NITROGEN FIXATION BY *RHIZOBIUM LEGUMINOSARUM* PRE; A GENETICAL AND BIOCHEMICAL APPROACH

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STELLINGEN

1. De hypothese dat de genen van het *fix*ABC operon koderen voor eiwitten die zijn betrokken bij het electronentransport naar nitrogenase berust op een onderschatting van het aantal *fix*-genen in aeroob stikstofbindende bacteriën.

   Gubler en Hennecke (1986). FEBS Lett. *200*, (1), p. 186.

2. De conclusie van Kaminski *et al.*, dat het *fix*ABC operon waarschijnlijk niet betrokken is bij het electronentransport naar nitrogenase, kan niet getrokken worden aan de hand van de door hen gepubliceerde resultaten.

   Kaminsky, Norel, Desnoues, Kushk, Salzano en Elmerich (1988) Mol.Gen.Genet. *214*, p. 496.

3. De door Maier en Graham bepaalde kinetische parameters voor de opname van molybdeen door geisoleerde *B.japonicum* bacteroïden gelden zeer waarschijnlijk niet voor molybdeenopname door bacteroïden in de wortelknol.

   Maier en Graham (1988) J. Bacteriol. *170* (12), p. 5613.

4. De conclusie van Batut *et al.*, dat *nif*A negatief gereguleerd wordt door *fix*K is in strijd met de door hen gepubliceerde experimentele gegevens.

   Batut, Daveran-Mingot, David, Jacobs, Garnerone en Kahn, EMBO J. *8*, (4), p. 1279.

5. De onzekerheid over de afstand tussen genetische loci op chromosomen zal het fysisch karteren van die chromosomen ernstig bemoeilijken.

6. Het is twijfelachtig of het door Knox *et al.* beschreven JIM4 antigeen, in een ontwikkelingsstadium waarin de differentiatie van meristematische cellen tot xyleemcellen nog niet is begonnen, een marker is voor de positie van toekomstige xyleenelementen in de wortel van *Daucus carota*.

   Knox, Day en Roberts (1989) Development *106*. p. 47.

7. Voor het oplossen van de mondiale milieuproblemen is het essentieel dat de derde-wereldlanden hun schulden worden kwijtgescholden.

8. Het gebrek aan compatibiliteit van verschillende computersystemen vormt een ernstig obstakel voor het voltooien van een proefschrift.

9. De ontdekking van koude kernfusie blijkt een storm in een glas zwaar water te zijn.

10. In de gemiddelde schoenwinkel wekt het assortiment schoenen groter dan maat 46 de foutieve indruk dat er een omgekeerd evenredig verband bestaat tussen schoenmaat en het hebben van een goede smaak.

Stellingen behorende bij het proefschrift:

NITROGEN FIXATION BY *RHIZOBIUM LEGUMINOSARUM* PRE;
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Wageningen, 19 september 1989 René Klein Lankhorst

René Klein Lankhorst

NITROGEN FIXATION BY *RHIZOBIUM LEGUMINOSARUM* PRE; A GENETICAL AND BIOCHEMICAL APPROACH

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus
dr. H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 19 september 1989
des namiddags te vier uur in de aula
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Scope of this thesis

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Nitrogen fixation by *Rhizobium* and *Bradyrhizobium* bacteria in symbiosis with their leguminous host plants forms an attractive alternative for the industrial production of nitrogenous fertilizers, both from an economic as well as an environmental point of view, and is the topic of many scientific research programs nowadays. Ultimate goals in many of these programs are improving the efficiency of nitrogen fixation, the extension of the host range of the bacteria to important, non-leguminous crops and the transfer of the nitrogen fixing ability from the bacterium to the plant. A thorough knowledge of the biochemical processes and the genetic determinants involved in both plant and bacterium, however, is a prerequisite for the achievement of any of the above mentioned goals.
In this thesis a genetic and biochemical analysis of *R.leguminosarum* PRE is described. The organization of the *nif* and *fix* genes on the *sym* plasmid, involved in the reduction of dinitrogen, was studied by the construction of extended physical maps of parts of this plasmid (chapter 2). Furthermore, the region upstream of the *fix*ABC operon was studied in detail. A novel *fix* gene (*fix*W), located immediately upstream of *fix*A, was identified and the expression of this gene was studied (chapter 3). Four regions on the PRE *sym* plasmid were found to contain reiterations of (parts) of functional *nif* and *fix* genes.
To elucidate the route by which the enzyme nitrogenase is supplied with electrons, the membrane fraction of *R.leguminosarum* bacteroids was analyzed for NADH dehydrogenase activity and a bacteroid specific NADH dehydrogenase complex, DH1, was isolated (chapter 4). With the aid of specific antisera directed against the different subunits in this dehydrogenase complex, it was shown that only one subunit, with a molecular weight of 35 kD, is bacteroid specific. To investigate a possible role of complex DH1 in the electron transport to nitrogenase, an attempt was made to construct *R.leguminosarum* mutants disturbed in the synthesis of this 35K subunit (chapter 5). Therefore, this protein was isolated and the N-terminal amino acid sequence was determined. Based on this sequence oligodeoxynucleotide probes were synthesized and used to screen a cosmid library in order to identify the gene encoding the 35K subunit. Although this gene was not found, several other DNA fragments were cloned showing a high degree of homology to the deduced 35K-gene nucleotide sequence, which indicates that the experimental procedure followed eventually can lead to the isolation of this gene.
In chapter 6 an analysis of proteins of the peribacteroid space (PBS) is described. This symbiotic compartment forms the interface between the *Rhizobium* bacteroid and the plant

host, and the proteins of this space may have an important role in transport processes and in signal transduction between the two partners in the symbiosis. The bulk of the proteins (about 90%) was found to be excreted by the bacteroid into the PBS, whereas the remaining 10% probably is of plant origin. About one third of the PBS proteins appeared to occur also in the periplasmic space of free-living bacteria. Four bacteroid encoded PBS proteins were identified, which are not present in free-living *R.leguminosarum* bacteria and thus might play a role in nitrogen fixation. These proteins might lead to the identification of novel symbiotic loci on the *R.leguminosarum* genome.

Chapter 1

Symbiotic nitrogen fixation by *Rhizobiaceae*

CHAPTER 1: Symbiotic nitrogen fixation by Rhizobiaceae

1.1. Introduction.

Although nitrogen is one of the most abundant elements on earth, only a small part of it is available in a form that can be used by living organisms: dinitrogen gas, which makes up approximately 80% of the elements present in the earth's atmosphere, is a very inert chemical which can only participate in chemical reactions under rather extreme conditions and thus cannot be incorporated directly into biologically important compounds. Most of the nitrogen present in biological systems, as for instance in plants and animals, forms part of complicated chemical structures like proteins and nucleic acids. Eventually, due to plant and animal decay or waste production, the nitrogen will reach the soil. In the soil bacteria convert the complicated nitrogen compounds to simple ones like ammonia and nitrate, which can be taken up from the soil by plants. In the plants the nitrogen is incorporated again in proteins etc., which can be used for instance as animal fodder. The nitrogen present in biological systems thus is constantly recycled. Through the action of denitrifying soil bacteria however, part of the nitrogen is converted into dinitrogen gas and leaks out from this cycle. Also the removal of crops from the fields exhausts the nitrogen pool in the soil. It is therefore of great agricultural importance that the amount of nitrogen constantly is replenished, which can be accomplished by the conversion of dinitrogen gas into ammonia. This process is known as "nitrogen fixation".
In general, there are two major sources of fixed nitrogen. One forms the chemical industry, where yearly about 10^8 tons of fertilizer are produced using the Haber-Bosch process (166), at the expense of an enormous amount of fossil fuels. In this process dinitrogen is reacted with hydrogen under conditions of high pressure and temperature (106), see eq. 1.

$$N_2 + 3\,H_2 \rightarrow 2\,NH_3 \qquad T \geq 400°C\,,\ P \geq 10^4\ kPa$$

Equation 1. Nitrogen fixation by the Haber-Bosch process.

The other major source of fixed nitrogen forms biological nitrogen fixation by diazotrophic bacteria. These microorganisms are able to carry out the reduction of dinitrogen gas into ammonia at atmospheric pressure and moderate temperatures, using the enzyme nitrogenase, see eq. 2.

$$N_2 + 8\,H^+ + 8\,e^- + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16\,P_i$$

nitrogenase

$T \cong 25°C,\ P = 10^2\ kPa$

Equation 2. Nitrogen fixation by diazotrophic microorganisms.

The energy crises in the first half of the seventies and the increasing concern about the pollution of the environment, have caused a major interest in the process of biological nitrogen fixation as a substitute for the chemical production of nitrogenous fertilizers.

Nitrogen fixing microorganisms can be divided into two distinct groups. One comprises bacteria which can fix nitrogen in the free-living state. Examples of these are the obligate aerobic *Azotobacter vinelandii*, the facultative aerobic *Klebsiella pneumoniae*, the anaerobic *Clostridium pasteurianum*, cyanobacteria like *Anabaena variabilis* and *Plectonema boryanum*, and photosynthetic bacteria like *Rhodobacter sphaeroides*. The second group consists of diazotrophs which can only fix nitrogen when closely associated or in symbiosis with particular plants. This group is exemplified by *Anabaena azollae*, which fixes nitrogen in close contact with the waterfern *Azolla*, by some members of the genus *Azospirillum*, which fix nitrogen in close contact with the roots of plants of the genus *Gramineae*, and by members of the genus *Rhizobium* and *Bradyrhizobium* reducing dinitrogen only in root nodules on their host plants of the genus *Leguminosae*. The symbiosis of *Rhizobium* and *Bradyrhizobium* bacteria with their leguminous host plants is farmost the most important source of biologically fixed nitrogen. Estimations based on FAO statistics show that as much as 25 % (139×10^6 tons/annum) (30) of the biologically fixed nitrogen, representing a value of about $\$\ 6.4 \times 10^{11}$ (*), must be accounted for by leguminous plants. Legumes, like soja, alfalfa and pea, therefore are not only important as food and fodder, but also as a natural fertilizer.

The *Rhizobium*-legume symbiosis has been the subject of intensive scientific research during the past two decennia. Ultimate goals in many of these projects are the improvement of biological nitrogen fixation, for instance by increasing the amount of fixed nitrogen in the *Rhizobium*-legume symbiosis, and the extension of the *Rhizobium* host specificity to other, non-leguminous, crops (see 167,129 and ref. therein). In the next paragraphs an overview will be given about our present knowledge concerning the role of the *Rhizobium*

(*) Based at 1986 prices for industrial fertilizers.

the symbiosis, with an emphasis on the genetics and the biochemical processes involved the reduction of dinitrogen.

1.2.1. Taxonomy

Rhizobiae are Gram-negative, rod shaped, soil bacteria which are capable of inducing the formation of nodules on the roots of leguminous plants. These nodules are inhabited by the *Rhizobium* bacteria and form a highly specialized surroundings in which the bacteria fix atmospheric dinitrogen. Different species of *Rhizobium* only invade a limited range of host plants, which is referred to as "host specificity". The species *R. meliloti* for instance infects host plants of the genera *Medicago*, *Melilotus* and *Trigonella*, while *R. loti* only forms nitrogen fixing nodules on *Lupinus*, *Lotus*, *Anthyllis* and *Ornithopus*. Host plants, which are infected by one species of *Rhizobium* belong to a so called "cross-inoculation" group. These cross-inoculation groups have for long been the basis of a classification system for *Rhizobium* bacteria. Nowadays a classification system is used based on a phenotypical characterization, biochemical properties and DNA/DNA and DNA/RNA hybridizations. The *Rhizobiaceae* family consists of three genera of nitrogen fixing bacteria, *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (see table 1), of the genus *Agrobacterium*, members of which induce tumors or hairy roots on plants, and of the genus *Phyllobacterium*, containing bacteria which form leaf nodules (48, 119, 95).

The genus *Rhizobium* comprises four different species; *R. meliloti*, *R. loti*, *R. leguminosarum* and *R. fredii*. To the species *R. leguminosarum* now belong the three former species *R. phaseoli*, *R. trifolii* and *R. leguminosarum*. (These former names will be used in this thesis however in order to make proper use of literature references and citations).

All members of the genus *Rhizobium* are fast growing bacteria, with a generation time of 2-4 hours, and harbor the genetic information for nodulation and nitrogen fixation (*nod* and *nif* genes, see paragraph 1.2.4.) on large plasmids, the so called *sym* plasmids. An exception form some strains of *R. fredii*, which carry the *nif* and *nod* genes on the chromosome (9,5). In general *Rhizobium* species are only capable of nitrogen fixation in symbiosis with their host plants and not in the free living state, although in chemostat cultures of *Rhizobium* a very low level of nitrogen fixation can be measured under conditions of ammonia starvation and an extremely low oxygen concentration (168).

The genus *Bradyrhizobium* consists of slow growing bacteria with a generation time of 6-8 hours. Although high molecular weight plasmids are present in some *Bradyrhizobium*

species, the *nif* and *nod* genes are located on the bacterial chromosome (2,82). This genus contains only one well-defined species, *B. japonicum*, and further all the species that were previously referred to as slow-growing *Rhizobia*. In contrast to the *Rhizobium* species, some Bradyrhizobium species are capable of free-living nitrogen fixation when cultured under microaerobic conditions.

The genus *Azorhizobium* comprises only one species, *A. caulinodans*. This is the only member of the *Rhizobiaceae*, which induces beside root nodules nitrogen fixing nodules on the stem of its host plant, the tropical legume *Sesbania rostrata*. *A. caulinodans* bacteria have a generation time of 3-5 hours and are capable of both free-living and symbiotic nitrogen fixation (47). Since no plasmids have been detected in *A. caulinodans* (87), the genes involved in nodulation and nitrogen fixation probably are located on the chromosome.

In the following paragraphs some aspects of symbiotic nitrogen fixation will be discussed, exemplified by the *Rhizobium*-legume symbiosis. Unless specified, similar processes also take place in *Bradyrhizobium*. The *Azorhizobium-Sesbania* symbiosis differs in several aspects from the *Rhizobium* and *Bradyrhizobium* symbioses and will not be further described in this introduction.

Table 1. Taxonomy of nitrogen fixing *Rhizobiaceae*.

Bacteria	Genera of host plants
Rhizobium meliloti	*Medicago, Melilotus, Trigonella*
Rhizobium loti	*Lupinus, Lotus, Anthyllis, Ornithopus*
Rhizobium leguminosarum	
biovar. *trifolii*	*Trifolium* spp.
biovar. *phaseoli*	*Phaseolus vulgaris, P. multiflores*
biovar. *vicea*	*Pisum, Lathyrus, Lens, Vicia*
Rhizobium fredii	*Glycine soja, G. max* cv. Peking
Bradyrhizobium japonicum	*Glycine max*
Bradyrhizobium spp	other genera and species
Azorhizobium caulinodans	*Sesbania rostrata*

1.2.2. Formation of nitrogen fixing root nodules

One of the earliest steps in root nodule formation is the colonization of the tips of newly developing root hairs by *Rhizobium* bacteria (20). Possibly, the bacteria are attracted towards the root by flavonoids excreted by the plant since *Rhizobia* exhibit positive chemotaxis towards these compounds (18,31,3). Flavonoids were also shown to induce the expression of the bacterial *nod* genes (145,148,144), which play a role in nodule development (see below) and host range specificity (163,86).

Binding of the bacteria causes a deformation of the root hairs, referred to as "shepherds crook". The bacteria entrapped within this crook enter the root hair by partly dissolving the plant cell wall (32) In the plant cell an infection thread is formed by the plant host in which the bacteria reside. Simultaneously to this infection process, a process of dedifferentiation is induced in the root cortex, resulting in the formation of new meristems (118) which will grow out to form the nodule. The infection thread filled with bacteria ramifies and grows towards these meristems. Upon contact with the newly formed plant cells in the central tissue of the nodule, the bacteria are released from the infection thread into the cells by an endocytosis-like process (11). During this the bacteria become enclosed in a plant derived membrane, called the peri- bacteroid membrane (PBM, see paragraph 1.2.5.). The bacteria continue to divide and differentiate into so-called bacteroids, the nitrogen fixing forms of *Rhizobium*, which ultimately fill the entire plant cell. In this process the size of the *Rhizobia* increases; also the DNA content increases by a factor estimated between 8 and 15 (177,21). Furthermore the bacteria undergo some major morphological changes. The central tissue of a fully grown nodule consists for the larger part of cells filled with bacteroids. In between and in the surroundings of these cells, plant cells are found which are not invaded by bacteria, but have a supporting function in the process of nitrogen fixation (135).

During nitrogen fixation and nodule development both in the bacterium and in the plant host a specific set of proteins is synthesized. The plant encoded nodule specific proteins are referred to as nodulins (178). These are distinguished in early and late nodulins (see for instance 134 and ref. therein). The early nodulins play a role in root nodule development, and the expression of the early nodulin genes is controlled by the bacterial *nod* genes (68). Late nodulin genes are expressed when the *Rhizobium* bacteria are released into the plant cell and play a role in the support of the nitrogen fixation process in the bacteroids. Examples of late nodulins are leghemoglobin, which transports oxygen towards the bacteroids, and a nodule

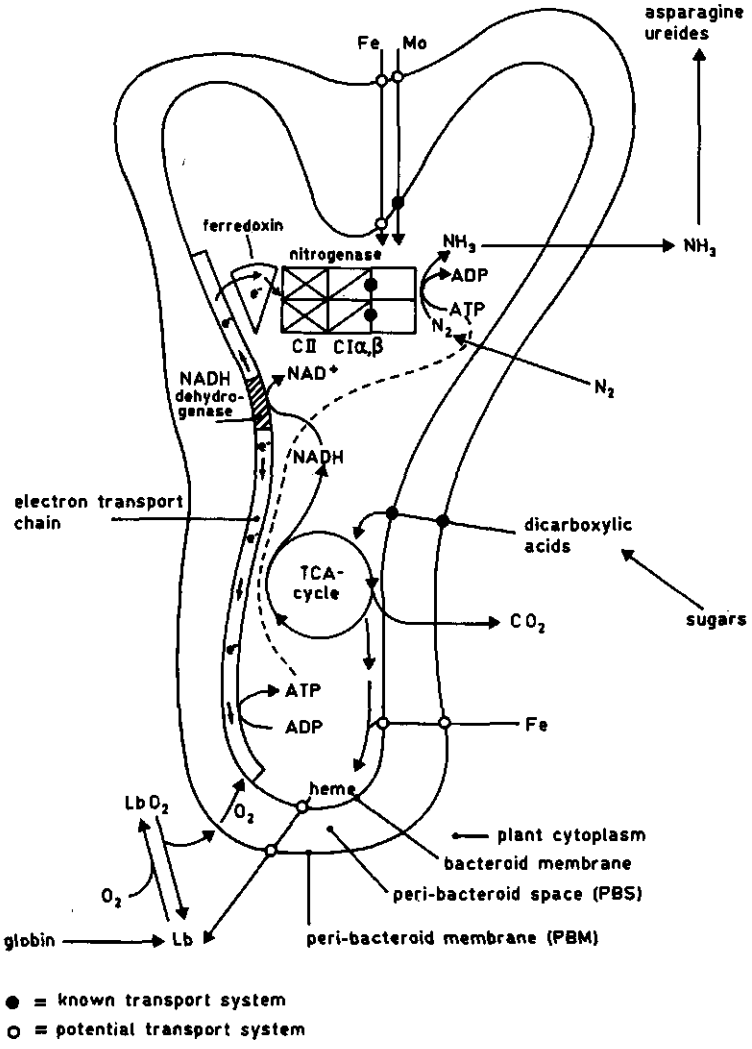

Figure 1 Major metabolic processes involved in nitrogen fixation.

The major metabolic routes in *Rhizobium* bacteroids resulting in the reduction of dinitrogen are shown. Some of these routes have been completely elucidated, whilst others, like the electron transport to nitrogenase and the transport of some metabolites, are drawn in accordance with published models. Nitrogenase and ferredoxin probably occur in one single membrane bound complex. For full details, see text.
Abbreviations: CIα,β: nitrogenase components Iα and Iβ, CII: nitrogenase component II, Lb: leghemoglobin, e^-: electron.

specific form of uricase, which is involved in the assimilation of the ammonia produced by the bacteroids. The *Rhizobium* proteins, which play a role in nitrogen fixation, and the processes they are involved in will be discussed in the following paragraphs.

1.2.3. Physiology and biochemistry of nitrogen fixing bacteroids

Inside the nodule, the nitrogen fixing bacteroids are dependent on the plant host for the maintenance of their metabolic processes. Through the plant cell, the bacteroid is supplied with carbon sources, oxygen, dinitrogen and metal ions, while fixed nitrogen and CO_2 must be transported to the plant (see fig. 1). Since the bacteroids are enveloped by the PBM, all these metabolites have to cross this membrane and the space between the PBM and the bacterial cell wall, which is referred to as the peri-bacteroid space (PBS). Some of these transports are passive, others require active transport systems, specifically adapted to or induced by the nitrogen fixing state.
The most important metabolic processes that take place in nitrogen fixing bacteroids will be discussed in the following paragraphs. More detailed information on these metabolic pathways can be found for instance in reviews by Bergersen (16) or Emerich *et al.* (56).

1.2.3.1. *Nitrogen fixation and metabolism*

The reduction of dinitrogen to ammonia is catalyzed by nitrogenase. This enzyme complex is present in all nitrogen fixing microorganisms known today and the polypeptide composition of the enzyme is highly conserved amongst them. The complex consists of two subunits referred to as component I or dinitrogenase and component II or dinitrogenase-reductase. Component I has a molecular weight of about 220 kDa and has an $\alpha_2\beta_2$ structure. The α and β subunits have molecular weights between 50 and 60 kDa, the β subunit being slightly larger. The complex contains two Fe-Mo clusters, known as the Fe-Mo cofactor or FeMoCo. These cofactors probably form the catalytic center of the enzyme complex where the actual reduction of dinitrogen takes place. Component II has a molecular weight of about 60 kDa and consists of two identical subunits of about 30 kDa. Both components I and II are extremely oxygen sensitive.

The enzyme complex nitrogenase catalyzes the following reaction

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

According to a generally accepted reaction mechanism (165,51), component II binds two molecules of MgATP and is reduced by low potential electron donors, either flavodoxins or ferredoxins (see paragraph 1.2.3.4.). In a one electron transfer reaction component II reduces component I with the concomitant hydrolysis of the bound MgATP. In eight reduction/oxidation cycles, which involve rapid association and dissociation of the components I and II, dinitrogenase accepts eight electrons. These electrons are used for the reduction of one molecule of dinitrogen and of two protons to yield two molecules of ammonia and one molecule of hydrogen gas. The production of hydrogen, which always accompanies the production of ammonia, seems to be a wasteful side reaction consuming about 25% of the energy available for nitrogen fixation. Some *Rhizobium* strains possess a hydrogenase (44) which is used to recover part of this loss by reduction of the hydrogen. In this reaction electrons are generated which can be used for the synthesis of ATP. Besides dinitrogen, several other molecules containing triple bounds, like acetylene, can act as a substrate for nitrogenase. The acetylene reduction can be measured easily using a gas chromatograph, and is a very accurate means of determining nitrogenase activity.

In contrast to free-living diazotrophs, which fix nitrogen solely for their own use, the ammonia fixed by *Rhizobium* bacteroids is exported to the plant cell (15). Although free-living *Rhizobium* bacteria exert an active ammonia transport mechanism (139,140), in bacteroids ammonia export probably does not involve carriers but merely is the result of diffusion (92). The way the ammonia is metabolized by the plant divides leguminous host plants into two classes. In one class of plants, as exemplified by *Pisum* and *Lupinus*, the end product of ammonia assimilation is asparagine. In the other class, exemplified by the genera *Glycine* and *Phaseolus*, ureides like allantoin or allantoic acid are formed. In both classes however, in the initial step of ammonia assimilation glutamine is formed by a plant encoded nodule specific glutamine synthetase (175,164). More detailed information concerning the ammonia assimilation in leguminous plants can be found for instance in the review by Pate and Atkins (141).

Whether or not part of the fixed nitrogen is metabolized by the bacteroids themselves is not clear. Although biochemical data show that all of the fixed nitrogen is exported towards the plant cytosol (15), *Rhizobium* and *Bradyrhizobium* species possess at least two genes

encoding glutamine synthetases which are expressed both in the free-living state and during symbiosis. For *B. japonicum*, it was reported that one of these genes, *gln*II, is under symbiotic and general nitrogen assimilation control. Intriguingly, this gene shows a high homology with eukaryotic glutamine synthetase genes and probably is of plant origin (33). The bacterial glutamine synthetases, however, are not indispensable for nitrogen fixation since mutants in both glutamine synthetase genes exhibit a normal Fix^+ phenotype on plants (102,39,124).

1.2.3.2. *Carbon metabolism*

In free-living *Rhizobium* bacteria, all the enzymes of the glycolysis, gluconeogenesis and TCA-cycle are present (170,58), which enables them to grow on a variety of carbon sources like sugars, e.g. glucose and fructose, and dicarboxylic acids like succinate, malate or fumarate. In symbiosis, dicarboxylic acids form the main carbon sources as mutants in hexose metabolism form normal nitrogen fixing nodules, whereas mutants which are disturbed in dicarboxylic acid uptake form Fix^- nodules (158,155). Furthermore, dicarboxylates were shown to stimulate nitrogen fixation in isolated bacteroids (17). The dicarboxylic acids used in bacteroid metabolism are supplied by the plant host. Ultimately, these carbon sources are the product of photosynthesis. The metabolic routes in the nodule by which the primary product of photosynthesis, sucrose, is converted into dicarboxylic acids, however, have not been elucidated completely yet.

Both free-living bacteria and bacteroids possess a high affinity dicarboxylic acid permease, which is encoded by the *dct*A locus. During free- living growth this permease is modulated by a membrane bound dicarboxylic acid sensor. During symbiosis however the *dct*A gene is probably regulated by a general nitrogen fixation control mechanism, as was suggested by results obtained with *R. meliloti* (153, see paragraph 1.2.4.3.). Also the peribacteroid membrane seems to be involved in the regulation of the carbon supply to the bacteroids. Saroso *et al.* (160) concluded from experiments with *Rhizobium* strain NGR234 that the PBM probably is impermeable to C_6 carbon sources like glucose. Furthermore, a transport system for succinate and malate was shown to be present on the PBM of soybean nodules (176).

In the bacteroids the tricarboxylic acids probably are metabolized in the TCA-cycle and gluconeogenesis, since mutants impaired in these processes, like succinate dehydrogenase

mutants and phosphoenolpyruvate carboxykinase mutants, show a Fix$^-$ or a reduced Fix$^+$ phenotype (58,66). These results, however, do not exclude the possibility that other carbon sources play a role in the bacteroid metabolism.

1.2.3.3. *Heme and Mo*

The enzyme complex nitrogenase is extremely sensitive to oxygen, and therefore bacteroids have to protect this enzyme against oxygen damage. On the other hand, *Rhizobium* is an obligate aerobic diazothroph depending on respiration for the generation of its energy. To be able to carry out these two processes, the bacteroid is supplied with oxygen by the oxygen carrier leghemoglobin. This protein forms an oxygen buffer in the plant cell which keeps the free oxygen concentration at a low level, and at the same time facilitates the diffusion of oxygen to the respiring bacteroids. The protein moiety of leghemoglobin is derived from the host plant (27,42), whereas most experimental data indicate that the heme group is synthesized by the bacteroids. *R. meliloti* and *Rhizobium* sp. NGR234 *hem*A mutants, which are impaired in the synthesis of δ-aminolevulinic acid (δ-ALA), the first intermediate in heme synthesis, give rise to white Fix$^-$ nodules containing only apo-leghemoglobin (116,169). In contrast, Guerinot *et al.* (71) have shown that a similar *Bradyrhizobium* mutant induces Fix$^+$ nodules containing normal leghemoglobin, indicating that the plant host must have been the source of the heme group. In this case, however, the plant might have provided the bacteroids with δ-ALA thereby bypassing the mutation. This latter idea is supported by results of O'Brian *et al.* (138), who have shown that *Bradyrhizobium* mutants impaired in later stages of heme synthesis give raise to white, Fix$^-$ nodules.
The synthesis of heme in bacteroids seems not to be under the control of a symbiosis specific regulation system (117). The export of heme towards the plant cytoplasm probably uses at least some symbiosis specific components when the heme is transported across the PBM, but the involvement of such a specific transport system has still to be demonstrated.

Another important metabolite in nitrogen fixation is Mo, which is present in the active center of the nitrogenase complex, the Fe-Mo cofactor. Little is known about the processes involved in the transport and metabolism of Mo in *Rhizobium*. Only for *Bradyrhizobium* experiments have shown that in the free-living state Mo is sequestered by two import systems, one with a high and one with a low affinity for Mo. The high affinity system probably includes a

specific Mo-chelator (123,142). *Bradyrhizobium* mutants impaired in this high affinity uptake system can no longer fix nitrogen in the free-living state. Experiments with isolated *Bradyrhizobium* bacteroids show the presence of a Mo-uptake system which is dependent on a pH gradient across the bacteroid membrane (122). Whether in this system chelators are involved and how the Mo is transported across the peribacteroid space is not clear.

1.2.3.4. *Electron transport to nitrogenase*

In *Rhizobium* bacteroids, as in other bacteria fixing nitrogen under aerobic conditions, the metabolic processes generating electrons for the reduction of dinitrogen, and the routes by which these electrons are transferred to nitrogenase, have only partly been elucidated. The current knowledge about electron transport to nitrogenase in diazotrophs will be discussed in this paragraph, with an emphasis on aerobic nitrogen fixing organisms. More detailed information concerning electron generation and transport in anaerobic diazotrophs, as well as in nitrogen fixing cyanobacteria and photosynthetic bacteria can be found for instance in reviews by Haaker *et al.* (74,72).

