression.
Insertion of a constitutive promoter in front of *fnr*N does not affect microaerobic induction of the target gene *fix*N, demonstrating that in contrast to *R.meliloti* FixK the *R.leguminosarum* FnrN protein is only active under low oxygen conditions.
Despite the fact that there is a putative anaerobox in the *fix*C coding region upstream of the *R.leguminosarum nif*A gene, FnrN apparently is not involved in modulating *nif*A expression.

Literature:

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SALINITY EFFECT ON NITROGENASE ACTIVITY IN LEGUME NODULES

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The effect of sodium chloride salinity (100 mM NaCl) was tested on four cultivars of **Phaseolus vulgaris** inoculated by **Rhizobium phaseoli** CIAT 899. Experiments were conducted in a growth chamber on 30 day-old-nodulated plants.

Nitrogenase activity estimated by acetylene reducting activity (ARA) was strongly inhibited by salt. The decrease of nitrogenase activity was observed in the first hour after salt application. This inhibition has been observed in soybean and alfalfa nodules.

The influence of partial oxygen pressure (pO_2) on nitrogenase activity was measured in the presence or in the absence of NaCl, the ARA was stimulated by increasing pO_2 in the incubation medium. Although the stimulation of ARA by O_2 was higher in the presence that in the absence of NaCl.

These results suggest a direct and rapid effect of osmotic stress on nodular nitrogenase activity and the involvement of the oxygen resistance in the inhibition of nitrogenase activity by salinity.

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Localization of genes implied in syntesis of extracellular polisacarides.

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Bacteria of the genus *Rhizobium* produce an acidic exopolisaccharide required for the invasion of the nodule and possibly for its development. In *R. meliloti* 1021 there have been identified 13 *exo* genes which are in the second megaplasmid, they are involved in the syntesis of the exopolysaccharide and when mutated produce loss of the capacity for nitrogen fixation. For the location of the genes involved we used mutants of *R. leguminosarum* bv. *phaseoli* CIAT899, which present alterations on the production of EPS and in its simbiotic characteristics.

1. **Complementation of mutants with plasmids which bare *exo* genes of *R. meliloti* 1021 (pD2, pD5, pD15, pD34).** The complementation of these mutants was acomplished by a triparental conjugation with the *R. meliloti* plasmids and selecting the transconjugants by testing plants (*Phaseolus vulgaris* cv. Negro Jamapa). From the nodules which were fixing nitrogen we proceded to isolate and check the complementing plasmids.
2. **Isolation of the cosmids of CIAT899 by complementation of the mutants in plants.** For the complementation of the mutants with cosmids from the gene library of *R. leguminosarum* bv. *phaseoli* CIAT899, we proceded to inoculate all the conjugation mass in bean plants. From the fixing nodules appeared we isolated bacteria which had the complementing cosmids. Then we checked the antibiotic resistances and transformed them into *E. coli*. Finally, these cosmids have been mapped using different restriction endonucleases.
3. **Fine location of the region which complement of the isolated cosmids.** For the analysis of these cosmids we made deletions by digesting partially with restriction endonucleases. Afterwords the delated cosmids were tested in plants. From the nitrogen fixing nodules we reisolated these cosmids and analysed the fragment which carried. Finally we made a restricction map of these fragments.
4. **Isolation of the cosmids of CIAT899 which complement *exo* and *ndv* mutants of *R. meliloti* 1021.** As before, to isolate these plasmids, we made a triparental conjugation of the gene library of CIAT899 and *exo* and *ndv* mutants. Again, all the conjugation mass was inoculated into beans and from the plants which presented nitrogen fixing nodules we isolated the complementing transconjugants. This way we have isolated regions of *R. leguminosarum* bv. *phaseoli* which complement the mutants mentioned above.

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