As can be seen in equation 2, the reduction of one molecule of dinitrogen by nitrogenase requires eight electrons. These electrons should be transferred by carriers acting at a very low midpoint potential (E_m) since nitrogenases isolated from various nitrogen fixing organisms were shown to operate only at redox potentials below approx. -350 mV (72). For example, nitrogenase component II of the anaerobe *C.pasteurianum* has an E_m of -380 mV, whereas the E_m of component II of the aerobe *A.vinelandii* is as low as -435 mV. In bacteria only two types of electron carriers acting at very low redox potentials are known, ferredoxins and flavodoxins. Both kinds of electron carriers have been isolated from various diazotrophs and genes encoding these proteins have been identified (111,14,130,40,49).

In *Rhizobium* and *Bradyrhizobium* species, however, no flavodoxins have been reported yet, and ferredoxins are proposed to be the physiological electron donor for nitrogenase. Carter *et al.* (35) isolated a ferredoxin from *B. japonicum* bacteroids with a redox potential of -484 mV which is sufficiently low for the reduction of the *Bradyrhizobium* component II. Furthermore, a gene encoding a ferredoxin-like protein, *fix*X, has been identified in several *Rhizobium* species and in *B. japonicum* (52,93,149). This gene is only expressed during nitrogen fixation and mutations in this gene give rise to a Fix^- phenotype (149,50). Whether the product of *fix*X is really involved in electron transport to nitrogenase, however, remains

to be proven. In *B. japonicum* a second ferredoxin encoding gene, *frx*A, was identified which is cotranscribed with *nif*B (54). This gene is probably not involved in electron transport to nitrogenase since *frx*A-mutants showed a normal Fix^+ phenotype. In contrast, the expression of a ferredoxin-like protein encoding gene (*fdx*N), cotranscribed with *nif*B in *R. meliloti*, is reported to be essential in nitrogen fixation (108). Amino acid sequence analysis showed that neither *fix*X nor *frx*A encode the ferredoxin identified by Carter *et al.* (35).

The source of reducing equivalents for the reduction of dinitrogen differs in anaerobic and aerobic nitrogen fixing organisms. In anaerobic nitrogen fixing diazotrophs, as exemplified by *C. pasteurianum* and *K. pneumoniae*, electrons are generated by the oxidation of pyruvate in a thioclastic reaction (eq. 3).

pyruvate + CoASH + 2 ferredoxin/flavodoxin (ox) →
acetyl-CoA + CO_2 + $2H^+$ + 2 ferredoxin/flavodoxin (red)

Equation 3

In this process also the ATP necessary for the reduction of dinitrogen is generated; the degradation of one molecule of pyruvate results in two molecules of reduced electron carrier plus one molecule of ATP (via acetyl CoA and acetylphosphate).

For *K. pneumoniae* the entire electron transport route to nitrogenase has been elucidated (eq. 4). Electrons from pyruvate are transferred to flavodoxin, the gene product of *nif*F (*nif*Fgp), by a pyruvate:flavodoxin oxidoreductase encoded by *nif*J (85,22). Flavodoxin then donates the electrons to component II of nitrogenase (encoded by *nif*H, see paragraph 1.2.4.), by which they are transferred to component I (encoded by *nif*D and *nif*K), where finally dinitrogen is reduced to yield ammonia.

				2 NH_3
pyruvate →	flavodoxin →	nitrogenase C-II→	nitrogenase C-I →	↑
pfo* *nif*Jgp	*nif*Fgp	*nif*Hgp	*nif*Dgp,*nif*Kgp	N≡N

*pfo = pyruvate:flavodoxin oxidoreductase

Equation 4. Electron flow to dinitrogen in *K. pneumoniae*.

In aerobic nitrogen fixing organisms thioclastic reactions seem to be absent since neither pyruvate:flavodoxin (ferredoxin) oxidoreductase activity is detectable in cell-free extracts, nor has a gene homologous to *K. pneumoniae nifJ* been found by interspecies DNA hybridization. In this group of diazotrophs reduced pyridine nucleotides, either NADH or NADPH, form the major source of electrons for the reduction of dinitrogen (74). In general, reduced pyridine nucleotides are generated during glycolysis and in the TCA cycle. Since bacteroids in the nodule are supplied with dicarboxylic acids by the host plant (see paragraph 1.2.3.3), the TCA cycle probably forms the major source of reducing equivalents in *Rhizobium* and *Bradyrhizobium.*

In *Azotobacter* (77) and in *B. japonicum* bacteroids (78), the reversed electron flow from succinate or H_2 to site I of the respiratory chain in principle forms another source of NAD(P)H. The relative efficiency of these reactions, however, is low (79) and they probably do not contribute significantly to dinitrogen fixation. The route by which electrons from NAD(P)H are transferred to nitrogenase has not been resolved yet, but several models have been proposed. For *Azotobacter*, Benemann *et al.* (13) suggested a linear electron transport from NADPH via ferredoxin and/or flavodoxin to nitrogenase. In an *A. vinelandii* cell free extracts were a high NADPH/$NADP^+$ ratio of about 20 was maintained using a NADPH regenerating system, electron transport from NADPH to nitrogenase was shown to be possible. In nitrogen fixing *A. vinelandii*, however, the NADPH/$NADP^+$ ratio is about 0.4, which corresponds with an E_m = -350 mV (109). Under physiological conditions thus, the redox potential of NADPH seems to be too high to act as an electron donor to nitrogenase, since *A. vinelandii* component II acts at an E_m of approx. -500 mV.

An important observation was made by Haaker *et al.* (73), who showed that nitrogen fixation in *A. vinelandii* is dependent on a high proton motive force ($\Delta\mu$) across the cytoplasmic membrane. Later this was also shown to be true for *R. leguminosarum* bacteroids (113).

The $\Delta\mu$ consists of two parts, an electrochemical gradient ($\Delta\psi$) and a proton gradient (ΔpH) (see eq. 5), and is generated by respiration.

$$\Delta\mu = \Delta\psi - 59\,\Delta\text{pH} \quad (\text{mV})$$

Equation 5

Amongst others, $\Delta\mu$ is used for the synthesis of ATP. Lowering $\Delta\mu$ by small amounts of uncoupler of the respiratory chain causes an immediate decline in nitrogenase activity, whereas under the same conditions neither the respiration of the cell nor the ATP/ADP ratio is

affected. From this it was concluded that $\Delta\mu$ has a direct role in the electron transport to nitrogenase. Since a membrane bound NAD(P)H:flavodoxin oxidoreductase activity was detected in *A.vinelandii* membranes (76), a model was proposed in which electrons are transferred from NAD(P)H to flavodoxin via a membrane bound NAD(P)H dehydrogenase. The energy necessary to sufficiently lower the redox potential of the electrons was supposed to be derived from a $\Delta\mu$ dependent proton influx.

Laane *et al.* (114) showed that in *Rhizobium* bacteroids the effect of $\Delta\mu$ on nitrogenase activity was caused by the electrochemical gradient, $\Delta\psi$, and not by ΔpH; ionophores that decrease the $\Delta\psi$ cause a decrease in nitrogenase activity, while decreasing the proton gradient across the membrane results in an initial increase of nitrogenase activity. A similar effect was reported also for the photosynthetic bacterium *Rhodobacter sphaeroides* (75) and for the cyanobacteria *Anabaena variabilis* and *Plectonema boryanum* (80,81). These results indicate that $\Delta\mu$ may have a function in keeping the enzyme complex nitrogenase in a conformation in which reduction by NADP(H) is possible, rather than in the generation of reducing equivalents. This idea is supported by the observation that during nitrogen fixation in *Azotobacter* and *Rhizobium*, nitrogenase probably is attached to the cytoplasmic membrane (91,103).

Further evidence that the cytoplasmic membrane is involved in nitrogen fixation in aerobic nitrogen fixing bacteria was given by Klugkist *et al.* (110), who showed the presence of a linear relationship between the rate of electron transport to oxygen, and nitrogenase activity. Based on this, a model was proposed in which two electrons derived from NAD(P)H with an E_m of -320 mV are used to reduce a membrane bound dehydrogenase (see fig. 2). One of these electrons then transfers part of its energy to the other and enters the respiratory chain at the level of ubiquinone (E_m ~-140 mV). The other electron now has an E_m of ~-500 mV, which is sufficient for the reduction of flavodoxin or ferrodoxin. This model explains the observed correlation between the respiration rate and nitrogenase activity, since for every electron transferred to nitrogenase, another has to flow through the respiratory chain to oxygen. Also the requirement of electrons with a low E_m can be met in this way. Finally, the role of $\Delta\psi$ is explained as holding together a membrane bound complex consisting of a NAD(P)H dehydrogenase, flavodoxin or ferredoxin and nitrogenase. Although this model is very attractive, it is not supported by many experimental data.

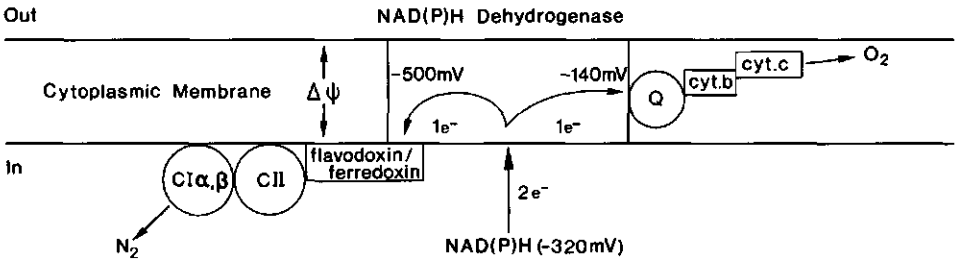

Figure 2. Model for the electron transport to nitrogenase.

The model according to Klugkist *et al.* (109) for the electron transport to nitrogenase is shown: two electrons derived from NAD(P)H with an E_m of -320 mV are used to reduce a membrane bound dehydrogenase. One of these electrons then transfers part of its energy to the other and enters the respiratory chain at the level of ubiquinone (E_m ~-140 mV). The other electron now has an E_m of ~ -500 mV, which is sufficient for the reduction of nitrogenase via either flavodoxin or ferredoxin. For full details, see text.
Abbreviations: CIα,β: nitrogenase components Iα and Iβ, CII: nitrogenase component II, Q: ubiquinone, cyt: cytochrome, e^-: electron, Δψ: electrochemical gradient.

In each of the models discussed, the first link in the electron transport chain to nitrogenase is a NAD(P)H dehydrogenase, which probably will be associated with the cytoplasmic membrane. This dehydrogenase will be either induced during nitrogen fixation, or alternatively, modified to meet the specific requirements imposed by nitrogen fixation. In *Azotobacter* indeed a membrane bound NADPH dehydrogenase was detected, which is only present during derepression of nitrogen fixation (110). Also in *R. leguminosarum* a membrane bound NADH dehydrogenase was detected, which is only present in bacteroids and not in free-living bacteria (107 and this thesis). Whether these dehydrogenases indeed are involved in electron transport to nitrogenase, however, remains to be proven.

1.2.4. Molecular genetic aspects of nitrogen fixation

In this paragraph the *Rhizobium* genes involved in the reduction of dinitrogen, the *nif* and *fix* genes, as well as a group of genes which probably are co-regulated with the *nif* genes, are discussed. The *nod* genes, which are involved in the infection process and root nodule formation, have been mentioned already in paragraph 1.2.2. and will not be discussed further. Since results obtained with the free-living nitrogen fixing *K. pneumoniae* have

contributed significantly to our present understanding of *Rhizobium* genetics, attention will be also paid to the relevant genetics of this species.

1.2.4.1. *Nif and fix genes*

In general, *nif* genes in nitrogen fixing organisms are genes exhibiting a structural and/or functional homology to the genes in the so-called *nif* cluster of *K. pneumoniae*. In *K. pneumoniae* this cluster comprises 20 genes (128,6) organized in 7 transcription units, which encode all the polypeptides involved in nitrogen fixation. The genes occur in the following order: *nif*JHDKTYENXUSVWZMFLABQ (see fig. 3).

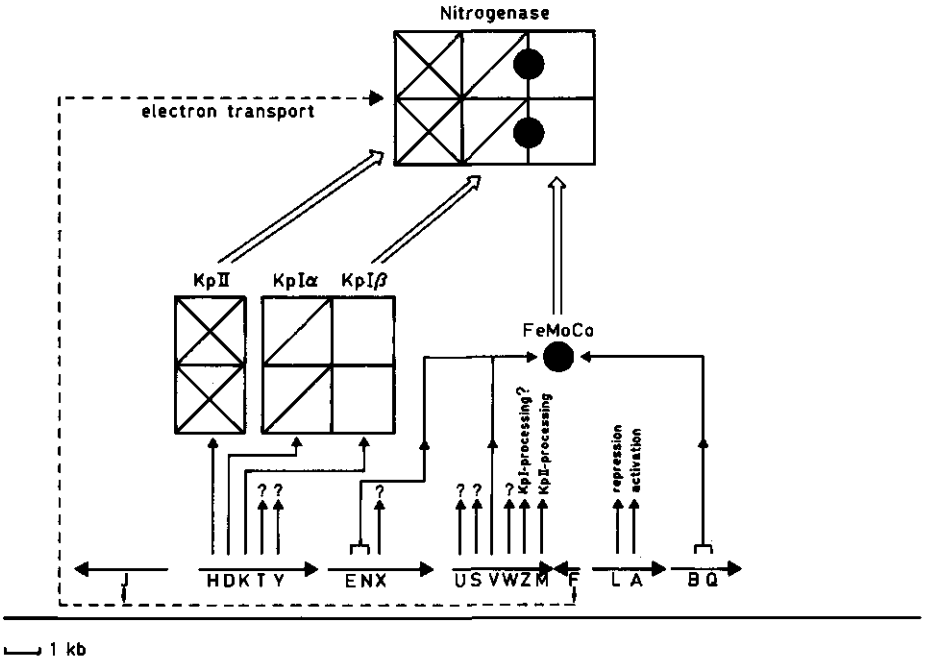

Figure 3. Map of the *K.pneumoniae nif* gene cluster.

The organization of the *K.pneumoniae nif* genes and *nif* operons, as well as the direction of transcription is indicated by horizontal arrows. Also the functions of the genes as far as known are indicated. For full details, see text.
Abbreviations: KpIα,β: *K.pneumoniae* nitrogenase components Iα and Iβ, KpII: *K.pneumoniae* nitrogenase component II, FeMoCo: iron-molybdenum cofactor.

The genes *nif*H, *nif*D and *nif*K, which are organized in one transcriptional unit, encode the subunits of the nitrogenase complex; component II (CII), component Iα (CIα) and component Iβ (CIβ). The *nif*M product is involved in the maturation of nitrogenase CII. *Nif*Q, *nif*B, *nif*E, *nif*N and *nif*V have been implicated in the synthesis of the FeMo-cofactor. The *nif*Q gene product probably is involved in Mo uptake, since *nif*Q mutants show a leaky Fix^- phenotype which can be suppressed by a high molybdate concentration (94). *Nif*E and *nif*N products show a structural homology to the nitrogenase components CIα and CIβ and were proposed to be the site where the FeMo cofactor is assembled (26). The Fix^- phenotype of a *nif*V mutant can be corrected by homocitrate (90), and the *nif*V gene product probably is homocitrate synthase. The function of homocitrate in FeMo cofactor synthesis as yet is unknown.

The genes *nif*J and *nif*F are involved in electron transport to nitrogenase; *nif*J encodes a pyruvate:flavodoxin oxidoreductase and *nif*F encodes a flavodoxin (22, 49). The genes *nif*A and *nif*L are involved in the regulation of nitrogen fixation. The product of *nif*A (*nif*Agp) is a transcription activator which directs the expression of the other *nif* genes except for the *nif*LA operon. The *nif*L gene product counteracts the *nif*Agp activity under conditions of nitrogen excess and high oxygen concentrations. The genes *nif*T, *nif*W, and *nif*Z were identified only recently upon sequencing the entire 24 kb *K. pneumoniae nif* cluster (128,6). In *K. pneumoniae*, *nif*W and *nif*Z mutants exhibit a Fix^+ phenotype, but are delayed in the derepression of nitrogen fixation (143). In contrast, the *A. vinelandii nif*Z gene was reported to be essential for nitrogen fixation (19), and a function in the maturation of CI was suggested. The *nif*X and *nif*Y genes of *K. pneumoniae* were reported not to be essential for nitrogen fixation; mutants in these genes show a reduced growth rate during diazotrophic growth (67). The functions of the genes *nif*T, *nif*Y, *nif*X, *nif*U, *nif*S and *nif*W are presently unknown.

By transposon mutagenesis, complementation analysis and interspecies DNA hybridization, several *nif* genes were shown to be present in a variety of *Rhizobium* and *Bradyrhizobium* species and in *Azorhizobium*. In *Rhizobium* the *nif* genes are present in several clusters located on a large plasmid, which is referred to as a *sym* plasmid (112,8,36). Also the *nod* genes are located on this plasmid (46,120,115). Curing of the *sym* plasmid results in the loss of nitrogen fixing ability. All essential genes involved in nitrogen fixation are present on the *sym* plasmid, as was shown by Martinez *et al.* (125) who transferred the *R. phaseoli sym* plasmid to a plasmid free *Agrobacterium tumefaciens*. The recipient *A. tumefaciens* strain by this plasmid transfer gained the ability to elicit nitrogen fixing nodules on *Pisum sativum* and

Leucaena esculentum. In *Bradyrhizobium* the *nif* genes are present on the bacterial chromosome instead of on a plasmid, located in at least two separated symbiotic clusters (see 83 and references therein).

Only a limited number of *nif* genes has been identified in *Rhizobium* and *Bradyrhizobium* species; the structural nitrogenase genes *nif*HDK (159,161,64), the regulatory gene *nif*A (59,162,173) and the genes *nif*B (29,63,69), *nif*N (53,4) and *nif*E (53,88). A *nif*S homologous gene has only been identified in *B. japonicum* (53). In *Rhizobium* species the relative position of the *nif* genes, as compared to *K. pneumoniae*, is relatively well conserved; the structural nitrogenase genes *nif*HDK form an operon which is transcribed from *nif*H towards *nif*K. The *nif*E gene is located downstream of this operon. Such an arrangement of *nif* genes was also found in *A. caulinodans* (41). As in *K. pneumoniae*, the position of *nif*B in all cases is downstream of *nif*A. The relative positions of the "*nif*HDK-cluster" and the "*nif*AB-cluster" on the *sym* plasmid may vary, however, between the different *Rhizobium* species.

In *B. japonicum* the *nif* genes are more scattered over the chromosome; cluster I contains amongst others (see below) the gene *nif*H, which is separated from *nif*DK, and *nif*B, *nif*E, *nif*N and *nif*S. *Nif*A is present in another cluster, referred to as cluster II, where also the *nod* genes are located (83,137).

Rhizobium genes involved in nitrogen fixation but not homologous to any of the *K. pneumoniae nif* genes, and for which no biochemical function has been assigned yet, are referred to as "*fix*" genes. Some of these genes are highly conserved amongst the symbiotic diazotrophs; the genes *fix*ABC were detected in several *Rhizobium* species, in *B. japonicum* and in *A. caulinodans* (63,69,147,45). In *Rhizobium*, the *fix*ABC genes are located upstream of *nif*A and form an operon which is transcribed from *fix*A towards *fix*C. Again, the situation in *B. japonicum* is more complex; *fix*BC constitutes an operon in cluster I, whereas the *fix*A gene is present in cluster II, downstream instead of upstream from *nif*A. The functions of the *fix*ABC genes are not known at present. However, since these genes are also present in the free-living aerobic nitrogen fixing *Azotobacter* (70), a role specific for aerobic nitrogen fixation seems obvious. Since in *Rhizobium* and *Bradyrhizobium* a gene encoding a ferredoxin-like protein (*fix*X, 52,93,55) is present directly adjacent to *fix*C, while no genes homologous to *nif*F and *nif*J have been found in these species, a role for the *fix*ABCX cluster in electron transport to nitrogenase has been proposed (70). Other *fix* genes that were recently identified in some of the rhizobial *nif* clusters are *fdx*N (*fix*Y), located downstream of *nif*B in *R. meliloti* (108) encoding a ferredoxin-like protein, a gene designated *nif*O upstream of the

*fix*ABC cluster in *A. caulinodans* (98), *fix*R, upstream of *nif*A in *B. japonicum* (174) and *fix*W, located upstream of the *fix*ABCX operon in *R. leguminosarum* (89, this thesis).
In *R. meliloti*, a novel *nif* cluster was found on the *sym* plasmid at 200 kb from the *nif*HDK-cluster (7,149). In this cluster at least eight *fix* genes are present; *fix*N, *fix*K, the *fix*LJ operon and *fix*GHIS, also forming a transcriptional unit. The *fix*GHIS operon probably encodes a membrane bound redox complex (97), whilst the *fix*LJ operon and *fix*K are involved in the regulation of nitrogen fixation (12,84) (see paragraph 1.2.4.2.). *Fix*N forms part of a repeated sequence on the *R. meliloti sym* plasmid, which is also present 40 kb downstream from the *nif*HDK cluster (149,96,37).

1.2.4.2. *Regulation of nitrogen fixation genes*

The regulation of the *nif* and *fix* genes in *Rhizobium* and *Bradyrhizobium* resembles the *nif* gene regulation in *K. pneumoniae*, but also some major differences are evident. In *K. pneumoniae*, the *nif* genes are regulated by a cascade system comprising the general nitrogen regulatory genes *ntr*B and *ntr*C and the nitrogen fixation specific regulatory genes *nif*A and *nif*L (see 152 and references therein). The *ntr*B and *ntr*C gene products belong to a family of regulatory proteins that consist of pairs of receptors and regulators. In general, the receptor protein senses an environmental stimulus thereby activating the regulator protein. The activated regulator then directs the transcription of relevant genes. Examples of these are the rhizobial *dct*B/*dct*D genes, which sense dicarboxylate concentrations outside the cell and direct transcription of a dicarboxylate transport gene encoded by the *dct*A locus, and the *Agrobacterium* genes *vir*A/*vir*G, involved in regulating virulence in response to a plant exudate (for a review, see 157).
Upon nitrogen starvation, the *Klebsiella ntr*B gene product activates the *ntr*C protein, which directs the transcription of several genes involved in nitrogen assimilation like *hut* (histidine utilization) and *put* (proline utilization) and also the *nif*A gene. The activation of the *nif*A gene requires beside the presence of activated *ntr*C protein, the presence of RNA polymerase which is equipped with a special sigma factor encoded by the *ntr*A gene. (The gene product of the *ntr*A gene, *ntr*Agp, is also known as RpoN, RpoE and σ^{54}.
The *nif*A product activates, again in concert with the *ntr*A product, the transcription of all other *nif* genes, which are equipped with a promoter recognized by the RNA polymerase-RpoN complex. This promoter has the consensus sequence CTGG-N8-TTGCA and is

located about 10 nucleotides upstream of the transcription start site. The *nif*A gene product binds to a so-called upstream activator sequence (UAS) with the consensus sequence TGT-N6-10-ACA, located more than 100 bp upstream of the transcription start. The exact mechanism by which the bound *nif*A protein regulates the RNA polymerase-RpoN complex is at the moment not known, but some possible modes of action of the *nif*A gene product are discussed in a review by Roelvink and Van den Bos (152).

The *nif*L gene product is a negative regulator and counteracts the *nif*A gene product under conditions of high oxygen concentration in the cell (which damages the nitrogenase polypeptides) or nitrogen excess. Also the *ntr*C gene is deactivated under conditions of a nitrogen excess by the reversed action of the *ntr*B gene product.

As in *K. pneumoniae*, in *Rhizobium* and *Bradyrhizobium* the activation of the *nif* genes is dependent on the *nif*A gene product, but the mode of activation of the *nif*A gene differs between *Klebsiella* and *(Brady)rhizobium*. The rhizobial *nif* genes or *nif* operons are equipped with the consensus *nif* promoter CTGG-N8-TTGCA and also an UAS has been found upstream of several *nif* genes (28). This promoter is also present upstream of all the *fix* operons, except for the *fix* genes present in the newly identified cluster in *R. meliloti* 200 kb downstream of the *nif*HDK cluster (38). Another exception forms the *nif*A promoter itself. For *R. meliloti* it was shown that the genes having these promoters are transcribed by a RNA polymerase which is equipped with a sigma factor encoded by the *ntr*A gene (156). This transcription is dependent on the presence of the *nif*A gene product (173). Although *R. meliloti* possesses a *ntr*C gene (172), during symbiotic nitrogen fixation the *nif*A gene is not induced by a deficiency of metabolizable nitrogen, and a *ntr*C mutant exhibits a normal Fix^+ phenotype (172). Instead, in *R. meliloti* the expression of the *nif*A gene was shown to be induced by a low oxygen concentration (43). The oxygen dependent expression of the *nif*A gene is mediated by the *fix*LJ genes (37,181), present in the *fix* cluster located at 200 kb from the *nif*HDK operon. Interestingly, the *fix*LJ proteins also belong to the family of receptor-regulator proteins as do the *ntr*C/*ntr*B proteins. Therefore David *et al.* (37) suggested that the *fix*L protein is a sensor of the oxygen concentration outside the cell. During microaerobiosis, this protein activates the *fix*J protein which then directs the transcription from the *nif*A promoter. Although this model is very attractive, no solid evidence in favor of it has been presented yet. The *fix*LJ genes also direct the transcription of *fix*K. The *fix*K gene product then positively regulates the transcription of *fix*N, which was shown to be transcribed independently from *nif*A (37,12).

In addition to the *fix*LJ mediated *nif*A expression, in *R. meliloti* about 50% of the *nif*A transcipts are the result of read-through transcription from the *fix*ABC operon into *nif*A (104). Since the *fix*ABC operon is preceded by a *nif*Agp dependent promoter, 50% of the *nif*A expression thus is dependent on autoregulation directed by the *nif*A protein. Whether in the other *Rhizobium* species the *nif*A expression is regulated similarly to *R. meliloti* is not known at present.

The regulation of *nif* and *fix* genes in *B. japonicum* differs from that in *R. meliloti*. Although transcription of the *B. japonicum nif*H and *nif*DK genes is only monitored under conditions of oxygen limitation (1), the *nif*A gene itself is not regulated by oxygen (174). In contrast to *Rhizobium* species, the expression of the *B. japonicum nif*A gene is directed by the promoter of a gene, *fix*R, which is situated immediately upstream of *nif*A. The expression of this *fix*R*nif*A cluster was detected during symbiosis as well as during free-living, aerobic growth. The *fix*R gene has a *nif* promoter but lacks the consensus UAS. Since the *B. japonicum nif*A protein is inactivated by oxygen (60), it is excluded that during aerobic growth the *fix*R*nif*A operon is (auto)regulated by the *nif*A gene product. Therefore, an unknown regulatory protein was hypothesized to be involved in *nif*A activation in *B. japonicum* (174). Also the environmental stimulus that triggers this activation is not known.

A final difference between *K. pneumoniae* and *Rhizobium* is the absence of a *nif*L homologous gene. This seems logical, since during symbiotic nitrogen fixation the oxygen concentration inside the nodule is kept at low levels by the action of leghemoglobin, making a protection mechanism against high oxygen concentrations superfluous.

1.2.4.3. *Other rhizobial genes controlled by the ntrA gene product*

A group of genes that is of special interest are those under control of the nitrogen regulatory sigma factor (*ntr*Agp), suggesting a role in nitrogen fixation.

Besides the already mentioned *nif* and *fix* genes, the following genes are currently known to possess a *ntr*A regulated promoter: *dct*A in *R. meliloti* (156), *gln*II in *B. japonicum* (34), *mos* in *R. meliloti* (133) and *mel*A in *R. phaseoli* and *R. leguminosarum* (23).

The *dct*A gene belongs to the *dct*ABD cluster, which is involved in the uptake of dicarboxylic acids. In *R. meliloti*, this cluster is located on the second megaplasmid, where also genes involved in exopolysaccharide synthesis (*exo* genes) are situated (182,57). The *dct*A gene codes for a membrane bound dicarboxylate permease which is regulated by the *dct*B and *dct*D

genes. The latter code for a receptor-regulator couple which senses the dicarboxylic acid concentration outside the cell and regulates transcription of *dct*A (154). During nitrogen fixation, dicarboxylic acids form the major carbon source for the bacteroids (see paragraph 1.2.3.2.) and *dct*A mutants exhibit a Fix$^-$ phenotype (182). In the free-living state, the *dct*A gene is not expressed in *dct*B or *dct*D mutants. These mutants, however, form normally nitrogen fixing root nodules, indicating that during symbiosis the *dct*A gene is activated in a different manner, possibly by the *nif*Agp (153).

The *gln*II gene identified in several *Rhizobium* species and *B. japonicum* codes for glutamine synthetase. In these species also other glutamine synthetase genes were identified, but only the *gln*II gene seems to be preceded by a *ntr*A dependent promoter. For *B. japonicum* it was shown that during free-living growth, the *gln*II gene is regulated by the product of the nitrogen regulatory gene *ntr*C. However, during microaerobiosis and *in planta* nitrogen fixation, the *gln*II gene is activated by a thusfar unknown mechanism (39,124). The role of the *gln*II gene product in symbiotic nitrogen fixation is not known at the moment. The induction of a glutamine synthetase in bacteroids seems superfluous since the bacteroids in the plant cell export all the fixed ammonia instead of catabolizing it. Furthermore, *gln*II mutants exhibit a normal Fix$^+$ phenotype even when combined with mutations in the other GS genes (39).

In *R. meliloti* a cluster of genes situated on the sym plasmid was described which is involved in the synthesis and catabolism of an opine-like compound, a rhizopine (132,133). This cluster comprises at least two genes involved in rhizopine catabolism, *moc* genes, and four genes which are involved in the synthesis of this compound, which are designated as *mos* genes. In the 5' region of the first *mos* gene (ORF 1), a perfect *nif*A dependent promoter is present, containing the *ntr*A dependent promoter structure at position -20 as well as an *nif*Agp binding site (UAS) at position -120. Intriguingly, this gene shows a very high homology with the promoter region, the 5'-untranslated leader sequence and the first 57 bp of the coding region of the *R. meliloti nif*H gene. These features show that the *mos* gene(s) are directly regulated by the *nif*A gene product. The *mos* genes, however, are not indispensable for symbiotic nitrogen fixation since a *mos* mutant shows a normal Fix$^+$ phenotype. The function of rhizopine is not known, but it has been hypothesized to serve as a selective growth substrate for *Rhizobium* bacteria present in the infection thread or in the rhizosphere. Finally, also the *mel*A gene, identified in *R. phaseoli*, possesses a *ntr*A dependent promoter and is regulated by the *nif*A gene product. The *mel*A gene is involved in melanin production,

both during free- living growth and in the nodule. The function of melanin in nitrogen fixation is unknown.

1.2.5. Development and function of peribacteroid membrane and peribacteroid space

As depicted in the previous paragraphs, during the *Rhizobium*-legume symbiosis both the endosymbiont and the host plant adapt their metabolisms to the nitrogen fixation process, and a constant exchange of metabolites takes place. The PBM and PBS, surrounding the bacteroids, probably play an important role in this, since they form the interface between plant and bacterium. In this paragraph the development and properties of the peribacteroid membrane and the peribacteroid space will be discussed.

Inside the infection thread the bacteria are surrounded by an infection thread wall. On the outside of this wall the so-called infection thread membrane is situated. The wall consists of material resembling plant cell wall material, whilst the membrane is derived from the plant cell cytoplasmic membrane, which is connected to the infection thread membrane.

The *Rhizobium* bacteria are released into the cells of the developing central tissue from the top of the penetrating infection thread, where the infection thread wall appears to be absent. Here the bacteria are in direct contact with the infection thread membrane, which forms small vesicles enclosing separate bacteria. These vesicles then bud off from the membrane and release the bacteria from the thread in a way preventing a direct contact between the bacteria and the plant cytoplasm. The release of the bacteria is probably caused by the concerted action of degradative enzymes derived from both the bacterium and the plant host (179,121,180).

After release, the bacteroids actively divide with a concomitant synthesis of the PBM. In early stages of infection, the synthesis of PBM-material is closely synchronized with the division of the *Rhizobium*, which results in the enclosure of separate bacteroids in one membrane envelope. This synchronization is probably mediated by points of adherence between the PBM and the bacteroid outer membrane. Such points of adherence were detected in a cytological study by Robertson *et al.* (150). Biochemical evidence for a connection between PBM and the bacteroid outer membrane came from Bradley *et al.* (24), who showed that lipopolysaccharides specific for the bacteroid outer membrane can also be found in small amounts in the PBM. In later stages of infection, a difference in bacteroid enclosure and development can be seen between plants, which form indeterminate nodules, like pea, and plants, which form determinate nodules, like soybean (171). In pea-like nodules, the

synchronization between bacteroid division and PBM synthesis is maintained until the bacteroids cease dividing. This results in nodules predominantly filled with bacteroids individually enclosed by a PBM. After the division has stopped, the bacteroids increase in volume by factors estimated from 10 to 40 (see 171 and references therein), and undergo morphological changes.

In determinate nodules the proliferation of the PBM is arrested at an earlier stage of nodule development than the division of the *Rhizobia*, resulting in the occurrence of 5 to 20 bacteroids within one single membrane envelope. The early stop in PBM proliferation is probably caused by a reduction of the number of adherence points between the PBM and the bacteroid outer membrane (150). An alternative explanation for the occurrence of multiple bacteroids within one envelope was given by Newcomb *et al.* (136), who propose that individually enveloped bacteroids may fuse to yield a single PBM, containing several bacteroids. Which of the two described processes actually takes place in determinate nodules is not known at the moment. In contrast to bacteroids in pea-like nodules, those in soybean-like nodules do not exhibit major morphological changes and resemble free-living bacteria (171).

During bacteroid division, the PBM, which is initially derived from the plant cytoplasmic membrane, is formed by fusion with membrane vesicles derived from the Golgi bodies (25) and the endoplasmatic reticulum (136). The vesicles derived from the Golgi bodies are possibly also involved in the synthesis of the infection thread membrane and the infection thread wall (136).

In a fully developed infected nodule cell, packed with PBM-enclosed bacteroids, the amount of membrane synthesized is 20 to 40 times as much as in an uninfected cell (179). For the *B. japonicum - Glycine max* symbiosis it has been shown that during nodule development a second form of the plant encoded enzyme choline kinase (CK II) is present, which is involved in PBM synthesis (127). Since no CK II activity could be detected in non-infected root tissue nor in tissue culture, choline kinase II probably is a nodulin. The *Bradyrhizobium* bacteria seem to regulate the CK II activity; several classes of Fix^- mutants were described which were not enveloped within a stable PBM. CK II activity in nodules induced by these mutants was reduced or even absent, indicating that a bacterial gene(s) influences the membrane building machinery of the host plant.

During development, proteins are targeted into the PBM. Most of these proteins are synthesized by the plant host (179), but the protein composition of the membrane and processing of some of these proteins is influenced by the endosymbiont. Werner *et al.* (184)

showed the presence of 27 major polypeptides in the PBM isolated from *B. japonicum*. Four of these proteins were absent from nodules induced by the Fix$^-$ mutant RH-31 Marburg, which also affects the glycosylation of several glycoproteins present in the PBM.

Two nodulins, nodulin-24 and nodulin-26, were detected in *Glycine max*, which are specifically targeted into the PBM (101,61,62). Also the expression of these nodulins appears to be influenced by the bacterium (131).

The PBS was shown to contain a specific set of polypeptides, different from both bacteroid and plant cytoplasm (62,99,151). In contrast to the PBM, most proteins present in the PBS are encoded by the bacteroid. Katinakis *et al.* (99, this thesis) showed that approx. 90% of the 40 major PBS proteins isolated from *R. leguminosarum* bacteroids are of bacterial origin. The plant host, however, seems to play a role in the level of accumulation of the different bacteroid encoded PBS proteins, since a *R. leguminosarum* strain used to induce nodules on both *Pisum sativum* and *Vicia faba* showed a difference in the relative amounts of several bacteroid encoded PBS proteins (100). Some of these proteins are only induced during symbiosis and therefore should be considered as bacteroidins (bacterial proteins specifically synthesized and involved in the process of nitrogen fixation, 178). The function of these bacteroidins is not known at present. The presence of nodulins in the PBS was demonstrated by Fortin *et al.* (62), who were able to precipitate poly A^+ RNA encoded polypeptides with antiserum directed against PBS proteins.

Not much is known about the function of the PBM and of the processes that take place in the PBS. Probably the PBM fulfills a double role. On the one hand this membrane isolates the bacteroid from the plant cell cytoplasm, preventing a defense reaction of the plant against the bacterium. Evidence for this was gained by Werner *et al.* (183), who showed a 10 to 100-fold stimulation in the production of glyceollin I (a phytoalexin of soybean) in root nodules induced by a *Bradyrhizobium* mutant that shows an early loss of the PBM. On the other hand the PBM is probably involved in transport of metabolites from and to the bacteroids. The PBM of soybean nodules shows both protein kinase and ATP-ase activities, which points to a role in active transport of metabolites and possibly in signal transduction (179, 10). Indeed, the membrane was shown to be involved in the transport of carbon sources to the bacteroid: the PBM is poorly permeable to carbon sources like glutamate, pyruvate and arabinose (146), and possesses an active transport system for dicarboxylic acids like succinate and malate (176).

Very little information is available on enzymatic activities present in the PBS. Only high levels of alpha-mannosidase activity have been reported to be present in the PBS of *Bradyrhizobium*

bacteroids, as well as a protease inhibition activity (126,105,65). The specific role, which these enzymes play in symbiotic nitrogen fixation is not clear presently. Since most of the metabolites designated to or derived from the bacteroid have to pass the PBS, specific carriers are supposed to be present. Such activities, however, have not been described yet, which is probably mainly due to the fact that not much interest currently is paid to this symbiotic compartment.

1.3. Literature cited

1. Adams, T.H. and Chelm, B. (1988). Effects of oxygen levels on the transcription of *nif* and *gln* genes in *Bradyrhizobium japonicum*. J. Gen. Microbiol. *134*, p.611.
2. Adams, T.H., McClung, C.R. and Chelm B. (1984). Physical organization of the *Bradyrhizobium japonicum* nitrogenase gene region. J.Bacteriol *159*, (3), p. 857.
3. Aguilar, M., Ashby, A.M., Richards, A.J.M., Loake, G.I., Watson, M.D. and Shaw, C.H. (1988). Chemotaxis of *Rhizobium leguminosarum* biovar *phaseoli* towards flavonoid inducers of the symbiotic nodulation genes. J.Gen.Microbiol. *134*, (10), p.2741.
4. Aguilar, M.O., Reiländer, H., Arnold, W. and Pühler, A. (1987). *Rhizobium meliloti nif*N (*fix*F) gene is part of an operon regulated by a *nif*A-dependent promoter and codes for a polypeptide homologous to the *nif*K gene product. J. Bacteriol. *169*, (12), p.5393.
5. Appelbaum, E.R., Johansen, E. and Chartrain, N. (1985). Symbiotic mutants of USDA191, a fast-growing *Rhizobium* that nodulates soybeans. Mol. Gen. Genet. *201*, p.454.
6. Arnold, W., Rump, A., Klipp, W., Priefer, B. and Pühler, A. (1988). Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. J. Mol. Biol. *203*, p.715.
7. Banvalvi, Z., Petkova, V., Lados, M., Slaska-Kiss, K., Putnoki, P., Ung, C.H. and Kondorosi, A. (1989). Identification of two *fix* loci controlling the expression of *nif* genes in *Rhizobium meliloti* 41. Mol. Gen. Genet. *215*, p.345.
8. Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. and Kondorosi, A. (1981). Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R.meliloti*. Mol. Gen. Genet. *184*, p.318.
9. Barbour, W.M., Mathis, J.N. and Elkan, G.H. (1985). Evidence for plasmid- and chromosome-born multiple *nif* genes in *Rhizobium fredii*. Appl. Environ. Microbiol *50*, (1), p. 41.
10. Bassarab, S. and Werner, D. (1987). Ca^{2+}-dependent protein kinase activity in the peribacteroid membrane from soybean root nodules. J. Plant Physiol. *130*, p.233.
11. Basset, B., Goodman, R.N. and Novacky, A. (1977). Ultrastructure of soybean nodules. Release of rhizobia from the infection thread. Can. J. Microbiol.*23*, p.573.
12. Batut, J., Daveran-Mingot, M.-L., David, M., Jacobs, J., Garnerone, A.M. and Kahn, D. (1989). *Fix*K, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation both positively and negatively in *Rhizobium meliloti*. EMBO J. *8*, (4), p.1279.
13. Benemann, J.R. and Valentine, R.C. (1972). The pathway of nitrogen fixation. Adv.Microbiol.Physiol. *8*, p. 59.
14. Bennet. L.T., Jacobson, M.R. and Dean, D.R. (1988). Isolation, sequencing, and mutagenesis of the *nif*F gene encoding flavodoxin from *Azotobacter vinelandii*. J.Biol.Chem. *263*, (3), p. 1364.
15. Bergersen, F.J. (1965). Ammonia - an early stable product of nitrogen fixation by

soybean root nodules. Aust.J.Biol.Sci. *18*, p. 1.

16. Bergersen, F.J. (1982). Root nodules of legumes: Structure and functions. Research Studies Press, Chichester, U.K.
17. Bergersen, F.J. and Turner, G.L. (1967). Nitrogen fixation by the fraction of breis of soybean root nodules. Biochim.Biophys. Acta *141*, p. 507.
18. Bergman, K., Gulash-Hoffee, M., Hovestadt, R.E., Larosiliere, R.C., Ronco, P.G. II and Lihe, S. (1988). Physiology of behavioral mutants of *Rhizobium meliloti*; evidence for a dual chemotaxis pathway J.Bacteriol. *170*,(7), p. 3249.
19. Beynon, J., Cannon, M., Cannon, F., Howard, J., Jacobson, M., Wilson, M. and Dean, D. (1988). Identification of new *nif*-specific genes from *A.vinelandii* and *K.pneumoniae*. p. 318. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.
20. Bhuvaneswari, T.V. and Solheim, B. (1985). Root hair deformation in the white clover/*Rhizobium trifolii* symbiosis. Physiol. Plant. *63*, p. 25.
21. Bisseling, T., Van den Bos, R.C., Van der Ploeg, M., Van Duijn, P. and Houwers, A. (1977). Cytofluorometric determination of the DNA contents of bacteroids and corresponding broth-cultured *Rhizobium* bacteria. J. Gen.Microbiol. *101*, p.79.
22. Bogusz, D., Houmard, J. and Aubert, J-P. (1981). Electron transport to nitrogenase in *Klebsiella pneumoniae*. Eur.J.Biochem. *120*, p. 421.
23. Borthakur, D., Lamb, J.W. and Johnston, A.W.B. (1987). Identification of two classes of *Rhizobium phaseoli* genes required for melanine synthesis, one of which is required for nitrogen fixation and activates the transcription of the other. Mol. Gen. Genet. *207*, (1), p.155.
24. Bradley, D.J., Butcher, G.W., Galfre, G., Wood, E.A. and Brewin, N.J. (1986). Physical association between the peribacteroid membrane and lipopolysaccharide from the bacteroid outer membrane in *Rhizobium*- infected pea root nodule cells. J. Cell Sci. *85*, p. 47.
25. Brewin, N.J., Robertson, J.G., Wood, E.A., Wells, B., Larkins, A.P., Galfre, G.and Butcher, G.W. (1985). Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea cross-react with plasma membranes and Golgi bodies. EMBO J. *4*, (3), p. 605.
26. Brigle, K.E., Weiss, M.C., Newton, W.E. and Dean, D.R. (1987). Products of the iron-molybdenum cofactor-specific biosynthetic genes, *nif*E and *nif*N, are structurally homologous to the products of the nitrogenase molybdenum-iron protein genes, *nif*D and *nif*K. J. Bacteriol. *169*, (4), p.1547.
27. Broughton, W.J. and Dilworth, M. (1971). Control of leghemoglobin synthesis in snake beans. Biochem.J. *125*, p. 1075.
28. Buck, M., Miller, S., Drummond, M. and Dixon, R. (1986). Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature *320*, p.374.
29. Buikema, W.J., Klingensmith, J.A., Gibbons, S.L. and Ausubel, F.M. (1987). Conservation of structure and location of *Rhizobium meliloti* and *Klebsiella pneumoniae nif*B genes. J. Bacteriol. *169*, (3), p.1120.
30. Burns, R.C. and Hardy, R.W.F. (1975). Nitrogen fixation in bacteria and higher plants, p.53. Springer Verlag, Berlin Heidelberg, New York.
31. Caetano-Anolles, G., Crist-Estes, D.K. and Bauer, W.D. (1988). Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J.Bacteriol. *170*, (7), 3164.
32. Callaham, D.A. and Torrey, J.G. (1981). The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. Can.J.Bot. *59*, p. 1647.
33. Carlson, T.A. and Chelm, B.K. (1986). Apparent eukaryotic origin of glutamine synthetase II from the bacterium *Bradyrhizobium japonicum*. Nature *322*, p. 568.
34. Carlson, T.A., Martin, G.B. and Chelm, B.K. (1987). Differential transcription of the two glutamine synthetase genes of *Bradyrhizobium japonicum*. J.Bacteriol. *169*, (12), p.5861.
35. Carter, K.R., Rawlings, J., Orme-Johnson, W.H., Becker, R.R. and Evans, H.J. (1980). Purification and characterization of a ferredoxin from *Rhizobium japonicum* bacteroids. J.Biol.Chem. *255*, p.4213.
36. Christensen, A.H. and Schubert, K.R. (1983). Identification of a *Rhizobium*

trifolii plasmid coding for nitrogen fixation and nodulation genes and its interaction with pJB5JI, a *Rhizobium leguminosarum* plasmid. J. Bacteriol. *156*, (2), p.592.

37. David, M., Daveran, M.-L., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P. and Kahn, D. (1988). Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. Cell *54*, p.671.
38. David, M., Domergue, O., Pognonec, P. and Kahn, D. (1987). Transcription patterns of *Rhizobium meliloti* symbiotic plasmid pSym: Identification of *nif*A-independent *fix* genes. J. Bacteriol. *169*, (5), p.2239.
39. De Bruijn, F.J., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W.W., Ausubel, F.M. and Schell, J. (1989). *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. J. Bacteriol. *171*, (3), p.1673.
40. Deistung, J., Cannon, F.C., Cannon, M.C., Hill, S. and Thorneley, R.N.F. (1985). Electron transfer to nitrogenase in *Klebsiella pneumoniae*. Biochem.J. *231*, p. 743.
41. Denèfle, P., Kush, A., Norel, F., Paquelin, A. and Elmerich, C. (1987). Biochemical and genetic analysis of the *nif*HDKE region of *Rhizobium* ORS571. Mol. Gen. Genet. *207*, p.280.
42. Dilworth, M.J. (1969). The plant as genetic determinant of leghemoglobin production in the legume nodule. Biochim.Biophys. Acta. *184*, p. 432.
43. Ditta, G., Virts, E., Palomares, A. and Kim, C.-H. (1987). The *nif*A gene of *Rhizobium meliloti* is oxygen regulated. J. Bacteriol. *169*, (7), p.3217.
44. Dixon, R.O.D. (1972). Hydrogenases in legume root nodule bacteroids: occurrence and properties. Arch.Microbiol. *85*, p.289.
45. Donald, R.G.K., Ness, D.W., Raymond, C.K., Loroch, A.I. and Ludwig, R.A. (1986). Characterization of three genomic loci encoding *Rhizobium* sp. strain ORS571 N_2 fixation genes. J. Bacteriol. *165*, p.72.
46. Downie, J.A., Hombrecher, G., Ma, Q.-S., Knight, C.D., Wells, B. and Johnston, A.W.B. (1983). Cloned nodulation genes of *Rhizobium leguminosarum* determine host-range specificity. Mol. Gen. Genet. *190*, p.359.
47. Dreyfus, B.L. Elmerich, C. and Domergues, Y. (1983). Free living *Rhizobium* strain able to grow on N_2 as sole nitrogen source. Appl. Environ. Microbiol. *45*, 711.
48. Dreyfus, B., Garcia, .L. and Gilis, M, (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. *38*, (1), p.89.
49. Drummond, M.H. (1985). The base sequence of the *nif*F gene of *Klebsiella pneumoniae* and homology of the predicted amino acid sequence of its protein product to other flavodoxins. Biochem. J. *232*, p.891.
50 Dusha, I., Kovalenko, S., Banfalvi, Z. and Kondorosi, A. (1987). *Rhizobium meliloti* insertion element ISRm2 and its use for identification of the *fix*X gene. J.Bacteriol. *169*, (4), p. 1403.
51. Eady, R.R. (1986). Enzymology in free-living diazothrophs. In Broughton, W.J. and Pühler, A. (eds.) Nitrogen Fixation Volume 4: Molecular biology. Clarendon Press, Oxford, U.K. p. 1.
52. Earl, C.D., Ronson, C.W. and Ausubel, F.M. (1987). Genetic and structural analyses of the *Rhizobium meliloti fix*A, *fix*B, *fix*C, and *fix*X genes. J.Bacteriol. *169*, (3), p. 1127.
53. Ebeling, S., Hahn, M., Fisher, H.-M. and Hennecke, H. (1987). Identification of *nif*E-, *nif*N- and *nif*S-like genes in *Bradyrhizobium japonicum*. Mol. Gen. Genet. *207*, p.503.
54. Ebeling, S., Noti, J.D. and Hennecke, H. (1988). Identification of a new *Bradyrhizobium japonicum* gene (frxA) encoding a ferredoxin-like protein. J. Bacteriol. *170*, (4), p. 1999.
55. Ebeling, S., Zürcher,T., Gubler, M., Noti, J.D. and Hennecke, H. (1988). Identification of two genes in *Bradyrhizobium japonicum* coding for ferredoxin-like proteins. p. 366. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.
56. Emerich, D.W., Lepo, J.E. and Evans, H.J. (1983). Nodule metabolism, p. 211. In Broughton, W.J. (ed.) Nitrogen fixation,

Volume 2 Rhizobium. Clarendon Press, Oxford.

57. Finan, T.M., Kunkel, B., de Vos, G.F. and Signer, E.R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. *167*, (1), p.66.

58. Finan, T.M., Oresnik, I. and Bottacin, A. (1988). Mutants of *Rhizobium meliloti* defective in succinate metabolism. J.Bacteriol. *170*, (8), p. 3396.

59. Fischer, H.M., Alvarez-Moralez, A. and Hennecke, H. (1986). The pleiotropic nature of symbiotic regulatory mutants: *Bradyrhizobium japonicum nif*A gene is involved in control of *nif* gene expression and formation of determinate symbiosis. EMBO J. *5*, (6), p.1165.

60. Fisher, H.-M. and Hennecke, H. (1987). Direct response of *Bradyrhizobium japonicum nif*A-mediated *nif* regulation to cellular oxygen status. Mol. Gen. Genet. *209*, p.621.

61. Fortin, M.G., Morrison, N.A. and Verma, D.P.S. (1987). Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid membrane. Nucl. Acids Res. *17*, (2), p.813.

62. Fortin, M.G., Zelechowska, M. and Verma D.P.S. (1985). Specific targeting of membrane nodulins to the bacteroid-enclosing compartment in soybean nodules. EMBO J. *4*, (12), p.3041.

63. Fuhrmann, M., Fischer, H.-M. and Hennecke, H. (1985). Mapping of *Rhizobium japonicum nif*B-, *fix*BC-, and *fix*A-like genes and identification of the *fix*A promoter. Mol. Gen. Genet. *199*, p. 315.

64. Fuhrmann, M. and Hennecke, H. (1982). Coding properties of cloned nitrogenase structural genes from *Rhizobium japonicum*. Mol. Gen. Genet. *187*, p.419.

65. Garbers, C., Meckbach, R., Mellor, R.B. and Werner, D. (1988). Protease (thermolysine) inhibition activity in the peribacteroid space of *Glycine max* root nodules. J. Bacteriol. *169*, (12), p.5393.

66. Gardiol, A., Arias, A., Cervenansky, C. and Martinez-Dretz, G. (1982). Succinate dehydrogenase mutants of *Rhizobium meliloti*. J.Bacteriol. *151*, p. 1621.

67. Gosink, M., Franklin, N. and Roberts, G. (1988). Initial characterization of *nif*X and *nif*Y mutants in *Klebsiella pneumoniae*. p.308. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.

68. Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., van Kammen, A. and Bisseling, T. (1986). *Rhizobium nod* genes are involved in inducing an early nodulin gene. Nature *323*, p.564.

69. Grönger, P., Manian, S.S., Reiländer, H., O'Connell, M., Priefer, U.B. and Pühler, A. (1987). Organization and partial sequence of a DNA region of the *Rhizobium leguminosarum* symbiotic plasmid pRL6JI containing the genes *fix*ABC, *nif*A, *nif*B and a novel open reading frame. Nucleic Acids Res. *15*, (1), p.31.

70. Gubler, M. and Hennecke, H. (1986). *Fix*A,B and C genes are essential for symbiotic and free-living, microaerobic nitrogen fixation. FEBS Lett. *200* (1), p.186.

71 Guerinot, M.L. and Chelm, B.K. (1986). Bacterial δ-aminolaevulinic acid synthetase activity is not essential for leghemoglobin formation in the soybean/*Bradyrhyzobium japonicum* symbiosis. Proc.Natl.Acad.Sci. USA *83*, p. 1837.

72. Haaker, H. (1986). Electron transport to nitrogenase in diazotrophs. In Broughton, W.J. and Puhler, A. (eds.) Nitrogen fixation volume 4: Molecular biology. Clarendon Press, Oxford, U.K. p. 70.

73. Haaker, H., de Kok, A. and Veeger, C. (1974). Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. Biochim.Biohpys.Acta *357*, p. 344.

74. Haaker, H. and Klugkist, J. (1987). The bioenergetics of electron transport to nitrogenase. FEMS Microbiol.Reviews *46*, p. 57.

75. Haaker, H., Laane, C., Hellingerwerf, K., Houwer, B., Konings, W.N. and Veeger, C. (1982). Sort-term regulation of the nitrogenase activity in *Rhodopseudomonas sphaeroides*. Eur.J.Biochem. *127*, p. 639.

76. Haaker, H. and Veeger, C. (1977). Involvement of the cytoplasmic membrane in nitrogen fixation by *Azotobacter vinelandii*. Eur. J. Biochem. *77*, p.1.

77. Häger, K-P. and Bothe, H. (1987). Reduction of NAD^+ by reversed respiratory electron flow in *Azotobacter vinelandii*. Biochim. Biophys.Acta *892*, p. 213.
78. Häger, K-P., Gätz, A. and Bothe, H. (1987). Succinate-dependent NAD^+ reduction by the reversed respiratory electron transport in *Bradyrhizobium japonicum*. J.Plant Physiol. *133*, p. 91.
79. Häger, K-P., Hundeshagen, B. and Bothe, H. (1988). Nitrogen fixation and reversed electron transport in *Azotobacter* and *Bradyrhizobium*. p. 249. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.
80. Hawkesford, M.J., Reed, R.H., Rowel, P. and Steward, W.D.P. (1981). Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Anabaena variabilis*. Eur.J.Biochem. *115*, p.519.
81. Hawkesford, M.J., Reed, R.H., Rowel, P. and Steward, W.D.P. (1982). Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Plectonema boryanum*. Eur.J.Biochem. *127*, p. 63.
82. Hennecke H., Fisher, H.-M., Ebeling, S., Gubler, M., Thöny, B., Göttfert, M., Lamb, L., Hahn, M., Ramseier, T., Regensburger, B., Alvarez-Moralez, A. and Studer D. (1987). *Nif*, *fix* and *nod* gene clusters in *Bradyrhizobium japonicum*, and *nif*-A mediated control of symbiotic nitrogen fixation, p. 191. In D.P.S.Verma and N.Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
83. Hennecke, H., Fisher, H.-M., Gubler, M., Thöny, B., Anthamatten, D., Kullik, I., Ebeling, S., Fritsche, S. and Zürcher, T. (1988). Regulation of *nif* and *fix* genes in *Bradyrhizobium japonicum* occurs by a cascade of two consecutive gene activation steps of which the second one is oxygen sensitive. p.339. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart.
84. Hertig, C., Li, R.Y., Louarn, A.-M., Garnerone, A.-M., David, M., Batut, J., Kahn, D. and Boistard, P. (1989). *Rhizobium meliloti* regulatory gene *fix*J activates transcription of *R. meliloti nif*A and *fix*K genes in *Escherichia coli*. J. Bacteriol. *171*, (3), p.1736.
85. Hill, S. and Kavanagh, E.P. (1980). Roles of *nif*F and *nif*J gene products in electron transport to nitrogenase in *Klebsiella pneumoniae*. J.Bacteriol. *141*, (2), p. 470.
86. Hirsch, A.H., Drake, D., Jacobs, T.W. and Long, S. (1985). Nodules are induced on alfalfa roots by *Agrobacterium tumefaciens* and *Rhizobium trifolii* containing small segments of the *Rhizobium meliloti* nodulation region. J. Bacteriol. *161*, (1), p.223.
87. Holsters,M., Van den Eede, G., Goethals, K., Van Montagu, M. and Dreyfus, B. (1986) Nodulation genes of the stem nodulating *Sesbania rostrata* symbiont, strain ORS571, p. 208.In D.P.S.Verma and N.Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
88. Hontelez, J.G.J., Klein Lankhorst, R.M., Jansma, J.-D., Jacobsen, E., Van den Bos, R.C. and Van Kammen, A. (1987). Characterization of symbiotic genes and regulation of their expression in *Rhizobium leguminosarum* PRE. p.241. In D.P.S.Verma and N.Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
89. Hontelez, J.G.J., Klein Lankhorst, R.M. Van Kammen, A. and Van den Bos, R.C. (1988). Evidence for a novel gene *fix*W, upstream of *fix*ABC in *R.leguminosarum*. p.371. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart.
90. Hoover, T.R., Imperial, J., Ludden, P.W. and Shah, V.S. (1988). Homocitrate cures the $NifV^-$ phenotype in *Klebsiella pneumoniae*. J. Bacteriol. *170*, (4), p.1978.
91. Howard, K.S., Hales, B.J. and Socolofsky, M.D. (1985). In vivo interactions between nitrogenase molybdenum-iron protein and membrane in *Azotobacter vinelandii* and *Rhodospirillum rubrum*. Biochim. Biophys.Acta *812*, p. 575.
92. Howitt, S.M., Udvardi, M.K., Day, D.A. and Gresshoff, P.M. (1986). Ammonia transport in free-living and symbiotic

Rhizobium sp.ANU289. J.Gen.Microbiol. *132*, p. 257.

93. Iismaa, S. and Watson, J.M. (1987). A gene upstream the *Rhizobium trifolii nif*A gene encodes a ferredoxin-like protein. Nucleic Acids Res. *15*, (7),p.3180.
94. Imperial, J., Ugalde, R.A., Shah, V.K. and Brill. W. (1985). Mol$^-$ mutants of *Klebsiella pneumoniae* requiring high levels of molybdate for nitrogenase activity. J. Bacteriol. *163*, (3), p.1285.
95. Jordan, D.C. (1982). Transfer of *Rhizobium japonicum* to *Bradyrhyzobium japonicum*. A slow growing root nodule bacterium from leguminous plants. Int.J. Syst. Bacteriol. *32*, p.136.
96. Kahn, D., Batut, J., Boistard, P., Daveran, M.-L., Domergue, O., Garnerone, A.M., Ghai, J., Hertig, C., Infante, D. and Renalier, M.H. (1987). Molecular analysis of a *fix* cluster from *Rhizobium meliloti*. p.258. In D.P.S.Verma and N.Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
97. Kahn, D., David, M., Domergue, O. Daveran, M.-L., Ghai, J., Hirsch, P.R. and Batut, J. (1989). *Rhizobium meliloti fix*GHI sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J. Bacteriol. *171*, (2), p.929.
98. Kaminski, P.A., Norel, F., Desnoues, N., Kush, A., Salzano, G. and Elmerich, C. (1988). Characterization of the *fix*ABC region of *Azorhizobium caulinodans* ORS571 and identification of a new nitrogen fixation gene. Mol. Gen. Genet. *214*, p.496.
99. Katinakis, P., Klein Lankhorst, R.M., Louwerse, J., Van Kammen, A. and Van den Bos, R.C. (1988). Bacteroid-encoded proteins are secreted into the peribacteroid space by *Rhizobium leguminosarum*. Plant Mol. Biol. *11*, p.183.
100. Katinakis, P., Van Kammen, A. and Van den Bos, R.C. (1988). Protein composition of the peribacteroid space in root nodules of *Pisum sativum* and *Vicia faba* induced by *Rhizobium leguminosarum* biovar *viciae*. Lett. Appl. Microbiol. *7*, p.115.
101. Katinakis, P. and Verma, D.P.S. (1985). Nodulin-24 gene of soybean codes for a peptide of the peribacteroid membrane and was generated by tandem duplication of a sequence resembling an insertion element. Proc. Natl. Acad. Sci. USA *82*, p.4157.
102. Keister, D.L., Jones, B.L., Kondorosi, A. and Svab, Z. (1987). Characteristics of glutamine synthetase I and II mutants of *Rhizobium*. J. Gen. Appl. Microbiol. *33*, (3), p. 267.
103. Kieber, J., Clover, R., Finan, T.M. and Signer, E.R. (1986). Surface properties of *Rhizobium meliloti* associated with symbiosis. p. 182. In D.P.S.Verma and N.Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
104. Kim, C.-H., Helinsky, D.R. and Ditta, G. (1986). Overlapping transcripts of the *nif*A regulatory gene in *Rhizobium meliloti*. Gene *50*, p.141.
105. Kinnback, A., Mellor, R.B. and Werner, D. (1987). Alpha-mannosidase II isoenzyme in the peribacteroid space of *Glycine max* root nodules. J. Exp. Bot. *38*, (193), p.1373.
106. Kirk-Othmer Encyclopedia of chemical technology (1980) 3rd edition, vol. *12*, p.946.
107. Klein Lankhorst, R.M., Katinakis, P., Van Kammen, A. and Van den Bos, R.C. (1988). Identification and characterization of a bacteroid specific dehydrogenase complex in *Rhizobium leguminosarum* PRE. Appl.Environ.Microbiol. *54*, (12), p. 3008.
108 Klipp, W., Reiländer, H., Schlüter, A., Krey, R. and Pühler, A. (1989). The *Rhizobium meliloti fdx*N gene encoding a ferredoxin-like protein is necessary for nitrogen fixation and is cotranscribed with *nif*A and *nif*B. Mol.Gen.Genet. *216*, p. 293.
109. Klugkist, J. (1985). Electron transport to nitrogenase in *Azotobacter vinelandii*. PhD Thesis, Agricultural University Wageningen, Wageningen, The Netherlands.
110. Klugkist, J., Haaker, H. and Veeger, C. (1986). Studies on the mechanism of electron transport to nitrogenase in *Azotobacter vinelandii*. Eur.J.Biochem. *155*, p. 41.
111. Klugkist, J., Voorberg, J., Haaker, H. and Veeger, C. (1986). Characterization of three different flavodoxins from

Azotobacter vinelandii Eur.J.Biochem. *155*, p. 33.

112. Krol. A.J.M., Hontelez, J.G.J. and Van Kammen, A. (1982). Only one of the large plasmids in *Rhizobium leguminosarum* strain PRE is strongly expressed in the endosymbiotic state. J. Gen. Microbiol. *128*, p.1839.
113. Laane, C., Haaker, H. and Veeger, C. (1978). Involvement of the cytoplasmic membrane in nitrogen fixation by *Rhizobium leguminosarum* bacteroids. Eur.J.Biochem. *87*, p. 147.
114. Laane, C., Krone, W., Konings, W.N., Haaker, H. and Veeger, C. (1979). The involvement of the membrane potential in nitrogen fixation by bacteroids of *Rhizobium leguminosarum*. FEBS Lett. *103*, (2), p. 328.
115. Lamb, J.W., Downie, J.A. and Johnston, A.W.B. (1985). Cloning of nodulation (*nod*) genes of *Rhizobium phaseoli* and their homology to *R.leguminosarum nod* DNA. Gene *34*, p.235.
116. Leong, S.A., Ditta, G.S. and Helinski, D.R. (1982). Heme biosynthesis in *Rhizobium*. J.Biol.Chem. *257*, p. 8724.
117. Leong, S.A., Williams, P.H. and Ditta, G.D. (1985). Analysis of the 5' regulatory region of the gene for δ-aminolevulinic acid synthase of *Rhizobium meliloti*. Nucl.Acids Res. *13*, (16), p. 5965.
118. Libbenga, K.R. and Harkes, P.A.A.(1973). Initial proliferation of cortical cells in the formation of root nodules in *Pisum sativum* L. Planta *114*, p. 17.
119. Lim, G. and Burton, J.C. (1982) Nodulation status of the *Leguminosae*, p.1. In Broughton, W.J. (ed.) Nitrogen fixation, Volume 2 Rhizobium. Clarendon Press, Oxford.
120 .Long, S.R., Buikema, W.J. and Ausubel, F.M. (1982). Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of *nod*- mutants. Nature *298*, p.485.
121. Lopez, M.F. and Signer, E.R. (1987). Degradative enzymes in *Rhizobium meliloti*. p. 185. In D.P.S.Verma and N.Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
122. Maier, R.J. and Graham, L. (1988). Molybdate transport by *Bradyrhizobium* bacteroids. J.Bacteriol.*170*,(12), p. 5613.
123. Maier, R.J., Graham, L, Keefe, R.G., Pihl, T. and Smith, E. (1987). *Bradyrhizobium japonicum* mutants defective in nitrogen fixation and molybdenum metabolism. J.Bacteriol. *169*,(6), p. 2548.
124. Martin, G.B., Chapman, K.A. and Chelm, B.K. (1988). Role of the *Bradyrhizobium ntr*C gene product in differential regulation of the glutamine synthetase II gene (*gln*II). J.Bacteriol. *170*, (12), p. 5452.
125. Martinez, E., Palacios, R, and Sanchez, F. (1987). Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. J. Bacteriol. *169*, (6), p.2828.
126. Mellor, R.B., Mörschell, E. and Werner, D. (1983). Legume root response to symbiotic infection. Enzymes of the peribacteroid space. Z. Naturforsch. *39*c, p.123.
127. Mellor, R.B., Thierfelder, H., Pausch, G. and Werner, D. (1986). The occurrence of choline kinase II in the cytoplasm of soybean root nodules infected with various strains of *Bradyrhizobium japonicum*. J.Plant Physiol. *128*, p. 169.
128. Merrick, M.J. (1988). Organization and regulation of nitrogen fixation genes in *Klebsiella* and *Azotobacter*. p.293. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart.
129. Merrick, M and Dixon, R. (1984). Why don't plants fix nitrogen? Trends Biotechnol.2, (6), 162.
130. Morgan, T.V., Lundell, J. and Burgess, B.K. (1988). *Azotobacter vinelandii* ferredoxin I: cloning, sequencing and mutant analysis. J.Biol.Chem. *263*, (3), p. 1370.
131. Morrison, N. and Verma, D.P.S. (1987). A block in the endocytosis of *Rhizobium* allows cellular differentiation in nodules but affects expression of some peribacteroid membrane nodulins. Plant Mol. Biol. *9*, p.185.
132. Murphy, P.J., Heycke, N., Banfalfi, Z., Tate, M.E., de Bruijn, F., Kondorosi, A., Tempe, J. and Schell, J. (1987). Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the sym plasmid. Proc. Natl. Acad. Sci. USA *84*, p.493.
133. Murphy, P., Heycke, N., Trenz, S.P., Ratet, P., de Bruijn, F.J. and Schell, J.

(1988). Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. Proc. Natl. Acad. Sci. USA *85*, p.9133.

134. Nap, J-P. (1987). Nodulins in root nodule development. Ph.D. thesis, Agricultural University, Wageningen, The Netherlands.
135. Newcomb, W. (1981). Nodule morphogenesis and differentiation, p.247 In Giles, K.L. and Atherley, A.G. (eds.) Biology of *Rhizobiaceae*. Int. Rev. Cytol. Suppl. 13 Academic Press, New York.
136. Newcomb, W. and McIntyre, L. (1981). Development of root nodules of mung bean (*Vigna radiata*): a reinvestigation of endocytosis. Can.J.Bot. *59*, p. 2478.
137. Nieuwkoop, A.J., Banfalvi, Z., Deshmane, N., Gerhold, D., Schell, M.G., Sirtokin, K.M. and Stacy, G. (1987). A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*. J. Bacteriol. *169*, (6), p.2631.
138. O'Brian, M.R., Kirshbom, P.M. and Maier, R.J. (1987). Bacterial heme synthesis is required for expression of the leghemoglobin holoprotein but not the apoprotein in soybean root nodules. Proc.Natl.Acad.Sci. USA *84*, p. 8390.
139. O'Hara, G.W., Riley, I.T., Glenn, A.R. and Dilworth, M.J. (1985). The ammonium permease of *Rhizobium leguminosarum* MNF3841. J.Gen.Microbiol. *131*, p. 757.
140. Pargent, W. and Kleiner, D. (1985). Characteristics and regulation of ammonium (methylammonium) transport in *Rhizobium meliloti*. FEMS Microbiol. Lett. *30*, p. 257.
141. Pate, J.S. and Atkins, C.A. (1983). Nitrogen uptake, transport and utilization. p.245. In Broughton, W.J. (ed.) Nitrogen fixation volume 3: Legumes. Clarendon Press, Oxford, U.K.
142. Patel, U., Baxi, M.D. and Modi, V.V. (1988). Evidence for the involvement of iron siderophore in the transport of molybdenum in cowpea *Rhizobium*. Curr.Microbiol. *17*, (3), p. 179.
143. Paul, W. and Merrick, M. (1988). Analysis of the *nif*W,Z,M genes of *Klebsiella pneumoniae*. p.315. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.
144. Peters, N.K., Frost, J.W. and Long, S.R. (1986). A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science *233*, p.977.
145. Peters, K.N. and Long, S.R. (1988). Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiol. *88*, p.396.
146. Price, G.D., Day, D.A. and Gresshoff, P.M. (1987). Rapid isolation of intact peribacteroid envelopes from soybean nodules and demonstration of selective permeability to metabolites. J. Plant Physiol. *130*, p.157.
147. Pühler, A., Aguilar, M.O., Hynes, P., Klipp, W., Priefer, U., Simon, R. and Weber, G. (1984). Advances in the genetics of free-living and symbiotic nitrogen fixation bacteria, p.609. In Veeger, C. and Newton, W.E. (eds.), Advances in nitrogen fixation research. Nijhoff-Junk/ Pudoc, The Hague.
148. Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. (1986). Flavones induce expression of nodulation genes in *Rhizobium*. Nature *323*, p.632.
149. Renalier, M.-H., Batut, J., Ghai, J., Terzaghi, B., Gherardi, M., David, D., Garnerone, A.-M., Vasse, J., Truchet, G., Huguet, T. and Boistard, P. (1987). A new symbiotic cluster on the pSym megaplasmid of *Rhizobium meliloti* 2011 carries a functional *fix* gene repeat and a *nod* locus. J. Bacteriol. *169*, (5), p. 2231.
150. Robertson, J.G. and Lyttleton, P. (1984). Division of peribacteroid membranes in root nodules of white clover. J. Cell Sci. *69*, p.147.
151. Robertson, J.G., Warburton, M.P., Lyttleton, P., Fordyce, A.M. and Bullivant, S. (1978). Membranes in lupin root nodules. II. Preparation and properties of peribacteroid membranes and bacteroid envelope inner membranes from developing lupin nodules. J. Cell Sci. *30*, p.151.
152. Roelvink, P. and Van den Bos. R.C. (1989). Regulation of nitrogen fixation in diazotrophs: The regulatory *nif*A gene and its characteristics. Act. Bot. Neerl., in press.
153. Ronson, C.W. (1988). Genetic regulation of C_4-dicarboxylate transport in *Rhizobia*,

p. 547. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.

154. Ronson, C.W., Astwood, P.M., Nixon, T.B. and Ausubel. F.M. (1987). Deduced products of C_4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res. *15*, (19), p.7921.

155. Ronson, C.W., Lyttleton, P. and Robertson, J.G. (1981). C_4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. Proc.Natl.Acad.USA *78*, (7), p. 4284.

156. Ronson, C.W., Nixon, T.B., Albright, L.M. and Ausubel. F.M. (1987). *Rhizobium meliloti ntr*A (*rpo*N) gene is required for diverse metabolic functions. J. Bacteriol. *169*, (6), p. 2424.

157. Ronson, C.W., Nixon, T.B. and Ausubel. F.M. (1987). Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell *49*, p. 579.

158. Ronson, C.W. and Primrose, S.B. (1979). Carbohydrate metabolism in *Rhizobium trifolii*: identification and symbiotic properties of mutants. J.Gen.Microbiol. *112*, p. 77.

159. Ruvkun, G.B. and Ausubel, F.M. (1980). Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. USA *77*, (1), p.191.

160. Saroso, S., Dilworth, M.J. and Glenn, A.R. (1986). The use of activities of carbon catabolic enzymes as a probe for the carbon nutrition of snakebean nodule bacteroids. J.Gen.Microbiol. *132*, (2), p. 243.

161. Schetgens, T.M.P. Bakkeren, G., Van Dun, C., Hontelez, J.G.J., Van den Bos, R.C. and Van Kammen. A. (1984). Molecular cloning and functional characterization of *Rhizobium leguminosarum* PRE structural *nif*-genes by site-directed transposon mutagenesis and expression in *Eschericha coli* minicells. J. Mol. Appl. Genet. *2*, p.406.

162. Schetgens, T.M.P., Hontelez, J.G.J., Van den Bos, R.C. and Van Kammmen, A. (1985). Identification and phenotypical characterization of a cluster of *fix* genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE. Mol. Gen. Genet. *200*, p.368.

163. Schofield, P.R., Ridge, R.W., Rolfe, B.G., Shine, J. and Watson, J.M. (1984). Host-specific nodulation is encoded on a 14 kb DNA fragment in *Rhizobium trifolii*. Plant Molec. Biol. *3*, p.3.

164. Sengupta-Gopalan, C. and Pitas, J.W. (1986). Expression of nodule-specific glutamine synthetase genes during nodule developement in soybeans. Plant. Mol. Biol. *7*, (3), 189.

165. Smith, B.E., Buck, M., Eady, E.E., Lowe, D.J., Thorneley, R.N.F., Ashby, G., Deistung, J., Eldrich, M., Fisher, K., Gormal, C., Ioannidis, I., Kent, H., Arber, J., Flood, A., Garner, C.D., Hasnain, S. and Miller, R. (1988). Recent studies on the structure and function of molybdenum nitrogenase, p. 91. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.

166. Sprent, J.I. (1986). Benefits of *Rhizobium* to agriculture. Trends Biotechnol. *4*, (5), p.124.

167. Stacey, G. and Upchurch, R.C. (1984). *Rhizobium* inoculation of legumes. Trends Biotechnol.*2*, (3), 65.

168. Stam, H., van Verseveld, H.W. and Stouthamer, A.H. (1983). Derepression of nitrogenase in chemostat cultures of the fast growing *Rhizobium leguminosarum*. Arch. Microbiol. *135*, p.199.

169. Stanley, J., Dowling, D.N. and Broughton, W.J. (1988). Cloning of *hem*A from *Rhizobium* sp. NGR234 and symbiotic phenotype of a gene-directed mutant in diverse legume genera. Mol.Gen.Genet. *215*, p. 32.

170. Stowers, M.D. (1985). Carbon metabolism in *Rhizobium* species. Annu.Rev.Microbiol. *39*, p. 89.

171. Sutton, W.D. (1983). Nodule development and senescence. p.144. In Broughton, W.J. (ed.) Nitrogen fixation volume 3: legumes. Clarendon Press, Oxford, U.K.

172. Szeto, W.W., Nixon, T.B., Ronson, C.W. and Ausubel, F.M. (1987). Identification and characterization of the *Rhizobium meliloti ntr*C gene: *R.meliloti* has separate regulatory pathways for activation of nitrogen fixation genes in free-living and symbiotic cells. J. Bacteriol. *169*, (4), p.1423.

173. Szeto, W.W., Zimmerman, .L., Sundaresan, V. and Ausbel, F.M. (1984). A *Rhizobium meliloti* symbiotic regulatory gene. Cell *36*, p.1035.

174. Thöny, B., Fisher, H.-M., Anthamatten, D., Bruderer, T. and Hennecke, H. (1987). The symbiotic nitrogen fixation regulatory operon (*fix*R*nif*A) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nif*A-independent type of activation. Nucleic Acids Res. *15*, (20), p. 8479.

175. Tingey, S.V. Walker, E.L. and Coruzzi, G.M. (1987). Glutamine synthetase genes of pea encode distinct polypeptides which are differentially expressed in leave, roots and nodules. EMBO J. *6*, (1), p. 1.

176. Udvardi, M.K., Price, G.D., Gresshoff, P.M. and Day, D.A. (1988). A dicarboxylate transporter on the peribacteroid membrane of soybean nodules. FEBS Lett. *231*, (1), p. 36.

177. Van den Bos, R.C., Bisseling, T. and Van Kammen, A. (1978). Analysis of DNA content, nitrogenase activity and *in vivo* protein synthesis of *Rhizobium leguminosarum* bacteroids on sucrose gradients. J. Gen. Microbiol. *109*, p.131.

178. Van Kammen, A. (1984). Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Mol. Biol. Rep. *2*, p. 43.

179. Verma, D.P.S., Kazazian, V., Zogbi, V. and Bal, A.K. (1978). Isolation and characterization of the membrane envelope enclosing the bacteroids in soybean root nodules. J.Cell Biol. *78*, p. 919.

180. Verma, D.P.S., Zogbi, V. and Bal, A.K. (1978). The cooperative action of plant and *Rhizobium* to dissolve the host cell wall during developement of root nodule symbiosis. Plant Sci.Lett. *13*, p. 137.

181 Virts, E.L., Stanfield, S.W., Helinsky, D.R. and Ditta, G. (1988). Common regulatory elements control symbiotic and microaerobic induction of *nif*A in *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA *85*, p.3062.

182. Watson, R.J., Chan, Y.-K., Wheatcroft, R., Yang, A.-F. and Han, S. (1988). *Rhizobium meliloti* genes required for C_4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. J. Bacteriol. *170*, (2), p.927.

183. Werner, D., Mellor, R.B., Hahn, M.G. and Grisebach, H. (1985). Soybean root response to symbiotic infection. Glyceollin I accumulation in an effective type of soybean nodule with an early loss of the peribacteroid membrane. Z. Naturforsch. *40c*, p.179.

184. Werner, D., Mörschel, E., Garbers, C., Bassarab, S. and Mellor, R. (1988). Particle density and protein composition of the peribacteroid membrane from soybean root nodules is affected by mutation in the microsymbiont *Bradyrhizobium japonicum*. Planta *174*, p.263.

Chapter 2

Physical and genetic mappig of parts of the *Rhizobium leguminosarum* PRE *sym* plasmid and identification of reiterated *nif*- and *fix*-gene sequences.

CHAPTER 2: Physical and genetic mapping of parts of the *Rhizobium leguminosarum* PRE *sym* plasmid and identification of reiterated *nif*- and *fix*-gene sequences

2.1. Summary

A cosmid library, containing 40-50 kb *R.leguminosarum sym* plasmid DNA fragments, was constructed and used to produce two extended physical maps of *sym* plasmid regions known to contain genes involved in symbiotic nitrogen fixation. One characterized region is located around the *nif*HDK operon and is about 140 kb in size. The other mapped region, containing amongst others the *nif*A gene, the *fix*ABC operon and the *nod* genes, spans over 105 kb.
Within the mapped regions sequences occur with a strong homology to respectively parts of *nif*H, *fix*A and *fix*W. The nucleotide sequence of the fragment of the *nif*A region homologous to *nif*H is presented. The distance between *nif*A and the *nif*HDK operon on the *sym* plasmid could not be determined, but is between 60 and 175 kb.

2.2. Introduction

In previous studies, Krol *et al.* (10) showed that genes located on the 350 kb *R.leguminosarum* PRE *sym* plasmid are specifically expressed during symbiotic nitrogen fixation. These genes are mainly clustered in two separate parts of the plasmid. One region, which will be referred to as the "*nif*HDK region", contains amongst others the structural nitrogenase genes *nif*H, *nif*D, *nif*K (19), and *nif*E (8). The second region, called the "*nif*A region", contains the regulatory *nif*A gene (20), the *fix*ABC operon (8), *fix*W (this thesis) and the nodulation (*nod*) genes (18). Although physical maps of both regions have been constructed each spanning over 50 kb of DNA (18), the relative positions of the two regions on the *sym* plasmid could not be determined.
To further study the organization of the *R.leguminosarum* PRE *sym* plasmid, a cosmid library was constructed from purified *sym* plasmid DNA. The library was used to extend the existing physical maps of the *nif*HDK region and the *nif*A region, and to investigate the organization of *nif* and *fix* genes on the *sym* plasmid. Upon screening this library with various *nif* and *fix* genes as probes, several reiterated sequences were identified. One of these is located upstream of *nif*A and shows a high degree of homology with part of *nif*H. The other identified sequences will be discussed in detail in chapter 3.

2.3. **Materials and Methods**

2.3.1. Bacterial Strains and Plasmids

Strains of *E.coli* and *R.leguminosarum* and cloning vectors are listed in table 1. All *E.coli* strains except DH5αF' were grown at 37°C in NZY medium (10 gr. NZ-amine, 5 gr. yeast extract, 5 gr. NaCl, 2 gr. $MgSO_4$/l), if necessary supplied with antibiotics at the following concentrations (μg/ml): ampicillin 100, chloramphenicol 25 and tetracycline 10. *E.coli* DH5αF' was grown in Brain Heart Infusion medium (Difco). *R.leguminosarum* PRE was grown at 29°C in TY medium (5 gr. tryptone, 3 gr. yeast extract, 1.3 gr. $CaCl_2$/l) supplemented with 125 μg/ml streptomycin.

2.3.2. Construction of a cosmid library

For the construction of a cosmid library, *sym* plasmid DNA was isolated according to Krol *et al.* (10) and partially digested with 0.02 units Sau 3A per μg of DNA for 60 min. The digestion was stopped by two extractions with phenol and one with diethyl ether. After ethanol precipitation the DNA (12.5 μg) was dissolved in 0.3 ml TE (10 mM Tris.HCl, 1 mM EDTA, pH 8.0) and size fractionated by electrophoresis on a 0.4 % preparative agarose gel at 0.5 V/cm for 72 h. Restriction fragments of 40-50 kb were isolated from the gel by electro-elution in a dialysis bag as described by Maniatis *et al.* (13). One hundred nanograms of these fragments were mixed with 250 ng of the pJB8 HindIII-BamHI-arm and 125 ng of the pJB8 SalI-BamHI-arm, prepared according to Ish-Horowicz and Burk (9). Ligation of the DNA fragments was carried out for 18 h at 11°C, followed by 3 h at 15°C. Packaging of the cosmids into phage particles and transfection of *E.coli* 1046 was carried out according to Maniatis *et al.* (13). A typical packaging experiment showed an efficiency of approx. 1.2×10^4 cfu/μg DNA. About 5×10^3 transfected *E.coli* 1046 were spread out on a 13 cm HATF filter (Millipore) layered on a NZY-bacto agar plate containing ampicillin, and 1 mm colonies were grown at 30°C for 16 h.

For storage, the HATF filter was transferred to a NZY bacto agar plate containing 15 % (v/v) glycerol and ampicillin, and incubated for 30 min at 37°C. Subsequently a second HATF filter, which had been moistened on a NZY plate containing 15 % (v/v) glycerol, was layered on top of the filter containing the colonies and the two filters were pressed firmly together.

The sandwiched filters were placed in the middle of a stack of six Whatmann 3MM filters, one of which was moistened with water, and sealed airtight in a plastic bag. The library was then stored at -80°C.

Table 1.

Designation	Relevantcharacter/genotype	Reference/Source
Bacterial strains		
R.leguminosarum PRE	Str^r	11
E.coli 1046	*RecA*$^-$,*supE*,*supF*,*hsdS*$^-$,*met*$^-$	3
DH1	F$^-$, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (rk$^-$,mk$^+$), *supE44*,λ^-	12
DH5αF'	Host for pTZ18 (pTZ19); production of single stranded DNA for sequencing	BRL,Gaithersburg, USA
BHB 2688	A gamma lysogen used to prepare packaging extracts	7
BHB 2690	A gamma lysogen used to prepare packaging extracts	7
Bacteriophages		
M13K07	Sequencing helper phage	BRL, Gaithersburg, USA
Cloning vectors		
pJB8	Amp^r, cosmid vector	9
pBR322	Amp^r, Tet^r	2
pACYC184	Tet^r, Cm^r	4
pTZ18R	Amp^r, sequencing vector	Pharmacia, Uppsala Sweden
pTZ19R	Amp^r, sequencing vector	"
pGB25	*R.leguminosarum nif*HDK	18
pRleH12	*R.leguminosarum nif*A	18
pRmR3	*R.meliloti fix*ABC	17

2.3.3. Replication and screening of the cosmid library

The sealed library was defrosted for one hour at room temperature. After opening the plastic bag the mother filter and the first replica filter were separated and placed on NZY-ampicillin plates. Further replicates were made by sandwiching the mother filter and a new HATF filter

by pressing them firmly together between several layers of Whatmann 3MM paper and two glass plates. The filters then were separated and put back on a NZY-ampicillin plate. The replica filters where incubated for 8-10 h at 37°C and then placed on Whatmann 3MM filters soaked with 0.4 N NaOH for 4 min to cause lysis of the bacteria. Subsequently, the filters were placed on Whatman 3MM filters soaked with 1 M Tris.HCl pH 7.0 for 4 min, transferred to the liquid surface of a tray containing 0.5 M Tris.HCl, 1,5 M NaCl, pH 7.0 and gently submerged by shaking the tray for 10 min. The replica filters then were washed for 2 min in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1 % (w/v) SDS. Residual bacterial debris was removed by gently wiping the filters with a piece of tissue paper. Finally, the replicas were rinsed for 2 min in 0.3 x SSC and baked for 1 hour at 80°C. Colony filter hybridization was carried out as described by Maniatis *et al.* (13).

2.3.4. DNA methodology

All further DNA methodology, like Southern blotting, labeling of DNA fragments, DNA hybridization and sequence analysis, was as described in chapter 3.

2.4. **Results and Discussion**

2.4.1. Mapping of the *nif*HDK region

To identify cosmids containing the *R.leguminosarum* PRE *nif*HDK operon, the library was screened by colony filter hybridization using as a probe the 8.5 kb XhoI insert from plasmid pGB25 (see fig. 1), encoding the *R.leguminosarum* structural nitrogenase genes. About 150 out of 5,000 colonies present in the library showed a positive signal. Ten cosmids (designated pKL) were chosen for further analyses. By restriction enzyme mapping of the cosmids and combination of the deduced restriction fragment order with an existing physical map constructed by Schetgens (18), the pKL cosmids were arranged as shown in Fig. 1. These cosmids span the entire region described previously (18) containing the *nif*HDK operon, *nif*E and the *fix* region downstream of *nif*E were several Tn*5* insertions conferring a Fix^- phenotype were mapped.

The physical map was extended by cosmid-hopping; restriction fragments located near the borders of the cosmid inserts were subcloned in pACYC184 or pBR322 and used to rescreen the library. Newly isolated cosmids were analyzed by restriction enzyme mapping. By comparing the restriction fragment patterns of overlapping sets of cosmids, terminally located insert fragments could be identified, without the need to construct extended physical maps of these cosmids.

By a procedure of repeated subcloning terminal insert fragments and screening of the library with these fragments as probes, a 140 kb *sym* plasmid region was characterized (Fig. 1). It turned out to be impossible to extend the physical map beyond the 7.4 kb BamHI fragment in cosmid W21 (Fig. 1). This cosmid is highly unstable as different isolations showed that large parts of the insert DNA were deleted during bacterial growth. In addition, all cosmids picked up with the 7.4 kb BamHI fragment as a probe contain inserts having this fragment as the right-hand border. Apparently, cosmid W21 contains a *R.leguminosarum* DNA fragment which cannot be stably maintained in *E.coli*. Also screening of genomic *R.leguminosarum* DNA libraries in phage lambda EMBL3 (described by Schetgens, 18) never yielded DNA fragments extending beyond this 7.4 kb BamHI fragment (results not shown).

To establish the distance between the *nif*HDK operon and the *nif*A region, all cosmids of the *nif*HDK region were analyzed by Southern hybridization using the *R.leguminosarum nif*A gene (pRleH12, Fig.2) or the *R.meliloti fix*A gene (a 1.2 kb EcoRI-SalI fragment of pRmR3, see Earl *et al.* (6)) as a probe (results not shown). Although a fragment hybridizing with the

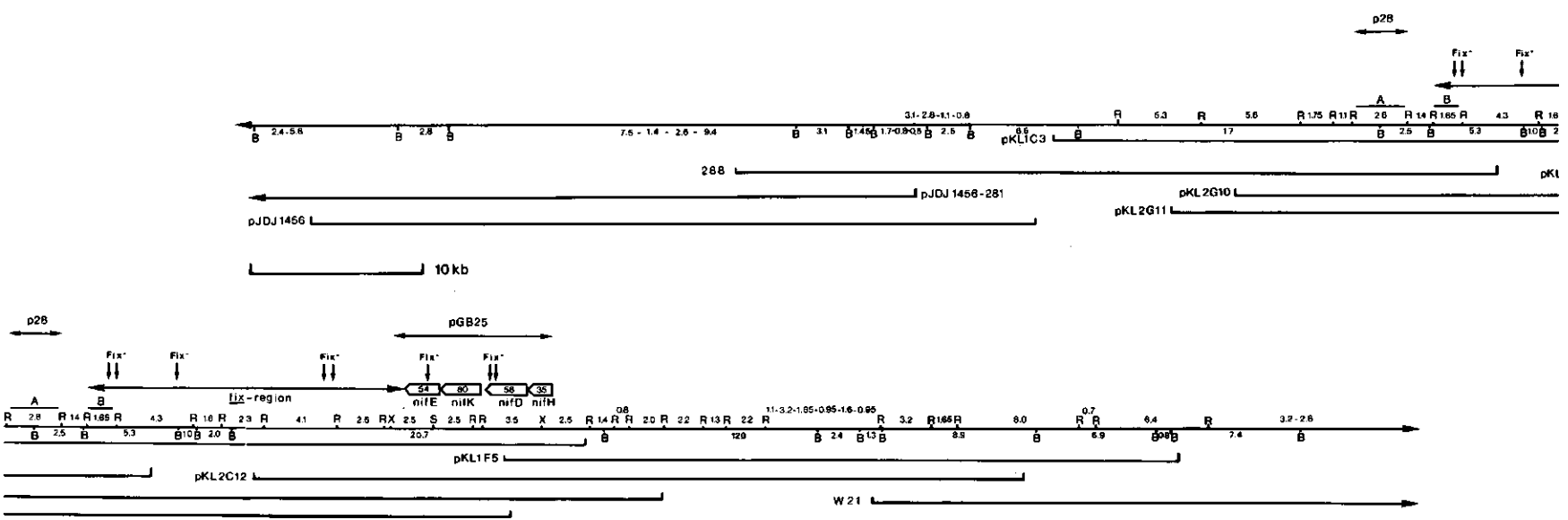

Fig.1 COSMID CLONES SPANNING THE nif-HDK REGION

Figure 1. Physical map of the 140 kb *R.leguminosarum sym* plasmid region containing the *nif*HDK operon.

The map is presented as two separate parts with approx. 10 kb overlap. Cosmids are indicated by horizontal lines, Tn*5* insertions by vertical arrows and by phenotypes of the corresponding mutants. All EcoRI(R) and BamHI(B) restriction sites are indicated except for regions where the exact order of the restriction fragments was not determined. In such cases, only the restriction fragment length is shown. Only the relevant SalI(S) and XhoI(X) sites (see text) are indicated.

*fix*A probe is present in some of the cosmids (see below), comparison of an existing physical map of the *nif*A region (18) with the restriction fragment pattern of the hybridizing cosmids showed that neither *nif*A nor *fix*A are located within the mapped 140 kb *sym* plasmid region.

2.4.2. Mapping the *nif*A region

Cosmids containing the *nif*A gene were isolated by screening the cosmid library with the 3.3 kb BamHI insert of plasmid pRleH12 (Fig. 2), which contains the *R.leguminosarum* PRE *nif*A gene and the 3'-coding region of *fix*C (18). About 50 hybridizing cosmids were identified, showing that this region is slightly underrepresented in the library compared to *nif*HDK. These cosmids, designated pKLA, cover the *sym* plasmid region containing *nif*A, *nif*B, the *fix*ABC operon, *fix*W (see chapter 3) and the region downstream of *nif*B were Tn*5*-insertions were mapped (18) conferring a Nod^- phenotype (Fig. 2).
By the method of cosmid-hopping described in the previous paragraph, 105 kb of *sym* plasmid DNA around *nif*A was physically mapped (Fig. 2). Also here, it showed to be impossible to extend the map beyond the right-hand border fragment shown in Fig. 2. Neither cosmids nor lambda EMBL3 phages, which contained DNA fragments located more than approx. 10 kb upstream of the *fix*ABC cluster, could be isolated.
To determine whether the 105 kb *sym* plasmid region containing *nif*A overlaps with the 140 kb region containing *nif*HDK, Southern blots containing EcoRI digested cosmid DNA spanning the 105 kb region were hybridized with various ^{32}P labeled cosmids derived from the 140 kb region (not shown). No evidence, however, could be found that these two *sym* plasmid regions are contiguous. Therefore, the distance between the *nif*HDK operon and *nif*A is still unknown. From the newly constructed physical maps, which together cover more than 70 % of the PRE *sym* plasmid, it can be deduced that this distance has a minimum value of approx. 60 kb. This would be the distance when the right-hand part of the mapped *nif*HDK region is located directly adjacent to the right-hand part of the *nif*A region. Since both characterized *sym* plasmid areas are bordered on the right-hand side by a region, which apparently can not be maintained in *E.coli*, it is tempting to speculate that this very area forms the connection between these two regions. Any direct evidence for this assumption however is lacking at the moment. Because of the reported size of the *R.leguminosarum sym* plasmid of approx. 350 kb (10), the distance between *nif*A and *nif*HDK will be at most 175 kb.
From these results it can be concluded that the organization of *nif* and *fix* genes on the *R.leguminosarum* PRE *sym* plasmid differs for example from that in *R.meliloti* 1021, were

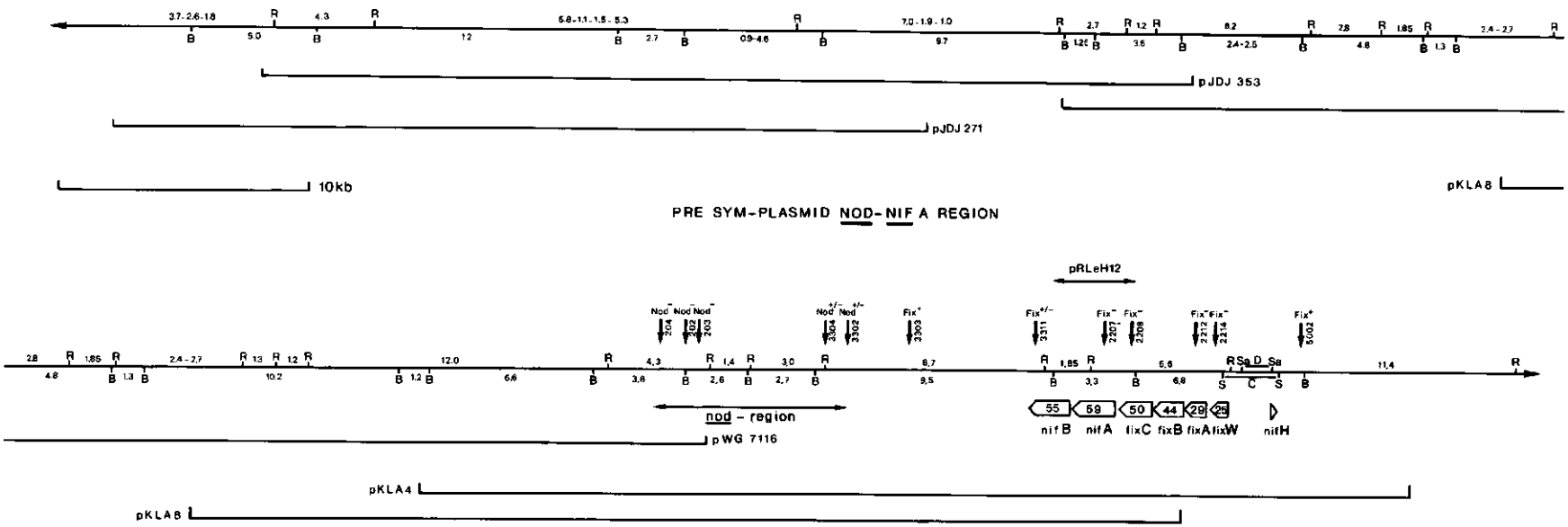

Figure 2. Physical map of the 105 kb *R.leguminosarum sym* plasmid region containing the *fix*ABC operon and the *nif*A gene.

The map is presented as two separate parts with approx. 5 kb overlap. Cosmids are indicated by horizontal lines, Tn*5* insertions by vertical arrows and by phenotypes of the corresponding mutants. All EcoRI (R) and BamHI (B) restriction sites are indicated except for regions where the exact order of the restriction fragments is not determined. In such cases, only the restriction fragment length is shown. Only the relevant SalI (S) and SacI (Sa) sites (see text) are indicated.

*nif*A is located 5.5 kb upstream from the *nif*HDK operon (21) and *R.leguminosarum* 248 (pRL1JI), where *nif*HDK is separated from *nif*A by a stretch of 27 kb of DNA (5).

2.4.3. Reiteration of part of the *nif*H gene

Screening of Southern blots containing EcoRI digested DNA derived from each of the cosmids of the 140 kb *nif*HDK region and the 105 kb *nif*A region with the *R.meliloti fix*A probe (the 1.2 kb EcoRI-SalI fragment) showed that besides the 5.6 kb EcoRI fragment present in cosmid pKLA4, encoding the *R.leguminosarum fix*ABC operon (Fig. 2 and 3b), two other fragments hybridize with this probe; a 11.4 kb EcoRI fragment directly adjacent to this 5.6 kb EcoRI fragment (Fig. 3b), and a 2.8 kb EcoRI fragment located downstream of *nif*HDK (fragment A, Fig. 3c) (results not shown). These fragments probably only contain partial or non-functional reiterations of *fix*A, since Schetgens *et al.* (20) showed that a single Tn*5* insertion in the *fix*A gene (Fig. 3b, Tn*5*-insertion 2212), located on the 5.6 kb EcoRI fragment, gives rise to a Fix$^-$ phenotype.
The *fix*A reiterations were further investigated on Southern blots containing EcoRI-digested genomic *R.leguminosarum* DNA which were hybridized with various DNA probes (Fig. 3a). As a control, EcoRI-digested genomic *R.meliloti* DNA was used. Hybridization of this control DNA with the *R.meliloti fix*A probe (the 1.2 kb EcoRI-SalI fragment) showed two hybridizing EcoRI fragments of 5.2 and 4.4 kb (Fig. 3a lane 1). These results are in agreement with data from Earl *et al.* (6), which show that the *R.meliloti fix*ABC operon is located on a 5.2 kb EcoRI restriction fragment, preceded by a partial *fix*A duplication present on a 4.4 EcoRI restriction fragment.
The hybridization pattern of *R.leguminosarum* DNA with the *R.meliloti fix*A probe (Fig. 3a lane 2) shows the 5.6 kb EcoRI fragment containing the *R.leguminosarum fix*ABC operon, and the two *fix*A reiterations located on the 11.4 kb EcoRI fragment in Fig. 3b and on the 2.8 kb EcoRI fragment (fragment A) in Fig. 3c. The 2.8 kb fragment was isolated and subcloned in plasmid p28. When this fragment was used to probe a blot containing EcoRI digested *R.leguminosarum* DNA (Fig. 3a, lane 4) again the 11.4 kb, the 5.6 kb and the 2.8 kb EcoRI fragments gave a signal. The hybridization of the *fix*A reiteration present on the 2.8 kb fragment with the 11.4 kb fragments appears to be much stronger than that with the 5.6 kb fragment, on which the *fix*A gene is located. This shows that the homology between the two *fix*A reiterations is greater than that of each of the two reiterations with the *fix*A gene. Hybridization of the *R.leguminosarum fix*A reiteration on the 2.8 kb fragment with EcoRI

digested *R.meliloti* DNA (Fig. 3a, lane 3) showed hybridization of the 5.2 kb fragment carrying *R.meliloti fix*A and the *R.meliloti fix*A reiteration on the 4.4 kb fragment.

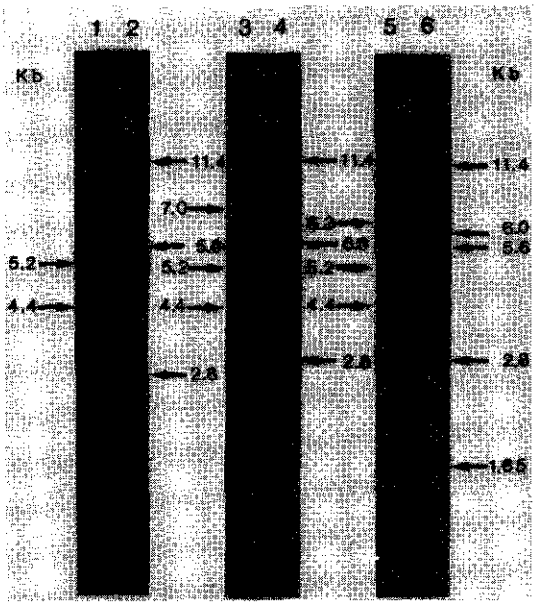

Figure 3A Hybridization of genomic *Rhizobium* DNA with *fix*A and *fix*A-homologous sequences.

Genomic DNA isolated from *R.meliloti* (lanes 1,3 and 5) and *R.leguminosarum* (lanes 2,4 and 6) was digested with EcoRI, separated by electrophoresis on a 1% agarose gel, transferred to a Gene Screen Plus filter and hybridized with ^{32}P-labeled DNA probes. The following probes were used: *R.meliloti fix*A (lanes 1 and 2,), the *R.leguminosarum fix* reiteration located on the 2.8 kb EcoRI fragment A (lanes 3 and 4) and the 1.85 kb SalI fragment C, containing the second *fix*A reiteration (lanes 5 and 6).
The resulting autoradiogram is shown.

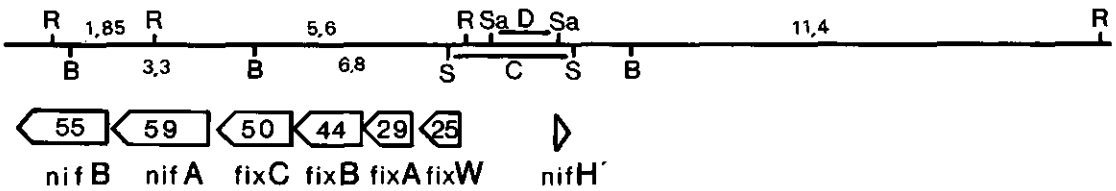

Figure 3B. Detailed physical map of the *R.leguminosarum nif*A/*fix*ABC region.

Abbreviations: R, EcoRI; B, BamHI; S, SalI; Sa, SacI. Only the relevant SalI and SacI fragments (see text) are drawn. For details, see text.

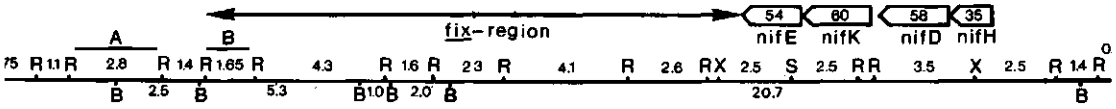

Figure 3C. Detailed physical map of the *R.leguminosarum nif*HDK region.

Abbreviations: R, EcoRI; B, BamHI; S, SalI; X, XhoI. Only the relevant SalI and XhoI fragments (see text) are drawn. For details, see text.

The *R.leguminosarum fix*A reiteration on the 11.4 kb EcoRI fragment was confined to a 1.85 kb SalI fragment (fragment C, Fig. 3b). When this fragment was used to probe a blot containing EcoRI digested *R.leguminosarum* total DNA, again the 5.6 kb *fix*A band and the two reiterations on the 2.8 kb and 11.4 kb EcoRI fragment were evident (Fig. 3a, lane 6). In addition, two hybridizing EcoRI fragments of 1.65 and 6.0 kb were observed (Fig. 3a, lane 6). The 1.65 kb fragment represents a duplication of part of *fix*W (see chapter 3), located downstream of *nif*HDK (fragment B, Fig. 3c) and the hybridization of this fragment with the 1.85 kb SalI fragment is caused by the presence of part of the *fix*W sequence within the probe. Hybridization of various cosmids with fragment C showed that the hybridizing 6.0 kb EcoRI fragment is identical to the 6.0 kb EcoRI fragment on which the genes *nif*H and *nif*D are located (Fig. 3c, results not shown).

Further analysis showed that the homology of fragment C with this 6.0 kb fragment is confined to a 1.2 kb SacI fragment present within fragment C, which is referred to as fragment D (see Fig. 3b). Nucleotide sequence analysis of fragment D showed a sequence on the right-hand border being 85 % homologous to the promoter region and the 5'-coding region of *nif*H (see Fig. 4).

```
NifH     1  GGGTGACAATCTATTTCGACATTGTCATCACCTTGTCGGTTACTTGACATAGCGTTTGTGTTCTCAATCCCCGTATTTTAC   80

NifH    81  GCCCACATAGCTGGAATCGCGGCGAATTATTGTTCGTCAGCTCAATCGGCCGCTTGGCACGAATCTTGAGAGCTATTGGAGA  160
NifH'       G CCACATA CTGC ATCGCAGC AAT AT GT CGTCATCTCAATCGGCTGCTTGGCACGAATCTTGAGAGCTATTGC GA

                                                                                  M  A  A
NifH   161  GGCAGCGGAACGGCCGCC GCATTCCGTTGCGGGT AACCAAATTGCTTCGAACACATGAAGGAACGCCAAGCATGGCAGCT  238
NifH'       GGCA CGGAACGGCCGCTTGCAT CCGTT CGGGACAACCGCATTGCTTCGAACACATGAAGGAACGCCAAGCATGGCAGCT
                                                                                  M  A  A

             L  R  Q  I  A  F  Y  G  K  G  G  I  G  K  S  T  T  S  Q  N  T  L  A  A  L  Y  D
NifH   239  CTGCGTCAGATCGCATTCTATGGAAAAGGCGGAATTGGCAAGTCCACTACGTCCCAAAACACGCTGGCCGCCCTTGTCGAC  318
NifH'       CTGCGTCAGATCGCATTCTATGGAAAGGGCGGAATTGGCAAGTCCACTACGTCCCAAAACACGCTAGCGGCCCTCGTGGACC
             L  R  Q  I  A  F  Y  G  K  G  G  I  G  K  S  T  T  S  Q  N  T  L  A  A  L  Y  D

NifH'       ATCACGTACCACGGATACCCATGATCATCCGAATTGGGGGATACGCCCAATGAGCTC
            H  H  V  P  R  I  P  M  I  I  R  I  G  G  Y  A  Q  *
```

Figure 4. Nucleotide sequence of *nif*H'.

The homology with the *nif*H sequence is indicated. The *ntr*A dependent promoter and upstream activator sequences are boxed, a potential ribosome binding site is underlined.

The *nif*H homologous sequence, designated *nif*H', contains an exact copy of the *ntr*A-dependent *nif*H promoter (15) (Fig. 4, boxed sequence), and the *nif*H ribosome binding site (underlined). Furthermore, the first 30 codons of *nif*H' show 87 % homology at the nucleotide level and a 100 % homology at the amino acid level with the first 30 codons of the *nif*H coding sequence. The coding region of *nif*H' continues for another 52 base pairs and theoretically encodes a polypeptide with a molecular weight of 5095 Dalton. The function of this hypothetical protein is unknown; a computer aided search in the NBRF protein library only revealed homology of the first 30 amino acids of the *nif*H' protein with the first 30 amino acids of *nif*H gene products from various nitrogen fixing organisms, and no homology of the remaining part of the polypeptide with any known protein. At the moment it is not known how far upstream the homology of the *nif*H' sequence with that of *nif*H extends, and whether or not the *nif*H upstream activator sequence, TGT-N_{10}-ACA, (Fig. 4, boxed) is present in the promoter region of *nif*H'.

Hybridization of *R.meliloti* DNA with fragment C shows the presence of the 5.2 and 4.4 kb EcoRI fragments, containing respectively *fix*A and the *fix*A reiteration, and an additional 6.3 kb EcoRI fragment (Fig. 3a lane 5). The origin of this latter hybridization signal is unknown, since *nif*H in *R.meliloti* is not located on a 6.3 kb EcoRI restriction fragment (16).

Only for *R.meliloti*, two reiterations of the *nif*H promoter region and parts of the 5'coding sequence have been described (1). One, located approx. 1 kb upstream of *nif*H, comprises the promoter region and the first 47 bp of the coding region. The reading frame continues for another 22 bp before a termination codon is reached. Although this DNA sequence is expressed during symbiosis, it does not encode a gene essential for symbiotic nitrogen fixation since Tn*5* mutants in this region exhibit a Fix^+ phenotype (17). The second *nif*H reiteration in *R.meliloti* was found within the first gene of the *mos* gene cluster (14). This gene is involved in the synthesis of an opine-like compound and encodes a polypeptide with a molecular mass of 11.0 kDa. The promoter region of this gene and the first 57 bp of the coding region are highly homologous to *R.meliloti nif*H . Also this gene was shown not to be essential in symbiotic nitrogen fixation.

It is not known whether the *nif*H reiteration identified in *R.leguminosarum* is actively transcribed during nitrogen fixation, although the presence of a *ntr*A dependent promoter upstream of *nif*H' strongly suggests that this will be the case. Tn*5* mutagenesis might show whether this sequence plays a role in symbiotic nitrogen fixation.

2.5. Acknowledgement

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2.6 Literature cited

1. Better, M., Lewis, B., Corbin, D., Ditta, G. and Helinski, D.R. (1983). Structural relationships among *Rhizobium meliloti* symbiotic promoters. Cell *35*, p.479.
2. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker. H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multiple cloning system. Gene 2, p.95.
3. Cami, B. and Kourilsky, P. (1978). Screening of cloned recombinant DNA in bacteria by in situ colony filter hybridization. Nucleic Acids Res. *5*, p.2381.
4. Chang, A.Y.C. and Cohen, S.N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. *134*, p.1141.
5. Downie, J.A., Ma, Q.-S., Knight, C.D., Hombrecher, G. and Johnston, A.W.B. (1983). Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and a *nif*A-like gene. EMBO J. 2, p.947.
6. Earl, C.D., Ronson, C.W. and Ausubel, F.M. (1987). Genetic and structural analysis of the *Rhizobium meliloti fix*A, *fix*B, *fix*C, and *fix*X genes. J. Bacteriol. *169*, (3), p.1127.
7. Hohn, B. and Murray, K. (1977). Packaging recombinant DNA molecules into bacteriophage particles in vitro. Proc. Natl. Acad. Sci. USA *74*, p.3259.
8. Hontelez, J.G.J., Klein Lankhorst, R.M., Jansma, J.-D., Jacobsen, E., Van den Bos, R.C. and van Kammen, A. (1987). Characterization of symbiotic genes and regulation of their expression in *Rhizobium leguminosarum* PRE. p.241. In D.P.S. Verma and N. Brisson (eds.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
9. Ish-Horowicz, D., and Burk, J.F. (1981). Rapid and efficient cosmid cloning. Nucleic Acids Res. *9* , (13), p.2989.
10. Krol. A.J.M., Hontelez, J.G.., Van den Bos, R.C. and van Kammen, A. (1982). Only one of the large plasmids in *Rhizobium leguminosarum* strain PRE is strongly expressed in the endosymbiotic state. J.Gen.Microbiol. *128*, p.1839.
11. Lie, T.A., Soe-Agnie, I.E., Muller, G.J.L. and Gökdan, D. (1979). Environmental control of symbiotic nitrogen fixation: limitation to and flexibility of the legume-Rhizobium system. In: Broughton W.J., John, C.K., Rajaro, J.C. and Lim, B. (eds.) Proc.Symp.Soil Microbiol. Plant Nutrition 1976. Kuala Lumpur: University of Malaya, p.194.
12. Low, B. (1968). Formation of merodiploids in matings with a class of Rec$^-$ recipient strains of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA *60*, p.160.
13. Maniatis, T., Fritsch, E. and Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, New York.
14. Murphy, P., Heycke, N., Trenz, S.P., Ratet, P., de Bruijn, F.J. and Shell, J. (1988). Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. Proc. Natl. Acad. Sci. USA *85*, p.9133.
15. Roelvink, P. (1989). Characterization of the *nif*A regulatory gene of *Rhizobium leguminosarum* PRE. PhD thesis, Agricultural University, Wageningen, The Netherlands.

16. Ruvkun, G.B. and Ausubel, F.M. (1980). Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. USA *77*, (1), p.191.
17. Ruvkun, G., Sundaresan, V. and Ausubel, F.M. (1982). Direct transposon Tn*5* mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell *29*, p.551.
18. Schetgens, T.M.P. (1986). Identification and characterization of symbiotic genes on the *Rhizobium leguminosarum* PRE *sym* plasmid. PhD-thesis, Agricultural University, Wageningen, The Netherlands.
19. Schetgens, T.M.P., Bakkeren, G., Van Dun C., Hontelez, J.G.J. Van den Bos, R.C. and Van Kammen, A. (1984). Molecular cloning and functional characterization of *Rhizobium leguminosarum* structural *nif* genes by site-directed transposon mutagenesis and expression in *Escherichia coli* minicells. J. Mol. Appl. Genet. 2, p.406.
20. Schetgens, T.M.P., Hontelez, J.G.J., Van den Bos, R.C. and Van Kammen, A. (1985). Identification and phenotypical characterization of a cluster of *fix* genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE. Mol. Gen. Genet. *200*, p.368.
21. Szeto, W.W., Zimmerman, J.L., Sundaresan, V., and Ausubel, F.M. (1984). A *Rhizobium meliloti* symbiotic regulatory gene. Cell *36*, p.1035.

Chapter 3

Characterization and nucleotide sequence of a novel gene *fix*W upstream of the *fix*ABC-operon in *Rhizobium leguminosarum*

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CHARACTERIZATION AND NUCLEOTIDE SEQUENCE OF A NOVEL GENE FIXW UPSTREAM OF THE *FIXABC*-OPERON IN *RHIZOBIUM LEGUMINOSARUM*

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Summary

On the *Rhizobium leguminosarum* PRE sym plasmid, *fixABC* and a novel gene *fixW* were identified upstream of the regulatory gene *nifA*. The molecular masses of FixABC, 29, 44 and 50 kdal respectively, were estimated by polyacrylamide gel electrophoresis and of FixW, 25 kdal, by PAGE and nucleotide sequencing. Hybridization studies using bacteroid mRNA as a probe showed that *fixABC* is one operon which can be transcribed independently of *fixW*. Nucleotide sequencing revealed that both *fixW* and *fixA* are preceded by a nif-consensus promoter. The *fixA* promoter partly overlaps the 3'-terminal coding region of *fixW*, indicating that readthrough from *fixW* into *fixA* is possible. Two open reading frames, ORF71 and ORF79, precede *fixW* and form one operon with *fixW*. ORF71 contains sequences homologous to the *fixA* promoter- and 5'-terminal coding region. One more duplication of *fixA* sequences was detected, also located within the sym plasmid *nif/fix* clusters. One duplication of *fixW* sequences was found. No *fixW* homologue could be found in other nitrogen fixing organisms except in a number of *R.leguminosarum* strains.

Key words: *Rhizobium leguminosarum* - nitrogen fixation - *nif/fix* genes - *E.coli* minicells - transcription regulation

Introduction

Nitrogen fixation in legume root nodules is the result of the concerted action of *Rhizobium* bacteria and leguminous plants, in which a number of specific genes both in the bacteria and in the host plants are involved. In fast-growing rhizobia essential symbiotic (*sym*) genes are located on large plasmids (Krol et al., 1980). Genes necessary for infection and nodule development are in the nodulation (*nod*) region; genes involved in the nitrogen fixation process are distinguished as *nif* and *fix* genes. The *Rhizobium nif* genes exhibit functional and structural homology to the *nif*- genes of *Klebsiella pneumoniae* (Dixon, 1984) and several have been characterized: the structural nitrogenase genes *nifHDK* (Ruvkun and Ausubel, 1980; Ausubel et al., 1982), the regulatory gene *nifA* (Szeto et al., 1984; Rossen et al., 1984; Fischer et al., 1985; Schetgens et al., 1985), *nifB* (Rossen et al., 1984), *nifE*, *nifN* (Ebeling et al., 1987; Aguilar et al., 1987) and *nifS* (Ebeling et al., 1987). Of the genes that have no known equivalent in *K.pneumoniae* (*fix* genes) the *fixABCX* genes seem to be the most common among nitrogen fixing organisms (Gubler and Hennecke, 1986). The *R.meliloti fixABCX* genes are located upstream of *nifA* and transcribed as one operon in the same orientation as *nifA* (Earl et al., 1987). An identical genetic organization has been found in *R.leguminosarum* (Grönger et al., 1987). Recently a number of novel open reading frames (ORF) in various organisms were reported to be located adjacent to *nifA* (*fixR*, Thöny et al., 1987), to *nifB* (*fixY*; Klipp et al., 1988; *frxA*., Ebeling et al., 1988) and to *fixABCX* (*nifO*, Kaminsky et al., 1988). All of these ORFs except *frxA* seem necessary for nitrogen fixation, and have in common with known *nif* and *fix* genes that their transcription is directed by a *nif*-consensus promoter resembling the sequence CTGG-N$_8$-TTGCA (Ausubel, 1984; Gussin et al, 1986) and by an upstream activator sequence (UAS) with the consensus sequence TGT-N$_{10}$-ACA (Buck et al., 1986).

Previously we reported on the identification and phenotypical characterization of two clusters of *nif* and *fix* genes in *R.leguminosarum* PRE. One contains the structural nitrogenase genes *nifHDK* (Schetgens et al., 1984) and *nifE* (Hontelez et al., 1987), the other bears the regulatory *nifA* gene and a homologue of *nifB* (Schetgens et al., 1985). The direction of transcription and coding properties of *nifA* were reported by Roelvink et al. (1988). In this paper we report on the further characterization of the *nifA*-upstream region where Tn*5* insertions resulted in Fix$^-$, NifHDK$^+$ phenotypes (Schetgens et al., 1985), and describe a novel gene *fixW*, that may form one operon with *fixABC*.

Materials and Methods

RNA isolation and labeling

Root nodules from fifty *Pisum sativum* plants were picked at 17 days after inoculation with *R.leguminosarum* PRE and immediately frozen in liquid nitrogen. Frozen nodules were ground in a 0.5 ml Eppendorf tube provided with a glass-wool plug and 1 ml icecold TE (10 mM TrisHCl, 1 mM EDTA, pH 8.0) was added. Bacteroids were collected in a 1.5 ml Eppendorf tube by centrifugation through a small hole pierced in the bottom of the 0.5 ml tube. The bacteroid pellet was resuspended in 500 µl icecold TE, SDS was added to a final concentration of 1% (w/v) and the lysate was extracted with phenol and chloroform (twice with each). After ethanol precipitation the pellet was dissolved in 100 µl TE and incubated for 30 min. with 10 µg/ml of RNAse-free DNAse (Boehringer, Mannheim, FRG) followed by phenol- chloroform extraction. After ethanol precipitation the RNA was dissolved in 10 mM phosphate buffer pH 7 and stored at -20°C. For hybridization experiments 0.2-0.5 µg of RNA was 5'-end labeled with ^{32}P using T4-polynucleotide kinase. Hybridization of Southern blots with labeled RNA was performed in 3 x SSC at 60°C (1 x SSC = 0.15 M NaCl, 0.015 M NaCitrate, pH 7).

DNA methodology

Isolation of total chromosomal and megaplasmid DNA from *R.leguminosarum* and isolation of plasmid DNA from *E.coli* have been described (Krol et al., 1982). The construction of a gene library in phage λEMBL3 was done according to Karn et al. (1980). Small scale phage DNA preparations were carried out according to Maniatis et al. (1982). Restriction endonuclease digestion, agarose gel electrophoresis, fragment elution, *E.coli* transformation and nick-translation were performed as described (Schetgens et al., 1985). For Southern blotting either nitrocellulose (Schleicher and Schüll, Dassel, FRG) was used and hybridization was as described (Schetgens et al., 1984) or Gene Screen Plus (New England Nuclear, Boston, USA) and hybridization was performed according to the manufacturers protocol.

Transposon mutagenesis

Transposon Tn*5*-mutagenesis was carried out as described (Ruvkun and Ausubel, 1981; Schetgens et al., 1985). Instead of the conjugative plasmid pRK290, the derivative pRK252 was used (Ditta et al., 1985).

Phenotypical characterization of R.leguminosarum mutants

Growth and inoculation of pea plants, measurement of acetylene reducing activity and analysis of bacteroid proteins by polyacrylamide gel electrophoresis (PAGE) have all been described (Schetgens et al., 1984).

Expression of R.leguminosarum genes and Tn5 derivatives in E.coli

For expression studies in minicells pACYC184 recombinant plasmids, carrying wild type or Tn5 mutated genes were transformed into *E.coli* DS410. Purification and ^{35}S-methionine labeling of minicells were performed essentially as described (Schetgens et al., 1984). Minor modifications were the following: sucrose gradient centrifugation was performed twice and the band of minicells was further purified by pelleting residual parental cells for 20 min. at 4,000 x g. The supernatant containing minicells was centrifuged at 10,000 x g for 5 min. and the cell pellet taken up in M9 incubation buffer. For SDS-PAGE 50,000 cpm of ^{35}S-methionine labeled proteins per lane were loaded (corresponding to 1 - 20 µl from 100 µl labeled minicell portions).

Expression of DNA inserts from a phage T7-promoter in *E.coli* was performed after cloning *Rhizobium* DNA fragments in vector pT7-5 or pT7-6 and transformation into *E.coli* K38, harbouring plasmid pGP1-2 encoding T7-polymerase. Plasmids and growth procedures for these expression experiments were as described by Tabor and Richardson (1985).

Sequencing techniques and S1-nuclease mapping

DNA fragments were digested with restriction enzymes and cloned in vectors pTZ18 and pTZ19 (Pharmacia, Uppsala, Sweden) and transformed into *E.coli* DH5 αF' (Bethesda Research Laboratories, Gaithersburg, USA). Single stranded DNA was isolated according to the Pharmacia protocol, and nucleotide sequences determined using the dideoxy-chain termination method (Sanger et al., 1977). In all sequencing reactions dGTP was replaced by 7-Deaza-dGTP (Boehringer Mannheim, FRG). Sequence data were analyzed on a Vax-computer using the ANALYSEQ and Database programs (Staden, 1980; Staden, 1984).

S1-nuclease experiments were performed as described by Berk and Sharp (1977), using PRE bacteroid RNA isolated from 17 days old nodules.

Microbiological techniques

The strains of bacteria, phages and plasmids used are listed in Table 1. Media, concentrations of antibiotics and growth conditions were as described (Schetgens et al., 1984).

TABLE 1: Bacterial strains, plasmids, bacteriophage.

Designation	Relevant character/genotype	Reference/source
- Bacterial strains		
R.leguminosarum PRE	wild type	Lie *et al* (1979)
R.leguminosarum PRE2107	$NifA^-$	Schetgens *et al* (1985)
R.leguminosarum PRE2112	$FixA^-$	Schetgens *et al* (1985)
R.leguminosarum PRE2114	$FixW^-$	Schetgens *et al* (1985)
R.leguminosarum TOM	wild type	Winarno and Lie (1979)
R.leguminosarum 248	Cm^r	Josey *et al* (1979)
R.leguminosarum 128c53	wild type	Brewin *et al* (1980)
R.leguminosarum Him	wild type	Winarno and Lie (1979)
R.trifolii ANU 843		Rolfe *et al* (1980)
R.meliloti 1021	Str^r	Meade *et al* (1982)
B.japonicum 110		Hennecke and Mielenz (1981)
A.caulinodans ORS571		Dreyfus and Dommergues (1981)
*A.vinelandii*ATCCOP	wild type	Haaker *et al* (1974)
E.coli DS410	minicell producer	Reeve (1979)
E.coli DH5αF'	host for pTZ18(pTZ19). Production of single stranded DNA for sequencing	BRL - USA
- Bacteriophage		
λEMBL3	derived from λ1059	Karn *et al* (1980)
- Plasmids		
pACYC184	Cm^rTc^r	Chang and Cohen (1978)
pRK252	Tc^r derived from pRK290	Ditta *et al* (1985)
pRmR3	*R.meliloti fixABC*	Ruvkun *et al* (1982)
pT7-5 (pT7-6)	T7-promoter	Tabor and Richardson (1985)
pGP1-2	T7-polymerase	Tabor and Richardson (1985)
pTZ18 (pTZ19)	sequencing vector	Pharmacia, Sweden

Results

Expression of pRleH21 in E.coli

Fragments of the *R.leguminosarum* PRE sym plasmid were cloned in phage λEMBL-3. Recombinant phages harbouring genes possibly involved in nitrogen fixation were selected by hybridization with bacteroid RNA isolated from 17 days old *Pisum sativum* nodules. Overlapping phage-inserts constituted two separate sym-plasmid regions, on which *nif/fix* and *nod* genes were identified by site-directed Tn*5*-mutagenesis and hybridization with heterologous DNA-probes (Fig. 1).

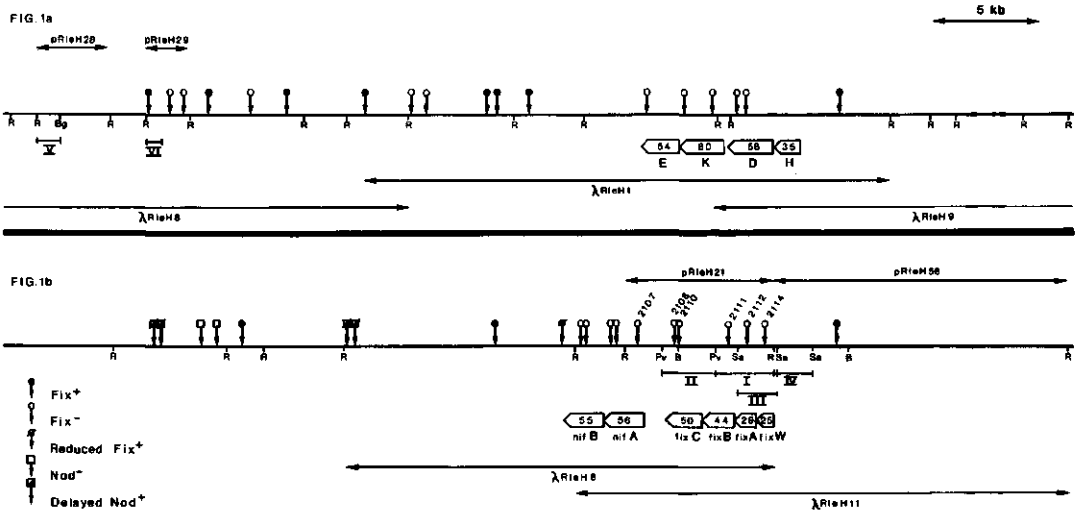

Figure 1. Physical map of *nif* and *fix* regions on the *R.leguminosarum* PRE sym plasmid.

The *nifHDK* region (1a) and *nifA* region (1b) are shown separately. Restriction endonuclease sites are abbreviated as R (*Eco*RI), B (*Bam*HI), Bg (*Bgl*II), Pv (*Pvu*I), S (*Sal*I), Sa (*Sac*I). Except for *Eco*RI only relevant sites are indicated. Relevant recombinant phages and subclones are indicated by designated horizontal arrows. Protein coding sequences and transcriptional directions are indicated by open arrows with gene designations and molecular masses (in kD). Positions of regions bearing sequences hybridizing with heterologous DNA probes are indicated by Roman numerals. The positions of Tn5 insertions are given by numbered vertical arrows.

Sequences homologous to *R.meliloti fixABC* were detected on a 5.6 kb EcoRI-fragment (subclone pRleH21, Fig. 1b), upstream of *nifAB*; *R.meliloti fixA* (a 0.8 kb *Mlu*I-*Sal*I-fragment of pRmR3, Earl et al., 1987) hybridized with a 2.2 kb *Eco*RI-*Pvu*I-fragment of pRleH21 (Fig. 1b, fragment I) and *R.meliloti fixC* (a 1.2 kb *Pst*I-*Hin*dIII-fragment of pRmR3) hybridized with a 1.9 kb *Pvu*I-fragment (Fig. 1b, fragment II) (results not shown).

Transposon Tn*5* insertions introduced in this PRE sym plasmid *nifIfix* gene cluster and the phenotypes of the resulting homogenotes upon inoculation on pea plants were all described earlier (Schetgens et al., 1985). All insertions, indicated as 2107-2114 (Fig. 1b) conferred a Fix$^-$ phenotype (including 2111, in contrast to earlier observations). To localize protein coding sequences, pRleH21 and derivatives carrying Tn*5* insertions 2107-2114 were expressed in *E.coli* DS410 minicells. Transcription of the inserted DNA fragments starts from the constitutive chloramphenicol acetyl transferase (CAT) promoter on the vector pACYC184. The results of these experiments are shown in the autoradiogram presented in Fig. 2a.

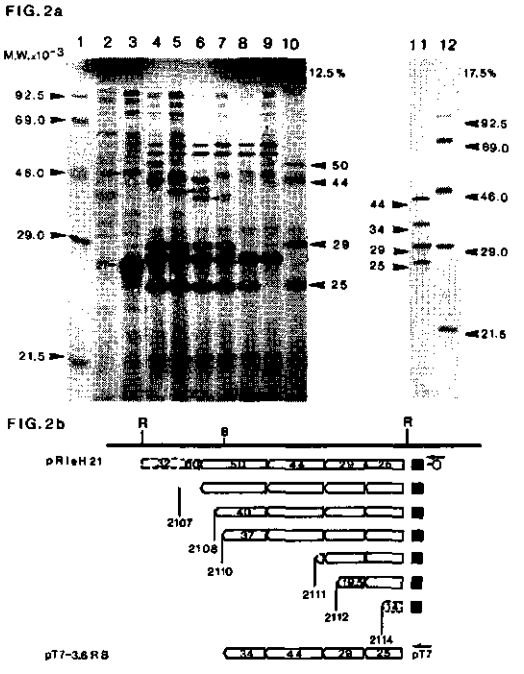

Figure 2a. Autoradiograms of ^{35}S-labeled proteins encoded by the *R.leguminosarum nifA*-upstream region synthesized in *E.coli* minicells (lanes 1-10) or T7-directed expression system (lanes 11-12), separated on SDS-polyacrylamide gels of 12.5% and 17.5% PAA respectively.
Lanes 1 and 12 ^{14}C-labeled marker proteins; lane 2 E.coli DS410 minicells without plasmid DNA; lane 3 pACYC184; lane 4 pRleH21-Tn*5* insertion 07; lane 5 pRleH21-Tn*5* insertion 08; lane 6 pRleH21-Tn*5* insertion 10; lane 7 pRleH21-Tn*5* insertion 11; lane 8 pRleH21-Tn*5* insertion 12; lane 9 pRleH21-Tn*5* insertion 14; lane 10 pRleH21; lane 11 3.6 kb *Eco*RI-*Bam*HI-fragment of pRleH21 in pT7-5.
Rhizobium encoded proteins and truncated products are indicated by arrows. Protein molecular masses are given in kD. Tn*5* encoded proteins in lanes 4-9 are 27.5, 54 and 58 kD.
Figure.2b. Gene products synthesized in *E.coli* minicells from the 5.6 kb *Eco*R1-insert and Tn*5*-derivatives are indicated by open arrows with protein molecular masses of complete or truncated products. Tn*5* insertions are shown by numbered vertical bars. The position of the CAT-promoter in vector pACYC184 and direction of transcription is given by ⊸ ; the N-terminal part (8 kD) of the CAT-protein is given by a black box (■).
Restriction endonuclease sites are abbreviated as in Fig. 1.

Proteins expressed in "empty" minicells represent a background pattern (Fig. 2, lane 2). The vector pACYC184 encodes proteins involved in tetracycline (46 kD) and chloramphenicol (27 kD) resistance (Fig. 2, lane 3). pRleH21 encodes polypeptides with molecular masses of 50, 44, 29 and 25 kD (Fig. 2, lane 10). The expression of *fixX* (10 kD polypeptide) and the 5'-end of *nifA*, expected to produce a fusion protein (with the C-terminal part of the CAT-protein) of at least 32 kD were not detectable. Tn*5* insertion 2107 (Fig. 2, lane 4) does not effect the expression

of any of the four polypeptides, but additionally three Tn*5* encoded polypeptides of 58, 54 and 27.5 kD (Rossetti et al., 1984) are visible. Tn*5* insertions 2108 and 2110 (Fig. 2, lanes 5 and 6) truncate the 50 kD protein to yield 40 and 37 kD polypeptides respectively. Tn*5* insertion 2111 (Fig. 2, lane 7) completely blocks the synthesis of the 50 kD as well as the 44 kD product, but no truncated protein is evident, probably because it is too small for detection.

Insertion 2112 (Fig. 2, lane 8) leaves only the 25 kD product intact and besides produces a 19.5 kD truncated protein derived from the 29 kD polypeptide. Insertion 2114 (Fig. 2, lane 9) blocks the synthesis of all four proteins. The truncated protein derived from the 25 kD polypeptide is not visible on this autoradiogram.

To establish whether the 25 kD product is a fusion polypeptide between the N-terminal part of the CAT-protein and the C-terminal part of a *Rhizobium* encoded polypeptide or a complete *Rhizobium* encoded protein, the right hand 3.6 kb *Eco*RI-*Bam*H1 part of the pRleH21 insert was cloned in expression vector pT7-5 and ^{35}S-methionine labeled proteins were produced (Tabor and Richardson, 1985). The resulting pattern of polypeptides with molecular masses of 25, 29, 44 and 34 kD (the N-terminal part of the truncated 50 kD protein; Fig. 2, lane 11) is consistent with the minicell pattern, and also shows that the 25 kD polypeptide is completely *Rhizobium* encoded. The four proteins fit within the coding capacity of this 3.6 kb *Eco*RI-*Bam*H1-fragment which is 133 kD. The deduced positions and transcriptional orientations of the four coding sequences are summarized in Fig. 2b. The positions of the 29, 44 and 50 kD protein encoding regions are in agreement with the hybridization results with *R.meliloti fixABC* and agree with data reported by Grönger et al. (1987). The 25 kD protein, which we denominate *fixW*, has not been described yet by others.

DNA sequence analysis of the fixW region

To establish the exact size, the position and the location of transcription regulatory elements of the *fixW* gene the nucleotide sequence of a 1.45 kb *Sac*I-fragment and part of the adjacent 1.2 *Sac*I-fragment (Fig. 1b, fragments III and IV) were determined. After digestion of both fragments with *Eco*RI, *Sal*I, *Xho*I and *Dra*I the resulting sub-fragments were cloned in both orientations in pTZ18 and pTZ19 and sequenced. The resulting nucleotide sequences were confirmed by shot-gun sequencing *Sau*3A subclones of both *Sac*I-fragments. The results are summarized in Fig. 3. Open reading frames were identified using the ANALYSEQ program (Staden, 1980); *FixW* starts at position 585 and codes for a 24.9 kD protein, which is in agreement with the results obtained in the expression studies. To establish the transcription start of *fixW* S1-nuclease protection experiments were performed using mRNA from bacteroids of 17 days old nodules, actively fixing nitrogen, and a single stranded 193 bases *Eco*RI-*Dra*I fragment (coordinates 406-598).

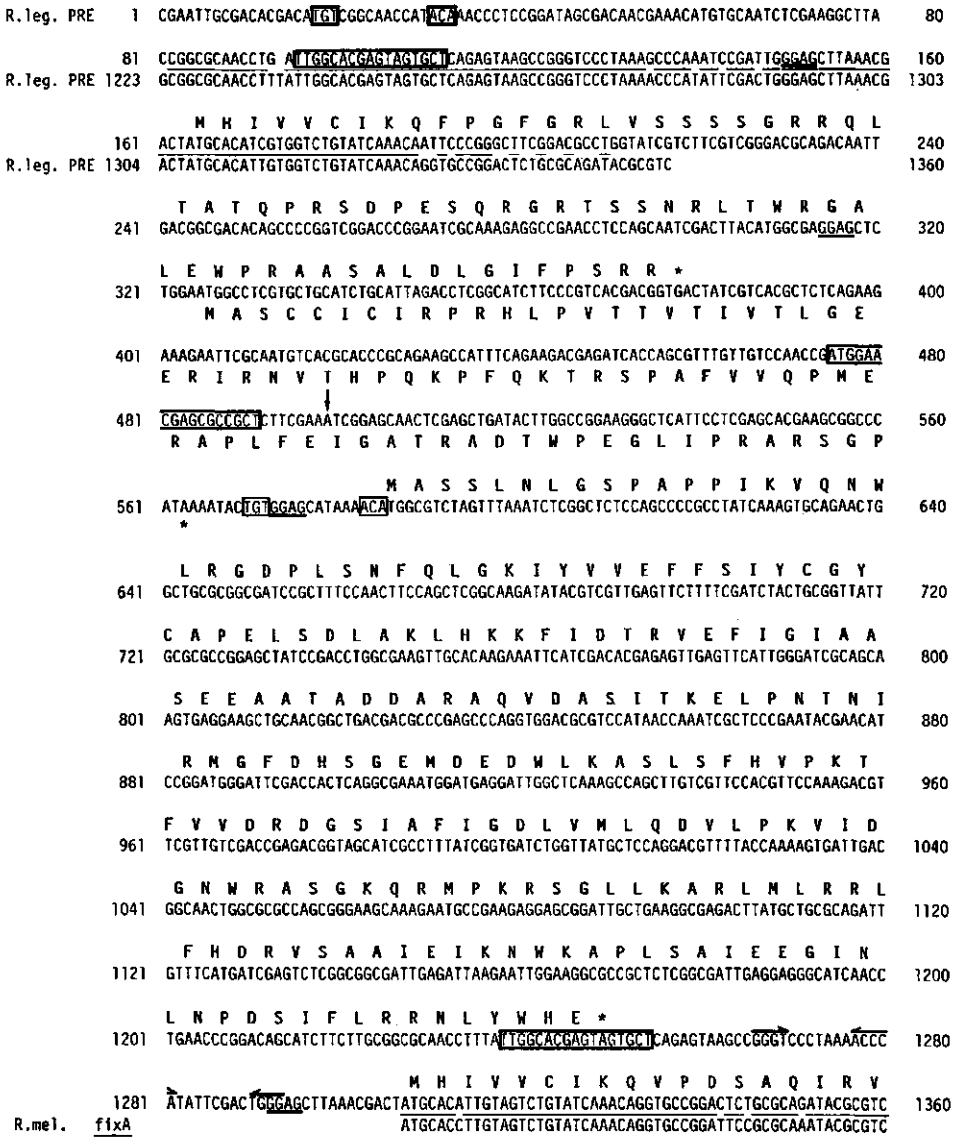

Figure 3. Partial nucleotide sequence of the 1.45 kb and 1.2 kb *Sac*I-fragments (Fig. 1b, fragments III and IV) including the amino acid sequences of ORF71, ORF79, FixW and N-terminus of Fix A. The latter is compared with the 5'-terminal nucleotide sequence of *R.meliloti fixA* (Earl *et al.*, 1987) ORF71 is compared with the nucleotide sequence of PRE *fixA*, starting from base 1223.
Underlined: ribosome binding sites. Boxed: *nif* promoters and upstream activator sequences. Overlined: possible transcription termination signals. Arrow: transcription start of *fixW*. *= translation stop codon.

A transcription start was found at coordinate 499 (Fig. 3, indicated by arrow). The ORF downstream of *fixW*, starting at position 1307, is the 5'-terminal part of *fixA* which is 90% homologous to the 5'-terminal part of *R.meliloti fixA* (Earl *et al.*, 1987), shown below the PRE sequence. Upstream of *fixW* two ORF's are present, denominated ORF71 (positions 164-376) and ORF79 (positions 325-561). The first one contains sequences homologous to the promoter and 5'-end of *fixA*.

Transcription regulation of fixA and fixW

Pea plants were inoculated with *R.leguminosarum* PRE strains carrying a Tn*5* insertion in *fixW* (2114), *fixA* (2112) or *nifA* (2107) respectively, resulting in all three cases in Fix$^-$ phenotypes. Nodules were red at days 17-19 after inoculation and turned green from day 19 to 20 due to early senescence. Bacteroid mRNA was isolated from 18 days old nodules and 5'-terminally ^{32}P-labeled. A Southern blot containing DNA fragments of subclone pRleH21 (5.6 kb *Eco*RI-fragment, Fig. 1b), digested with various restriction enzymes, was hybridized with ^{32}P-RNA from the three different mutants. Resulting autoradiograms (except for mutant 2107) are shown in Fig. 4a.

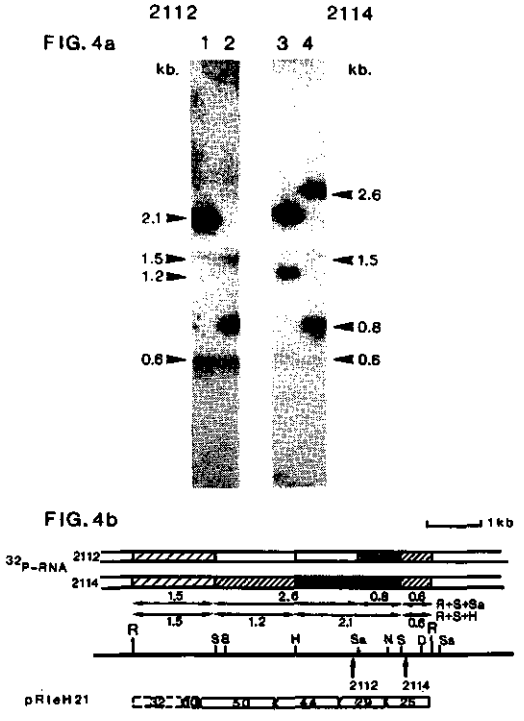

Figure 4a. Autoradiograms of a Southern blot with DNA-fragments of pRleH21 hybridized with ^{32}P-labeled RNA isolated from bacteroids of *R.leguminosarum* 2112 (lanes 1-2) and 2114 (lanes 3-4).
Lanes 1 and 3: pRleH21 x *Eco*R1-*Sal*I-*Hin*dIII.
Lanes 2 and 4: pRleH21 x *Eco*R1-*Sal*I-*Sac*I.
Relevant fragments are indicated by an arrow with fragment- length in kb.

Figure 4b. Detailed map of the 5.6 kb *Eco*RI-insert of pRleH21. Restriction endonuclease sites are abbreviated as D (*Dra*I), H (*Hin*dIII), N (*Nar*I) and as in Fig. 1.
Fragments resulting from incubation with *Eco*RI, *Sal*I, *Hin*dIII and with *Eco*RI, *Sal*I, *Sac*I respectively are given by arrows with fragment lengths in kb.
Intensities of hybridization with *R.leguminosarum* PRE mutant 2112 or 2114 bacteroid RNA are indicated by differentially shaded bars above the map. The more dense the shading the more intense the hybridization, as deduced from the autoradiograms shown in Fig. 4a. Positions of Tn*5*-insertions 2112 and 2114 are indicated by vertical arrows.

Tn*5* insertion 2112 in *fixA* blocks transcription of *fixBC* and the downstream part of *fixA*, as demonstrated by the absence of signal on the 1.2 kb *Hin*dIII-*Sal*I fragment (Fig. 4b) and on the 2.6 kb *Sac*1-*Sal*I fragment (Fig. 4b) in Fig. 4a, lanes 1 and 2. In contrast the mutation in *fixW* does not block *fixABC* transcription, as shown by the presence of signal on these same fragments (Fig. 4a, lanes 3 and 4). So in mutant 2114 (in fixW) the transcription of *fixW* is blocked, but no effect is apparent on the transcription of *fixABC*. Tn*5* insertion 2112 (in *fixA*) leaves *fixW* transcription intact (hybridization with the 0.6 kb *Eco*RI-*Sal*I fragment, Fig.4a lanes 1 and 2), but completely blocks the transcription of *fixABC*, proving at the same time that *fixABC* is one operon. Hybridization with the 1.5 kb *Eco*RI-*Sal*I-fragment (all four lanes) indicates that transcription of *nifA* is not blocked by either of the two mutations. The hybridization results are summarized in Fig. 4b by differentially shaded bars.
No expression of *fixW* and *fixABC* was detectable in mutant 2107 (*nifA*$^-$) (data not shown), suggesting that both are *nifA* regulated.

Presence of a fixW homologue in other nitrogen fixing organisms
A 0.7 kb *Eco*RI-*Nar*I fragment (Fig. 4b), carrying almost the complete *fixW* encoding region, was ^{32}P-labeled by nick-translation and used to probe a Southern blot of *Eco*RI-digested total DNA of several nitrogen fixing organisms. At low stringency hybridization conditions (6x SSC, 55°C) no hybridization was detected with *K. pneumoniae*, *Azotobacter vinelandii*, *Bradyrhizobium japonicum* and *Azorhizobium caulinodans* DNA while very faint signals were visible with *R. meliloti* and *R. trifolii* DNA. Using the same hybridization conditions with DNA from several *R.leguminosarum* strains, homology was found with strains TOM (pRl5JI), 248 (pRl3JI) and 128C53 (pRl6JI) but not with Him (data not shown). Apparently *fixW* is not highly conserved among nitrogen fixing organisms other than *R. leguminosarum* strains.

Duplication of fixW and fixA sequences in R. leguminosarum PRE
In the hybridization experiment described above the control lane containing *Eco*RI-digested DNA from *R. leguminosarum* PRE showed hybridization with a 5.6 kb fragment (*fixW* on pRleH21; Fig. 1b) and a 1.6 kb fragment. After screening the PRE genomic library the latter appeared to be located on phage RleH8 downstream of *nifHDK* (pRleH29, Fig. 1a). Two Tn*5* insertions within this 1.6 kb fragment caused Fix$^-$ phenotypes, but further analysis showed that these insertions are located close by, yet not within the *fixW* homologous sequences (Fig. 1a, fragment VI). Apparently the *fixW* homologous sequence is located adjacent to or is part of a functional *nifIfix* gene. This region also contains sequences homologous to *fixA*: pRleH28 (Fig. 1a, fragment V),

which is one of the two reiterations of *fixA* sequences we detected, both located within the *nif/fix* gene clusters. The other one, already mentioned (ORF 71), is upstream of *fixW* (pRleH56, Fig. 1b, fragment IV).

Discussion

In *R.leguminosarum* PRE the genetic organization of the *fixABC-nifAB* region, including *fixX* (Grönger et al., 1987), closely resembles that of *R.meliloti*, although the molecular masses of the *fixABC* protein products, 29, 44 and 50 kD respectively, differ somewhat from those of *R.meliloti* FixA, B and C (31, 38 and 47 kD, Earl et al., 1987). Unlike in *R.meliloti* however another open reading frame, *fixW*, encoding a 25kD polypeptide was found upstream of *fixA*. Furthermore nucleotide sequence analysis revealed that *fixW* is preceded by two smaller open reading frames. One, ORF71, starting at position 164 (Fig. 3), has a translation stop at base 376 and can produce a polypeptide of 71 amino acids (7.9kD). The second, ORF79 (positions 325-561), overlaps ORF71 and reads into the leader sequence of *fixW*. This second ORF has a potential ribosome binding site (Shine and Dalgarno, 1974), codes for a polypeptide of 79 amino acids (8.8kD) and forms one operon with ORF71.

In a NifA$^-$ mutant (Tn*5* insertion 2107) no transcrips of *fixW* and *fixABC* could be detected. This indicates that *fixABC* and *fixW* are NifA-regulated. The nucleotide sequence of *fixW* indeed shows a NifA-regulated promoter of the type CTGG-N8-TTGCA (Ausubel, 1984). The sequence between coordinates 475-491 (Fig. 3, boxed sequence) does not exactly fit the consensus but the crucial nucleotides GG at-25/-24 and GC at-13/-12 (Gussin et al., 1986) are present as well as the ACGA structure at -22 to -19, which is common in most *Rhizobium* NifA-regulated promoters (Alvares-Morales and Hennecke, 1985). The lack of this ACGA sequence in another promoter-like structure at position 500-516 rules out its role as a NifA-regulated promoter. Moreover, S1-nuclease mapping experiments showed transcription initiation at position 499 and thus proved the regulatory function of the 475-491 sequence, although the distance from promoter to transcription start appeared to be somewhat smaller (two basepairs) than in the consensus.

A similar *nif*-consensus promoter sequence is present upstream of *fixA*, at positions 1238-1254 (Fig. 3, boxed sequence), directing transcription of *fixABC*. This promoter overlaps the 3'-terminal coding region of *fixW* and can be regulated independently of *fixW*. This was proven by the RNA-hybridization experiments presented in Fig. 4. Tn*5* insertion 2114 in the *fixW* coding

region, blocks the CAT-promoter dependent transcription of *fixABC* in *E. coli* (Fig. 2), but not expression in *Rhizobium*. This further proves that the *fixABC* promoter is not activated in *E. coli*.

A third *nif*-consensus promoter sequence is present upstream of ORF71. Strikingly, from position 82 the promoter region, the leader sequence and the first 12 codons of ORF71 show an almost complete homology with those of *fixA* (Fig. 3, shown below the ORF71 sequence). Such a duplication of *fixA* sequences has also been reported for *R.meliloti* (Earl et al., 1987), but in that case the *fixA* duplicate does not have a proper *nif*-consensus promoter.

Almost without exception NifA-regulated promoters are preceded by an upstream activator sequence (UAS) with the consensus TGT-N_{10}-ACA (Buck et al., 1986). At position 570-585 (Fig. 3, boxed sequence) such a sequence is present upstream of *fixA*. However, because of the large distance (over 650 basepairs) and because Tn*5* insertion 2114 between this UAS-like sequence and *fixA* does not dramatically affect the *fixABC* messenger copy number (slightly less than in wild type, data not shown), it is unlikely that this sequence activates *fixA* transcription. This indicates that in *R.leguminosarum* a UAS is not necessary for transcription initiation by the *nif*A-product. A similar situation has been reported for *R.meliloti*, where a deletion of a UAS seems to have no effect on the level of transcription of *nifH* (Better et al., 1985; Albright et al., 1988).
It has to be noted that the messenger copy numbers of the *fixABC* operon were measured by hybridization of ^{32}P-RNA with *fixBC* only (1.2kb *Sal*I-*Hin*dIII-fragment, Fig. 4b), since the possible presence of *fixA* homologous messengers from ORF71 or the second *fixA* reiteration might interfere. In fact, the presence of these *fixA* homologous messengers could explain the earlier reported elevated level of *fixA* mRNA relative to *fixC* (Hontelez et al., 1987). Duplications of *fixBC* sequences have not been found in PRE.

No UAS was found directly upstream of *fixW*. However, ORF71 further upstream from *fixW*, has a UAS at positions 15-33 (Fig. 3, boxed sequence). Because no terminator is evident between ORF71 and *fixW*, it is possible that transcription of *fixW* is activated by this UAS and is initiated at the *nif*- consensus promoter of ORF71, rendering *fixW* part of one operon with ORF71 and ORF79. Albright et al. (1988) suggest a NifA-dosage effect on the activation of *nif* promoters. By this theory one could postulate that in the early stages of bacteroid development (low NifA concentration) transcription of this entire *nif/fix* region is initiated exclusively from the

ORF71 promoter, enhanced by its UAS. In later stages (high NifA concentration) transcription initiation occurs from all *nif*-promoters in this region.

RNA hybridization studies established that in the nitrogen fixing bacteroid a relatively large amount of messengers bearing *fixW-fixABC* sequences almost comparable to that of the *nifHDK* messengers (Hontelez et al., 1987). This can be explained by the presence of several possible transcription starts leading to a range of overlapping messengers. Although it was proven that in the FixW$^-$ mutant 2114 *fixABC* transcription is initiated from its own promoter, we cannot exclude the possibility that in wild type transcription is partly due to read-through from the *fixW* or even ORF71 promoter. In the intergenic region between *fixW* and *fixA* very small inverted repeats are present (Fig. 3, overlined sequences), but the stem- loop structures they may form have only a very low free-energy (G°37 = -3.8 Kcal/mol; Freier et al. 1986). If they would at all act as transcription termination signals for *fixW*, then at the same time they would terminate transcription from the *fixABC* promoter (theoretically starting at base 1265, as deduced from the nucleotide sequence). So we postulate that read-through from *fixW* into *fixABC* indeed occurs, implying that *fixWABC* form one operon, whereas *fixABC* can also be transcribed independent of *fixW*. More detailed studies have to prove this.

No function has been found as yet for the *fixABC* protein products, but it was proposed that they are involved in the electron transport of microaerobic nitrogen fixation (Gubler and Hennecke, 1986). The genes are well conserved among all microaerobic nitrogen fixing organisms. The N-terminus of PRE *fixA* is 90% homologous to that of *R. meliloti fixA* (Earl et al., 1987) and that of *A. caulinodans fixA* (Kaminski et al., 1988). But except for the *nif*-consensus promoter there is no significant homology between the upstream regions of these three *fixA* genes. *FixW* is only conserved among strains closely related to *R. leguminosarum* PRE. No relevant homology was found in a comparison of the *fixW* amino acid sequence, and those of ORF79 and ORF71, with data base libraries. So except for the fact that it is a *fix* gene we have no indications for the function of *fixW*. The position of ORF79 indicate that it is perhaps involved in posttranscriptional regulation of the ORF71-*fixW* operon. This resembles the mechanism proposed for *B.japonicum* (Gubler and Hennecke, 1988), where ribosomes translating an ORF within the leader region of *fixB* (ORF35) may lead to stabilization of that messenger. Whether or not the partial reiterations of *fixA* (ORF71 and Fig. 1a, fragment V) and *fixW* (Fig. 1a, fragment VI) are functional remains to be investigated. Like ORF71 these reiterations might be part of ORF's coding for proteins, which obviously do not exhibit the same functions as *fixA* or *fixW*, because of the Fix$^-$

phenotype of Tn*5* insertions in *fixA* and *fixW*. The same 1.2 kb *Sac*I-fragment (Fig. 1b, fragment IV) that harbours ORF71 also carries a small ORF (47 amino acids) of which the promoter region and first 30 codons are identical to those of *nifH* (data not shown). Perhaps all these duplications are non-functional as they may be the result of DNA-rearrangements. Remarkably they all are located on the borders of both *nif*/*fix* clusters, that originally could have been adjacent to one another.

A number of novel *fix* genes in various organisms have been reported to be located adjacent to *fixABC*. No homologues of these *fix* genes were found in other nitrogen fixing organisms. From this one could assume the existence of species specific *fix* genes, to which class *fixW* could be assigned. Alternatively other nitrogen fixing organisms may have a gene with a function similar to that of *fixW* but with only very litte sequence conservation.

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References

Aguilar OM, Reiländer H, Arnold W, Pühler A (1987) *Rhizobium meliloti nifN* (*fixF*) gene is part of an operon regulated by a *nifA*-dependent promoter and codes for a polypeptide homologous to the *nifK* gene product. J Bacteriol 169: 5393-5400.

Albright LM, Huala E, Gu Q, Ausubel FM (1988) Regulation of *R.meliloti* NifA function. In: Bothe H, de Bruijn F, Newton W (eds) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart, pp 345-349.

Alvarez-Morales A, Hennecke H (1985) Expression of *Rhizobium japonicum nifH* and *ifDK* operons can be activated by the *Klebsiella pneumoniae nifA* proteins but not by the product of *ntrC*. Mol Gen Genet 199: 306-314.

Ausubel FM (1984) Regulation of nitrogen fixation genes. Cell 37: 5-6.

Ausubel FM, Brown SE, de Bruijn FJ, Ow DW, Riedel GE, Ruvkun GB, Sundaresan V (1982) Molecular cloning of nitrogen fixation genes from *Klebsiella pneumoniae* and *Rhizobium meliloti*. In: Setlow JK, Hollaender A (eds) Genetic Engineering, Plenum Press, New York, pp 169-198.

Berk A, Sharp P (1977) Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1-endonuclease-digested hybrids. Cell 12: 721-732.

Better M, Ditta G, Helinski DR (1985) Deletion analysis of *Rhizobium meliloti* symbiotic promoters. EMBO J 4: 2419-2424.

Brewin NJ, de Jong TM, Phillips DA, Johnston AW (1980) Co-transfer of determinants for hydrogenase activity and nodulation ability in *Rhizobium leguminosarum*. Nature 288: 77-79.

Buck M, Miller S, Drummond M, Dixon R (1986) Upstream activator sequences are present in the promoters of nitrogen fixation genes. Nature 320: 374-378.

Chang AC, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA

cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 134: 1141-1156.

Ditta G, Schmidhauser T, Jakobson E, Lu P, Liang X-W, Finaly DR, Guiney D, Helinski DR (1985) Plasmids related to the broad host range vector pRK290, useful for gene cloning and for monitoring gene expression. Plasmid 13: 149-153.

Dixon RA (1984) The genetic complexity of nitrogen fixation. J Gen Microbiol. 130: 2745-2755.

Dreyfus BL, Dommergues YR (1981) Nitrogen fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. FEMS Microbiol Lett 10: 313-317.

Earl CD, Ronson CW, Ausubel FM (1987) Genetic and structural analysis of the *R.meliloti fixA*, *fixB*, *fixC* and *fixX* genes. J Bacteriol 169: 1127-1136.

Ebeling S, Hahn M, Fischer H-M, Hennecke H (1987) Identification of *nifE*-, *nifN*- and *nif*-like genes in *Bradyrhizobium japonicum*. Mol Gen Genet 207: 503-508.

Ebeling S, Zürcher T, Gubler M, Noti J, Hennecke H (1988) Identification of two genes in *Bradyrhizobium japonicum* coding for ferredoxin-like proteins. In: Bothe H, de Bruijn F, Newton W (eds) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart, p 366.

Fischer HM, Reber D, Hennecke H (1985) Localization and preliminary characterization of a *nifA*-like gene in *Bradyrhizobium japonicum*. In: Evans H, Bottomley P, Newton W (eds) Nitrogen Fixation Research Progress. Martinus Nijhoff Publishers, Dordrecht, p 183.

Freier S, Kierzek R, Jaeger J, Sugimoto N, Caruthers M, Neilson T, Turner D (1986) Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. USA 83: 9373-9377.

Grönger P, Manian SS, Reiländer H, O'Connell M, Priefer U, Pühler A (1987) Organization and partial sequence of a DNA region of the *Rhizobium leguminosarum* plasmid pRl6JI containing the genes *fixABC*, *nifA*, *nifB* and a novel open reading frame. Nucl Acids Res 15: 31-49.

Gubler M, Hennecke H (1986) *FixA*, *B* and *C* genes are essential for symbiotic and free-living, microaerobic nitrogen fixation. FEBS 200: 186-192.

Gubler M, Hennecke H (1988) Regulation of the *fixA* gene and the *fixBCX* operon in *Bradyrhizobium japonicum*: fine tuning by upstream activator sequences (UASs) and posttranscriptional control. In: Bothe H, De Bruijn F, Newton W (eds) Nitrogen Fixation:Hundred Years After, Gustav Fischer, Stuttgart, p 368.

Gussin G, Ronson C, Ausubel F (1986) Regulation of nitrogen fixation genes. Ann Rev Genet 30: 567-591.

Haaker H, de Kok A, Veeger C (1974) Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. Biochim Biophys Acta 357: 344-357.

Hennecke H, Mielenz J (1981) Molecular cloning of *Rhizobium japonicum* DNA in *E.coli* and identification of nitrogen fixation genes. In: Lyons et al (eds) Genetic engineering of symbiotic nitrogen fixation and conservation of fixed nitrogen. Plenum Press. New York, pp 41-53.

Hontelez J, Klein Lankhorst R, Jansma JD, Jacobsen E, van den Bos RC, van Kammen A (1987) Characterization of symbiotic genes and regulation of their expression in *Rhizobium leguminosarum* PRE. In: Verma D, Brisson N (eds) Molecular Genetics of Plant-Microbe Interactions. Martinus Nijhoff Publishers, Dordrecht, p 241-243.

Josey D, Beynon I, Johnston A, Beringer I (1979) Strain identification in *Rhizobium* using intrinsic antibiotic resistance. J Appl Bacteriol 46: 343-350.

Kaminski P, Norel F, Desnoues N, Kush A, Salzano G, Elmerich C (1988) Characterization of the *fixABC* region of *Azorhizobium caulinodans* ORS571 and identification of a new nitrogen fixation gene. Mol Gen Genet 214: 496-502

Kam I, Brenner S, Barnett L, Cesareni G (1980) Novel bacteriophage cloning vector. Proc Natl Acad Sci USA 77: 5172-5176.

Klipp W, Reiländer H, Schlüter A, Krey R, Pühler A (1988) A *Rhizobium meliloti* nitrogen fixation gene (*fixY*) encoding a ferredoxin-like protein forms a transcriptional unit with *nifA* and *nifB*. In: Bothe H, de Bruijn F, Newton W (eds) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart, p 375.

Krol A, Hontelez J, van den Bos RC, van Kammen A (1980) Expression of large plasmids in the endosymbiotic form of *Rhizobium leguminosarum*. Nucl Acids Res 8: 4337-4347.

Krol A, Hontelez J, van den Bos RC, van Kammen A (1982) Only one of the large plasmids in *Rhizobium leguminosarum* strain PRE is strongly expressed in the endosymbiotic state. J Gen Microbiol 128: 1839-1847.

Lie T, Soe-Agnie J, Muller G, Gökdan D (1979) Environmental control of symbiotic nitrogen fixation: limitation to flexibility of the legume-*Rhizobium* system. In: Broughton W et al (eds) Proc Symp Soil Microbiol Plants Nutrition.

University of Malaya, Kuala Lumpur, pp 194-212.

Maniatis T, Fritsch E, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York.

Meade H, Long S, Ruvkun G, Brown S, Ausubel F (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J Bacteriol 149: 114-122.

Reeve JC (1979) Use of minicells for bacteriophage-directed polypeptide synthesis. In: Wu R (ed) Methods in Enzymology. Vol 68, Recombinant DNA. Academic Press, New York, pp 493-503.

Roelvink P, van der Werf M, Hontelez J, van Kammen A, van den Bos RC (1988) Characterization of the regulatory *nifA* gene of *Rhizobium leguminosarum* PRE. In: Bothe H, de Bruijn F, Newton W (eds) Nitrogen Fixation: Hundred Years After. Gustav Fischer, Stuttgart, p 382.

Rolfe B, Gresshoff P, Shine J (1980) Rapid screening for symbiotic mutants of *Rhizobium* and white clover. Plant Sci Lett 19: 277-284.

Rossen L, Ma Q-S, Mudd E, Johnston A, Downie J (1984) Identification and DNA sequence of *fixZ*, a *nifB*-like gene from *Rhizobium leguminosarum*. Nucl Acids Res 12: 7123-7134.

Rossetti O, Altman R, Young R (1984) Kinetics of Tn*5* transposition. Gene 32: 91-98.

Ruvkun G, Ausubel F (1980) Interspecies homology of nitrogenase genes. Proc Natl Acad Sci USA 77: 191-195.

Ruvkun G, Ausubel F (1981) A general method for site-directed mutagenesis in prokaryotes. Nature 289: 85-89.

Ruvkun G, Sundaresan V, Ausubel F (1982) Directed transposon Tn*5* mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell 29: 551-559.

Sanger F, Nicklen S, Coulson A (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467.

Schetgens T, Bakkeren G, van Dun C, Hontelez J, van den Bos RC, van Kammen A (1984) Molecular cloning and functional characterization of *R.leguminosarum* structural *nif* genes by site-directed transposon mutagenesis and expression in *E.coli* minicells. J Mol Appl Genet 2: 406-421.

Schetgens T, Hontelez J, van den Bos RC, van Kammen A (1985) Identification and phenotypical charaterization of a cluster of *fix* genes, including a *nif* regulatory gene from *Rhizobium leguminosarum* PRE. Mol Gen Genet 200: 368-374.

Shine J, Dalgarno L (1974) The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc Natl Acad Sci USA 71: 1342-1346.

Staden R (1980) A new computer method for the storage and manipulation of DNA gel reading data. Nucl Acids Res 8: 3673-3694.

Staden R (1984) Graphic methods to determine the function of nucleic acid sequences. Nucl Acids Res 12: 521-567.

Szeto W, Zimmerman J, Sundaresan V, Ausubel F (1984) A *Rhizobium meliloti* symbiotic regulatory gene. Cell 36: 1035-1043.

Tabor S, Richardson C (1985) A bacteriophage T7 polymerase/promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci USA 82: 1074-1078.

Thöny B, Fischer H-M, Anthamatten D, Bruderer T, Hennecke H (1987) The symbiotic nitrogen fixation regulatory operon (*fixRnifA*) of *Bradyrhizobium japonicum* is expressed aerobically and is subject to a novel, *nifA*-independent type of activation. Nucl Acids Res 15: 8479-8499.

Winarno R, Lie T (1979) Competition between *Rhizobium* strains in nodule formation: interactions between nodulating and non-nodulating strains. Plant and Soil 51: 135-142.

Chapter 4

Identification and characterization of a bacteroid-specific dehydrogenase complex in *Rhizobium leguminosarum* PRE.

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Identification and Characterization of a Bacteroid-Specific Dehydrogenase Complex in *Rhizobium leguminosarum* PRE

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In membranes of *Rhizobium leguminosarum* bacteroids isolated from nitrogen-fixing pea root nodules, two different protein complexes with NADH dehydrogenase activity were detected. One of these complexes, with a molecular mass of 110 kilodaltons, was also found in membranes of free-living rhizobia, but the other, with a molecular mass of 550 kilodaltons, appeared to be present only in bacteroids. The bacteroid-specific complex, referred to as DH1, probably consists of at least four different subunits. Using antibodies raised against the separate polypeptides, we found that a 35,000-molecular-weight polypeptide (35K polypeptide) in the DH1 complex is bacteroid specific, while the other proposed subunits were also detectable in cytoplasmic membranes of free-living bacteria. Dehydrogenase complex DH1 is also present in bacteroids of a *R. leguminosarum nifA* mutant, indicating that the synthesis of the dehydrogenase is not dependent on the gene product of this *nif*-regulatory gene. A possible involvement of the bacteroid-specific DH1 complex in electron transport to nitrogenase is discussed.

Bacteria of the genus *Rhizobium* are able to fix atmospheric nitrogen in close concert with leguminous host plants. In this process nitrogen is reduced to ammonia by the bacterial enzyme complex nitrogenase. For its function, this enzyme requires the presence of a strong reductant with a redox potential of about −450 mV (5, 27). The only known bacterial electron carriers with a sufficiently low midpoint potential are flavodoxins and ferredoxins, which have indeed been identified in several nitrogen-fixing species (6, 14, 31, 34).

Little is known about the mechanism by which these low-potential electron carriers in diazotrophic bacteria are reduced. For the facultative anaerobic bacterium *Klebsiella pneumoniae*, which fixes nitrogen under anaerobic conditions, genetic and biochemical evidence indicates that a flavodoxin, encoded by the gene *nifF*, is reduced by electrons derived from pyruvate in a thioclastic reaction. The enzyme catalyzing this reaction is pyruvate:flavodoxin oxidoreductase, encoded by the *nifJ* gene (4, 14, 29). This thioclastic reaction is probably the source of reducing power for nitrogen fixation in anaerobic bacteria in general (9).

In *Rhizobium* spp. and other bacteria fixing nitrogen under aerobic conditions, the electron flow to nitrogenase probably follows a different route, since pyruvate:flavodoxin oxidoreductase activity has not been found in *Rhizobium* cell extracts (9), and genes homologous to *nifF* or *nifJ* of *Klebsiella* have not been detected by interspecies DNA hybridization. Furthermore, it was shown that in *Rhizobium* bacteroids, unlike diazotrophs fixing nitrogen under anaerobic conditions, the electron transport to nitrogenase is dependent on an energized cytoplasmic membrane (15, 21, 22, 23). This has also been reported for the strictly aerobic bacterium *Azotobacter vinelandii* (10, 20), the cyanobacteria *Anabaena variabilis* and *Plectonema boryanum* (12, 13), and the photosynthetic bacterium *Rhodobacter sphaeroides* (11).

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This observation and the observation that during nitroger fixation the enzyme nitrogenase probably is attached to the cytoplasmic membrane (16, 18) suggest that in *Rhizobium* bacteroids the electron transport chain to nitrogenase probably is located in the cytoplasmic membrane. Since the main source of electrons for nitrogen fixation in aerobic and facultatively anaerobic diazotrophs consists of reduced pyridine nucleotides (2, 9, 19, 33), NAD(P)H-dependent dehydrogenases supposedly form a part of the electron transport chain to nitrogenase. For *Azotobacter* it has been shown that during derepression of nitrogen fixation a membrane-bound NADPH dehydrogenase is induced (20); however, no function in the process of nitrogen fixation has been assigned to this enzyme yet.

In this paper we describe the identification of two membrane-bound NADH-dependent dehydrogenase complexes in *Rhizobium leguminosarum*, one of which, denoted DH1, is bacteroid specific. Partial purification of this complex showed that it probably consists of three different polypeptides which are present in both bacterial and bacteroid membranes and contains one bacteroid-specific polypeptide.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used: *nif*Agp, the regulatory *nifA* protein; NBT, 3,3'-(3,3'-dimethoxy-4,4'-biphenylylene)bis-2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PAA, polyacrylamide; FPLC, fast-protein liquid chromatography; kDa, kilodalton; SDS, sodium dodecyl sulfate.

Bacterial strains and growth conditions. Wild-type *R. leguminosarum* PRE (Str^r) (26) and *R. leguminosarum* 2107 (Str^r Km^r) carrying a Tn*5* insertion in the *nifA* locus (32) were grown at 29°C in TY medium (5 g of tryptone per liter, 3 g of yeast extract per liter, 1.3 g of $CaCl_2$ per liter) supplemented with 1 mg of uracil per liter. Antibiotics were added at the following concentrations: streptomycin, 250 μg/ml; kanamycin, 20 μg/ml.

Inoculation and growth of pea plants. Pea seeds (*Pisum sativum* L. cv. Rondo) were surface sterilized, inoculated

with 1 ml of *R. leguminosarum* culture per seed, and grown under greenhouse conditions. Nodules were harvested at day 18 after inoculation with strain 2107 or at day 21 after inoculation with wild-type strain PRE.

Isolation of bacteroids. Nodules were ground in a mortar at 0°C, suspended in 50 mM TES-KOH–5 mM $MgSO_4$ (pH 7.5), and homogenized. The homogenate was filtered through four layers of Miracloth (Calbiochem-Behring, La Jolla, Calif.), and the filtrate was centrifuged at 200 × *g* in a Heraeus desktop centrifuge for 2 min. The supernatant was centrifuged for 15 min at 4,000 × *g* at 4°C, and the bacteroid pellet was suspended in 50 mM TES-KOH–5 mM $MgSO_4$ (pH 7.5).

Isolation of bacteroid and bacterial membranes. Free-living bacteria harvested at an optical density at 550 nm of 0.3 or bacteroids, suspended in 50 mM TES-KOH–5 mM $MgSO_4$ (pH 7.5) at an optical density at 550 nm of 1.00, were broken in a French pressure cell at 1.4×10^5 kPa and centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was centrifuged at 140,000 × *g* for 1 h at 4°C. The resulting membrane pellet was suspended in 50 mM TES-KOH–5 mM $MgSO_4$ (pH 7.5) at a protein concentration of 10 mg/ml by using a Dounce glass homogenizer. The membrane suspension was frozen in liquid nitrogen and stored at −80°C.

Column chromatography of membrane proteins. Bacteroid membranes at a protein concentration of 8 mg/ml were extracted in 60 mM Tris hydrochloride–2 mM $MgSO_4$–5% (vol/vol) Triton X-100 (pH 8.8) for 15 min at room temperature and centrifuged at 10,000 × *g* in an Eppendorf desktop centrifuge for 1 min. Solubilized proteins were separated on a Sephadex G-200 column with 50 mM Tris hydrochloride–5 mM $MgSO_4$–0.1% Triton X-100 (pH 7.5), and fractions (0.5 ml) were analyzed for NADH-dehydrogenase activity. Active fractions were pooled, and proteins were separated by anion-exchange FPLC with a linear 0 to 2 M NaCl gradient in 50 mM TES-KOH–5 mM $MgSO_4$–0.1% (vol/vol) Triton X-100 (pH 7.5). Fractions containing NADH-dehydrogenase activity were further analyzed by nondenaturing PAA gel electrophoresis.

Analytical methods. Membranes at a protein concentration of 8 mg/ml were extracted in 60 mM Tris hydrochloride–2 mM $MgSO_4$–5% (vol/vol) Triton X-100–10% (vol/vol) glycerol (pH 8.8) for 15 min at room temperature and subsequently centrifuged for 1 min at 10,000 × *g*. Solubilized proteins were separated by electrophoresis on a 7.5% (wt/vol) nondenaturing PAA gel containing 0.1% (vol/vol) Triton X-100 or on a 4 to 20% (wt/vol) nondenaturing linear PAA gradient gel containing 0.1% Triton X-100 in a buffer containing 200 mM glycine, 25 mM Tris, and 0.1% (vol/vol) Triton X-100 (pH 8.6). Electrophoresis of 7.5% gels was carried out at 80 V for 18 h at 4°C; 4 to 20% gradient gels were run at 200 V for 27 h at 4°C. To detect NADH-dependent dehydrogenase complexes, we incubated gels with 0.25 mM NADH and 0.5 mM NBT in 50 mM TES-KOH–5 mM $MgSO_4$ (pH 7.5) after electrophoresis. To analyze the polypeptide composition of a dehydrogenase complex, a gel fragment containing enzymatic activity (localized by staining part of the gel) was incubated in 5% (wt/vol) SDS–10 mM β-mercaptoethanol for 5 min and fixed on top of an SDS-PAA gel with 1% (wt/vol) molten agarose; electrophoresis was performed by the method of Laemmli (24).

Protein concentrations were determined by the method of Lowry et al. (28) or with the BCA Protein Assay Reagent as specified by the manufacturer. Pyridine nucleotide dehydrogenase activities were determined by measuring the reduction of NBT spectrophotometrically at 560 nm at 24°C (samples contained 20 μg of protein per ml in 50 mM TES-KOH, 5 mM $MgSO_4$, 0.5 mg of NBT per ml, 0.05% [vol/vol] Triton X-100, 20 μg of catalase per ml, and 0.25 mM NADH [pH 7.5]).

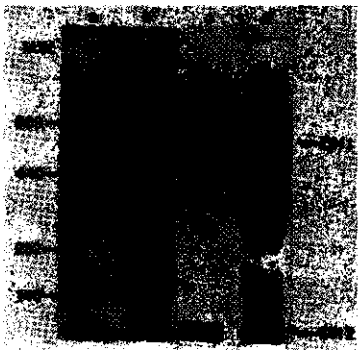

FIG. 1. Detection of extracted membrane dehydrogenases. Membranes of *R. leguminosarum* bacteria (lanes a and c) or bacteroids (lanes b and d) were incubated with 5% (vol/vol) Triton X-100, and extracted proteins were separated on a 4 to 20% nondenaturing PAA gradient gel and stained in 50 mM TES-KOH–5 mM $MgSO_4$–0.23 mM NADH–0.5 mM NBT (pH 7.5) for 1 h (lanes a and b) or 24 h (lanes c and d). DH2 and the bacteroid-specific dehydrogenase complex DH1 are indicated by arrows. M.W., Molecular weight (in thousands).

For antiserum production, dehydrogenase subunits present in active FPLC fractions were separated on a preparative SDS-PAA gel (15% [wt/vol] acrylamide), negatively stained in 2.5 M KCl, and cut out. The slices of PAA were mashed by being passed through a 21-gauge 1.5-in (3.8-cm) needle and mixed with equal volumes of Freund complete or incomplete adjuvant. Rabbits were injected subcutaneously with 50 μg of protein in Freund complete adjuvant and were given a boost of 50 μg of protein in Freund incomplete adjuvant after 28 days.

Western immunoblotting and immunological techniques were as described by Schetgens et al. (32).

Chemicals. NBT and TES were obtained from Sigma Chemical Co., St. Louis, Mo.; NADH was from Boehringer GmbH, Mannheim, Federal Republic of Germany; high-molecular-weight protein markers were from Pharmacia, Uppsala, Sweden; BCA Protein Assay Reagent was from Pierce Chemical Co. Rockford, Ill. All other chemicals were reagent grade from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS

Characterization of membrane dehydrogenases. Membranes of bacteroids and of free-living bacteria were isolated and treated with Triton X-100 to solubilize membrane-associated proteins. The solubilized proteins were separated by electrophoresis on a 4 to 20% nondenaturing PAA gradient gel, which was subsequently incubated with NBT and NADH to visualize dehydrogenase activity. After 1 h of incubation, two bands with dehydrogenase activity were visible in the lane containing bacteroid membrane proteins, whereas only one band was detectable in the bacterial membranes (Fig. 1). The dehydrogenase present only in the bacteroid membranes, denoted DH1, has a molecular mass

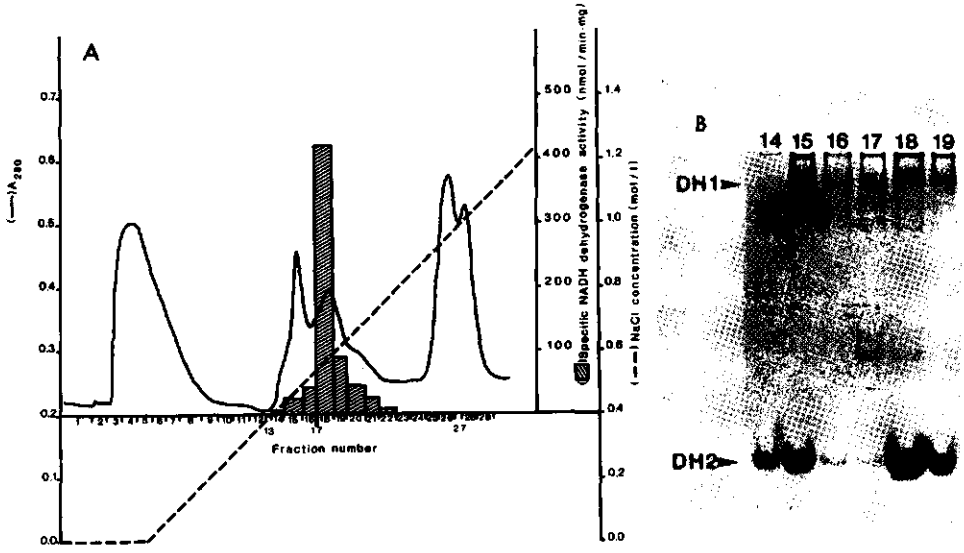

FIG. 2. (A) Chromatogram of NADH-dehydrogenases on a Mono-Q anion-exchange column. Extracted bacteroid membrane proteins (12.5 mg) were separated on a Sephadex G-200 column, and fractions (0.5 ml) were tested for NADH-dehydrogenase activity. Active fractions were pooled and applied to a Mono-Q anion-exchange column equilibrated with 50 mM TES-KOH–5 mM $MgSO_4$ (pH 7.5). The column was eluted with a gradient of 0 to 2 M NaCl at 1 ml/min, and fractions of 0.5 ml were collected. Fractions 1 to 29 were assayed for NADH-dehydrogenase activity; the specific dehydrogenase activity is indicated (▨). (B) Analysis of active FPLC fractions. Proteins in FPLC fractions 14 to 19 (20 μl) were separated by electrophoresis on a 7.5% nondenaturing PAA gel, and dehydrogenase activity was visualized by staining with NBT and NADH. The positions of the complexes DH1 and DH2 are indicated.

of 550 kDa, and the dehydrogenase activity denoted DH2, found in both bacteria and bacterial membranes, has a molecular mass of 110 kDa. Upon longer incubation the bands increased in intensity, but no dehydrogenase appeared at the position of DH1 in the lane with the bacterial membrane proteins. The detection of DH1 in bacteroid membranes and of DH2 in both bacteroid and bacterial membranes was reproducible in six separate experiments. Other minor bands visible in Fig. 1 were not found reproducibly and probably represent other NADH-dependent dehydrogenases which are more difficult to extract and thus occur in various amounts in the separate membrane extractions. When NADPH was used instead of NADH in the staining, the activity of DH1 and DH2 was reduced approximately 10-fold. No additional dehydrogenases were detected when NADPH was used (results not shown).

Polypeptide composition of DH1 and DH2. To study the polypeptide composition of the dehydrogenase complexes, we purified *DH1* and *DH2* by *FPLC*; therefore, Triton X-100-extracted high-molecular-weight membrane protein complexes were separated from low-molecular-weight proteins by chromatography on a Sephadex G-200 column. NADH-dependent dehydrogenase activity was detectable only in the void volume (results not shown). Active fractions were pooled, and protein complexes were separated on a Mono-Q anion-exchange column. Column fractions were assayed for NADH dehydrogenase activity (Fig. 2A), and portions of the active fractions were analyzed by electrophoresis on a 7.5% nondenaturing PAA gel and then stained with NBT and NADH. The bulk of complex DH1 was found in fraction 15 (Fig. 2B), whereas fraction 18 contained most of complex DH2. Two minor dehydrogenase complexes were detectable in fractions 17 and 18. Fractions 20 to 22 contained only small amounts of complex DH1 and DH2 (results not shown).

The partially purified DH1 and DH2 complexes, found in fraction 15 of a Mono-Q exchange column after separation on a nondenaturing gel, were cut out of the gel and subjected to electrophoresis to examine the polypeptide composition. Complex DH1 contained four polypeptides (Fig. 3, lane a), with molecular masses of 58, 35, 29, and 20 kDa (58K, 35K, 29K, and 20K polypeptides, respectively), which were reproducibly found in six separate experiments. Other polypeptides shown in Fig. 3, lane a, were not reproducibly found and probably are not related to DH1 activity. Complex DH2 contained two subunits with molecular masses of 58 and 75 kDa (Fig. 3, lane b).

To investigate whether any of the DH1 subunits are also present in bacterial membranes, antisera were raised against the separate 20K, 29K, 35K, and 58K polypeptides. Total bacterial and bacteroid membrane proteins were separated on an SDS–15% PAA gel, transferred to a nitrocellulose filter, and incubated with the different antisera. The Western blots showed that the 20K, 29K, and 58K polypeptides were present in both bacterial and bacteroid membranes (Fig. 4, lanes a, b, c, d, g, and h), whereas the 35K polypeptide was detectable only in bacteroid membranes (Fig. 4, lanes e and f). The 35K polypeptide thus appears to be a bacteroid-specific component.

Proteins in fraction 15 of the Mono-Q column (Fig. 2a) were separated on a 7.5% nondenaturing PAA gel and transferred to nitrocellulose. The blot was then incubated with antiserum directed against the 35K subunit (Fig. 5a). The 35K polypeptide appeared to be present in the bacte-

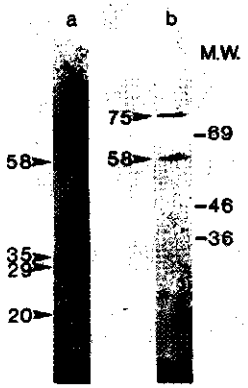

FIG. 3. Polypeptide composition of DH1 and DH2. Purified complexes DH1 (lane a) and DH2 (lane b) from FPLC fraction 15 (Fig. 2) were denaturated in 5% (wt/vol) SDS–10 mM β-mercaptoethanol and analyzed by electrophoresis on an SDS–12.5% PAA gel. The silver-stained pattern of the gel is shown. The positions of the major subunits are indicated M.W., Molecular weight (in thousands).

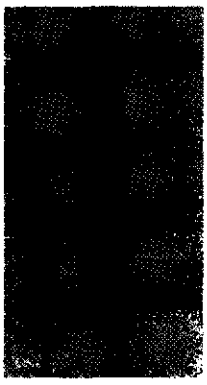

FIG. 5. Localization of the 35K subunit in bacteroid NADH-dehydrogenases. Proteins present in FPLC fraction 15 (8 μg) (Fig. 2) were separated on a 7.5% nondenaturing PAA gel, transferred to a nitrocellulose filter, and reacted with antiserum directed against the 35K subunit (lane a). Immunocomplexes were visualized by reaction with ^{125}I-protein A and autoradiography. A parallel gel was stained with NBT and NADH after electrophoresis to visualize dehydrogenase activity (lane b). Complexes DH1 and DH2 are indicated.

roid-specific complex DH1 but was not detectable in DH2. The positions of the DH1 and DH2 complexes in this experiment were determined by staining a parallel gel with NBT and NADH (Fig. 5b).

DH1 activity in *nifA* strain 2107. To investigate whether the synthesis of polypeptides in DH1 is under the control of the *nif* gene activating protein *nif*Agp, we prepared bacteroid membranes from 18-day-old pea root nodules induced by *R. leguminosarum* 2107 (32) carrying Tn*5* in the *nifA* locus. NADH dehydrogenase staining of extracted membrane proteins of bacteroids from strain 2107 after electrophoresis in a nondenaturing gel revealed that both DH1 and DH2 are present in membranes of the mutant in about the same amounts as in the wild-type bacteroid membranes. DH1 from the *nifA* mutant appeared to contain the same polypeptides as that from the wild type (results not shown). Apparently, neither NADH-dependent NBT reduction by DH1 nor the protein composition of DH1 depends on the presence of the regulatory *nifA* gene product.

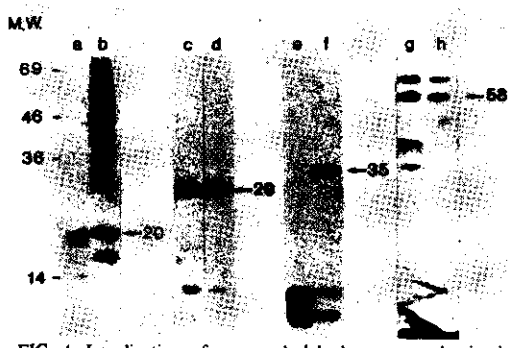

FIG. 4. Localization of proposed dehydrogenase subunits in bacterial and bacteroid membranes. Total bacterial membrane proteins (50 μg; lanes a, c, e, and g) and bacteroid membrane proteins (50 μg; lanes b, d, f, and h) were separated by electrophoresis on an SDS–15% PAA gel, transferred to a nitrocellulose filter, and incubated with antisera directed against the 20K polypeptide (lanes a and b), the 29K polypeptide (lanes c and d), the 35K polypeptide (lanes e and f), or the 58K polypeptide (lanes g and h). Immunocomplexes were visualized by reaction with ^{125}I-protein A and autoradiography. The 20K, 29K, 35K, and 58K polypeptides are indicated. M.W., Molecular weight (in thousands).

DISCUSSION

In this report we show the occurrence of two prominent membrane-bound NADH-dependent dehydrogenase complexes in bacteroids of *R. leguminosarum* PRE. Complex DH1, with a molecular mass of 550 kDa, is specific for bacteroid membranes, whereas complex DH2, with a molecular mass of 110 kDa, is present in both bacteroids and free-living bacteria.

After partial purification, analyses of complex DH1 consistently showed the presence of four polypeptides with molecular masses of 20, 29, 35, and 58 kDa. This indicates that these polypeptides probably form part of the active DH1 complex. Definite proof for this will depend on reconstitution of active DH1 from the individual components. This may also reveal whether more polypeptides present in small amounts are an intrinsic part of DH1.

Using polyclonal antisera raised against each of these four DH1 subunits, we showed that the 20K, 29K, and 58K polypeptides occurred in free-living bacteria as well as in bacteroids. The 35K polypeptide was found only in bacteroids and thus may be considered a bacteroid-specific component of this NADH-dehydrogenase complex. Since no reactions of the 35K antiserum with other bacteroid dehydrogenases could be detected, the 35K polypeptide probably is present only in DH1. The antiserum also reacted with a 35K polypeptide in bacteroids, but not in free-living bacteria, of *R. leguminosarum* PF2 (25) and RB1 (M. Van Mil, Ph.D. thesis, Agricultural University, Wageningen, The Netherlands, 1981), indicating that DH1 is probably common in *R. leguminosarum*.

Complex DH2, which is present in both bacteroids and free-living bacteria, is composed of at least two different polypeptides, with molecular masses of 58 and 75 kDa. The 58K subunit appears to be identical to the 58K subunit of DH1, since both proteins react with the antiserum directed against the 58K DH1 subunit (results not shown).

A bacteroid-specific NADH-dependent dehydrogenase containing one subunit which is present only during nitrogen fixation resembles the situation described for *Azotobacter vinelandii*. Klugkist et al. (20) showed that in *A. vinelandii* a high-molecular-weight membrane-bound dehydrogenase complex is specifically derepressed under nitrogen-fixing conditions. The *Azotobacter* dehydrogenase is dependent on NADPH and contains a specific 29K subunit. The antiserum directed against the 35K bacteroid-specific dehydrogenase component does not react with this 29K protein nor with any other *Azotobacter* protein specifically derepressed during nitrogen fixation. The function of this *Azotobacter* dehydrogenase has not been proved, but a role in electron transport to nitrogenase was suggested (20). The exclusive presence of DH1 in bacteroids suggests a similar role in nitrogen fixation in *Rhizobium* spp. Alternatively, it could be a part of the respiratory chain in bacteroids that is induced by the low oxygen concentration present in the root nodule (3); such an explanation seems less likely, however, since neither complex DH1 nor the 35K subunit was detectable in free-living bacteria grown under microaerobic conditions (<1% O_2 in the gas phase) (results not shown).

If DH1 is part of the electron transport chain to nitrogenase, the gene coding for the 35K polypeptide should be considered a *nif* or *fix* gene. Since DH1 and the 35K protein are also present in bacteroids of the *nifA* mutant strain 2107, the expression of the 35K gene is not regulated by the regulatory *nifA* gene product. This excludes the possibility that (part of) DH1 is encoded by the *nif*Agp-regulated *fixABCX* operon (30), which is hypothesized to have a role in electron transport to nitrogenase (8), or by any other *nif*Agp-regulated *fix* gene for which no function in the process of symbiotic nitrogen fixation has yet been assigned (1, 15). Recently the existence of genes essential for nitrogen fixation but not regulated by *nif*Agp has been exemplified by the identification of a cluster of *fix* genes on the *sym* plasmid of *R. meliloti* (7). Interestingly, sequence data for these *fix* genes show that some of these probably code for membrane proteins containing Fe-S clusters (17), as might be expected for proteins involved in membrane-located electron transport.

Currently we are isolating the gene coding for the 35K subunit. Mutagenesis and sequence analysis probably will reveal the function of this protein and that of dehydrogenase complex DH1 in symbiotic nitrogen fixation.

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LITERATURE CITED

1. **Aguilar, M. O., D. Kapp, and A. Pühler.** 1985. Characterization of a *Rhizobium meliloti* fixation gene (*fixF*) located near the common nodulation region. J. Bacteriol. **164**:245–254.
2. **Beneman, J. R., D. C. Yoch, R. C. Valentine, and D. I. Arnon.** 1971. The electron transport system in nitrogen fixation by *Azotobacter*. III. Requirement for NADPH-supported nitrogenase activity. Biochim. Biophys. Acta **226**:205–212.
3. **Bergersen, B. J., and G. L. Turner.** 1980. Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and of N_2-fixing bacteria grown in continuous culture. J. Gen. Microbiol. **118**:235–252.
4. **Bogusz, D., J. Houmard, and J.-P. Aubert.** 1981. Electron transport to nitrogenase in *Klebsiella pneumoniae*; purification and properties of the *nif* J protein. Eur. J. Biochem. **120**:421–426.
5. **Braaksma, A., H. Haaker, H. J. Grande, and C. Veeger.** 1982. The effect of the redox potential on the activity of the nitrogenase and on the Fe-protein of *Azotobacter vinelandii*. Eur. J. Biochem. **121**:483–491.
6. **Carter, K. R., J. Rawlings, W. H. Orme-Johnson, R. R. Becker, and J. H. Evans.** 1980. Purification and characterization of a ferredoxin from *Rhizobium japonicum* bacteroids. J. Biol. Chem. **255**:4213–4223.
7. **David, M., O. Domergue, P. Pognonec, and D. Kahn.** 1987. Transcription patterns of *Rhizobium meliloti* symbiotic plasmid *pSym*: identification of *nifA*-independent *fix* genes. J. Bacteriol. **169**:2239–2244.
8. **Gubler, M., and H. Hennecke.** 1986. *Fix* A, B and C genes are essential for symbiotic and free-living, microaerobic nitrogen fixation. FEBS Lett. **200**:186–192.
9. **Haaker, H.** 1986. Electron transport to nitrogenase in diazotrophs, p. 70–93. *In* W. J. Broughton and A. Puhler (ed.), Nitrogen fixation, vol. 4. Oxford University Press, Oxford.
10. **Haaker, H., A. de Kok, and C. Veeger.** 1974. Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*. Biochim. Biophys. Acta **357**:344–357.
11. **Haaker, H., C. Laane, K. Hellingerwerf, B. Houwer, W. N. Konings, and C. Veeger.** 1982. Short-term regulation of the nitrogenase activity in *Rhodopseudomonas sphaeroides*. Eur. J. Biochem. **127**:639–645.
12. **Hawkesford, M. J., R. H. Reed, P. Rowel, and W. D. P. Steward.** 1981. Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Anabaena variabilis*. Eur. J. Biochem. **115**: 519–523.
13. **Hawkesford, M. J., R. H. Reed, P. Rowel, and W. D. P. Steward.** 1982. Nitrogenase activity and membrane electrogenesis in the cyanobacterium *Plectonema boryanum*. Eur. J. Biochem. **127**: 63–66.
14. **Hill, S., and E. P. Kavanagh.** 1980. Roles of *nifF* and *nifJ* gene products in electron transport to nitrogenase in *Klebsiella pneumoniae*. J. Bacteriol. **114**:470–475.
15. **Hontelez, J. G. J., R. M. Klein Lankhorst, J.-D. Jansma, E. Jacobsen, R. C. van den Bos, and A. van Kammen.** 1987. Characterization of symbiotic genes and regulation of their expression in *Rhizobium leguminosarum* PRE, p. 241–243. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
16. **Howard, K. S., B. J. Hales, and M. D. Socolofsky.** 1985. In vivo interactions between nitrogenase molybdenum-iron protein and membrane in *Azotobacter vinelandii* and *Rhodospirillum rubrum*. Biochim. Biophys. Acta **812**:575–585.
17. **Kahn, D., J. Batut, P. Boistard, M. L. Daveran, M. David, O. Domergue, A. M. Garnerone, J. Ghai, C. Hertig, D. Infante, and M. H. Renalier.** 1987. Molecular analyses of a *fix* cluster from *Rhizobium meliloti*, p. 258–263. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
18. **Kieber, J., R. Clover, T. M. Finan, and E. R. Signer.** 1986. Surface properties of *Rhizobium meliloti* associated with symbiosis, p. 182–184. *In* D. P. S. Verma and N. Brisson (ed.), Molecular genetics of plant-microbe interactions. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
19. **Klucas, R. V., and H. J. Evans.** 1968. An electron donor system for nitrogenase-dependent acetylene reduction by extracts of

soybean nodules. Plant Physiol. 43:1458–1460.
20. **Klugkist, J., H. Haaker, and C. Veeger.** 1986. Studies on the mechanism of electron transport to nitrogenase in *Azotobacter vinelandii*. Eur. J. Biochem. **155**:41–46.
21. **Laane, C., H. Haaker, and C. Veeger.** 1978. Involvement of the cytoplasmic membrane in nitrogen fixation by *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **87**:147–153.
22. **Laane, C., H. Haaker, and C. Veeger.** 1979. On the efficiency of oxidative phosphorylation in membrane vesicles of *Azotobacter vinelandii* and of *Rhizobium leguminosarum* bacteroids. Eur. J. Biochem. **97**:369–377.
23. **Laane, C., W. Krone, W. N. Konings, H. Haaker, and C. Veeger.** 1979. The involvement of the membrane potential in nitrogen fixation by bacteroids of *Rhizobium leguminosarum*. FEBS Lett. **103**:53–57.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
25. **Lie, T. A.** 1971. Temperature-dependent root nodule formation in pea cv. Iran. Plant Soil **34**:751–752.
26. **Lie, T. A., I. E. Soe-Agnie, G. J. L. Muller, and D. Gokdan.** 1979. Environmental control of symbiotic nitrogen fixation: limitation to and flexibility of the legume-*Rhizobium* system, p. 194–212. *In* W. J. Broughton, C. K. John, J. C. Rajaro, and B. Lim (ed.), Proceedings of the Symposium on Soil Microbiology and Plant Nutrition, 1976. University of Malaya, Kuala Lumpur, Malaya.
27. **Lough, S., A. Burns, and G. D. Watt.** 1983. Redox reactions of the nitrogenase complex from *Azotobacter vinelandii*. Biochemistry **22**:4062–4066.
28. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
29. **Nieva-Gomez, D., G. P. Roberts, S. Klevickis, and W. Brill.** 1980. Electron transport to nitrogenase in *Klebsiella pneumoniae*. Proc. Natl. Acad. Sci. USA **77**:2555–2558.
30. **Pühler, A., M. O. Aguilar, M. Hynes, P. Müller, W. Klipp, U. Priefer, R. Simon, and G. Weber.** 1984. Advances in the genetics of free-living and symbiotic nitrogen fixing bacteria, p. 609–619. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Nijhoff/Junk, The Hague, The Netherlands.
31. **Robson, R. L.** 1979. O_2-Repression of nitrogenase synthesis in *Azotobacter chroococcum*. FEMS Microbiol. Lett. **5**:259–262.
32. **Schetgens, T. M. P., J. G. J. Hontelez, R. C. van den Bos, and A. van Kammen.** 1985. Identification and phenotypical characterization of a cluster of *fix*-genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE. Mol. Gen. Genet. **200**: 368–374.
33. **Schrautemeier, B., H. Böhme, and P. Börger.** 1985. Reconstitution of a light-dependent nitrogen-fixing and transhydrogenase system with heterocyst thylakoids. Biochem. Biophys. Acta **807**:147–154.
34. **Yates, M. G.** 1972. Electron transport to nitrogenase in *Azotobacter chroococcum*: azotobacter hydroquinone as an electron donor. FEBS Lett. **27**:63–67.

Appendix to chapter 4

Identification of the MAC115 antigen in the NADH dehydrogenase complex DH1.

APPENDIX TO CHAPTER 4. Identification of the MAC115 antigen in the NADH dehydrogenase complex DH1.

Recently, a monoclonal antibody, MAC115, was described that reacts with a 55 kDa membrane protein of *R.leguminosarum* strain 3814 (1,2). To study a possible relationship between this 55K protein and the 58K subunit of NADH dehydrogenase complex DH1 of strain PRE, this complex was partly purified from isolated *R.leguminosarum* PRE bacteroid membranes as described in chapter 4. Proteins present in an FPLC-fraction enriched for the 58K polypeptide (usually fraction 18, see Fig. 2A, chapter 4), were separated on a 12.5 % SDS.PAA gel and stained with Coomassie Brilliant Blue (Fig. 1, lane a) or blotted onto a nitrocellulose filter (Fig. 1, lanes b and c).

Figure 1. Reaction of the 56K and 58K DH1 subunits with MAC115 and 58K antiserum.

Proteins present in a FPLC fraction, enriched in the 58K polypeptide (usually fraction 18, see Fig. 2A, chapter 4) were separated on a 12.5 % SDS.PAA gel. Electrophoresis of proteins through the stacking gel was carried out at 6 V/cm, and polypeptide separation in the separating gel was accomplished at 15 V/cm. After separation, proteins were stained with Coomassie Brilliant Blue (lane 1), or blotted onto nitrocellulose and incubated with MAC115 for 2 h followed by an overnight incubation with rabbit anti-rat immunoglobulin G (Nordic, Tilburg, The Netherlands, dilluted 1:1000) (lane b) or 58K antiserum (lane c). Immunocomplexes were visualized by reaction with ^{125}I-protein A and autoradiography. The positions of the 56K and 58K polypeptides are indicated by arrows. Molecular masses are in kDa.

As can be seen in figure 1, lane a, a major polypeptide band is present just below the 58K polypeptide band. This second polypeptide band, with an estimated molecular mass of 56 kDa, had not been observed in the experiments described in chapter 4 of this thesis and is only evident when the electrophoresis is carried out at a high voltage of about 15 V/cm. When a low voltage of about 3 V/cm is applied, as in the experiments described previously, only a band corresponding to a polypeptide of 58 kDa is visible (results not shown). Incubation of the blots with antisera shows that MAC115 reacts strongly with this 56K polypeptide, and not with the 58K polypeptide (Fig. 1 lane b), whilst the 58K antiserum reacts with the 58K

polypeptide and not with the 56K polypeptide (Fig. 1 lane c). This result shows that the 56K polypeptide, and not the 58K protein, is related to the 55K protein from strain 3841. The conclusion that the 58K PRE-polypeptide is a subunit of DH1 is based on the fact that it consistently copurifies with DH1 and that the 58K antiserum reacts with the 550 kDa complex DH1 on a Western blot. Since MAC115 also reacts with the intact 550 kDa DH1 complex of strain PRE (not shown), the 56K polypeptide probably also forms part of this NADH dehydrogenase.

The DNA encoding the 55K polypeptide of strain 3841 recently has been cloned in cosmid pIJ1639 (2). When this cosmid is introduced into *R.meliloti* B287, this strain gains the ability to produce the 55K polypeptide (N. Brewin, pers. comm.). To determine whether further polypeptides, identical to the DH1 subunits, are encoded by this cosmid, a Western blot containing polypeptides from *R.meliloti* B287/pIJ1639 was incubated with antisera directed against the 20K, 29K, 35K and 58K subunits of DH1 and with MAC115.

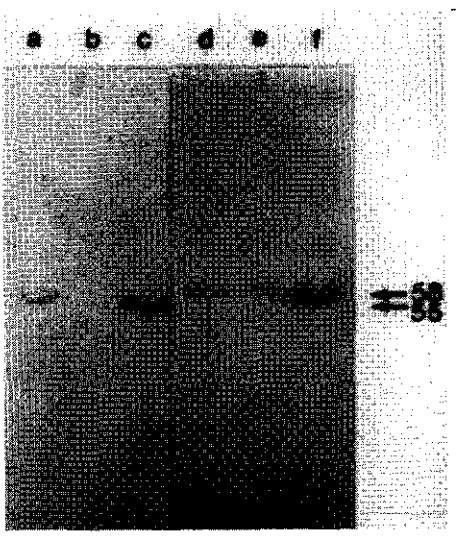

Figure 2. Reaction of proteins of *R.meliloti* B287/pIJ1639 with MAC115 and 58K antiserum.

Proteins present in *R.leguminosarum* 3841 (lanes a and d), *R.meliloti* B287 (lanes b and e) and *R.meliloti* B287/pIJ1639 (lanes c and f) were separated by SDS.PAGE on a 12.5% gel, transferred to nitrocellulose and incubated with MAC115 (lanes a,b and c) or 58K antiserum (lanes d, e and f). Immunocomplexes were visualized by reaction with goat anti-rat immunoglobulin G (IgG) coupled to alkaline phosphatase as described (1). The 55K and 58K polypeptides are indicated by arrows.

MAC115 recognizes a 55 kDa polypeptide of *R.leguminosarum* strain 3841 and of *R.meliloti* B287/pIJ1639, but not of *R.meliloti* B287 (Fig.2, lanes a-c). The 58K antiserum reacts with a 58 kDa polypeptide of all three strains (Fig.2 lanes d-f), but this polypeptide apparently is overproduced by *R.meliloti* B287/pIJ1639 harboring the cloned 55K-gene region.

Since the 58K antiserum does not react with the 56K polypeptide of strain PRE, these results show that in *R.leguminosarum* 3841 a 58 kDa polypeptide is present, which probably is

homologous to the 58K DH1 subunit of *R.leguminosarum* PRE and that this 58 kDa polypeptide is encoded on the same cosmid as the 55K polypeptide. No reactions of *R.meliloti* B287/pIJ1639 with the other antisera was observed (not shown), indicating that in strain 3841 polypeptides homologous to the other DH1 subunits of strain PRE are not encoded within the same cluster. The reaction of the 58K antiserum with a 58 kDa polypeptide of *R.meliloti* B287 (Fig. 2 lane e) shows that a polypeptide homologous to the 58K DH1 subunit supposedly is present also in *R.meliloti*. In contrast to the *R.leguminosarum* 3841 55K polypeptide (2), the *R.leguminosarum* PRE 58K polypeptide does not seem to be specific for *R.leguminosarum* strains. The 58K and the 56K polypeptides probably play an important role in *R.leguminosarum*, both during nitrogen fixation and in the free-living state. The 58K polypeptide was shown also to form part of a second NADH dehydrogenase, DH2, which is detectable both in bacteroids and free-living bacteria. The 56K polypeptide seems to be indispensable for free-living growth since no Tn5-mutants within the 55K-gene of *R.leguminosarum* 3841 could be obtained (N. Brewin, pers.comm.). Whether the 56K polypeptide also forms part of the second dehydrogenase DH2 is not known presently.

Literature cited

1. Bradley. D.J., Wood, E.A., Larkins, A.P., Galfre, G., Butcher, G.W. and Brewin, N.J.(1988). Isolation of monoclonal antibodies reacting with peribacteroid membranes and other components of pea root nodules containing *Rhizobium leguminosarum*. Planta *173*, p.149.
2. Brewin, N.J., Wood, E.A., Bradly, D.J., Harding, S.C., Sindu, S.S., Perotto, S., Kannenberg, E.L. and VandenBosch, K.A. (1988). The use of monoclonal antibodies to study plant-microbe interactions in the pea nodule, p.517. In Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds.) Nitrogen fixation: Hundred years after. Gustav Fischer, Stuttgart.

Chapter 5

A quest for the gene encoding the 35K DH1 subunit.

CHAPTER 5: A quest for the gene encoding the 35K DH1 subunit

5.1. Summary

In this chapter an attempt to isolate the gene encoding the 35K DH1 subunit (5) is described. The 35K polypeptide was purified and the sequence of the first 20 amino acids at the N-terminus was determined. Based on this sequence, oligodeoxynucleotide probes were synthesized and used to screen a *Rhizobium leguminosarum* PRE cosmid library. Four cosmids were isolated which showed a positive reaction with the oligos. None of these cosmids, however, appeared to encode the 35K polypeptide, but possibly code for polypeptides with sequence homology to part of the N-terminal sequence of the 35K-subunit. Computer analysis showed that one of these polypeptides is highly homologous to a cytoplasmatic NADH dependent ribitol dehydrogenase of *Klebsiella aerogenes*.

5.2. Introduction

Previously, we reported on the identification of a membrane bound bacteroid specific NADH dehydrogenase, DH1 (5, this thesis), in *R.leguminosarum* PRE. This enzyme complex consists of at least five different subunits, one of which, with a molecular weight of 35 kD (referred to as the "35K polypeptide"), appeared to be bacteroid specific. The other subunits were also identified in free-living bacteria.
The NADH dehydrogenase complex DH1 may play a role in electron transport to nitrogenase. This might be proven if *Rhizobium* mutants disturbed in the synthesis of the 35K polypeptide could be obtained. As a first step in the construction of such mutants, an attempt was made to isolate the gene encoding the 35K polypeptide. Even though we did not succeed in this, we show that the procedure followed can lead to the isolation of this gene.

5.3. Materials and Methods

5.3.1. Growth of bacterial strains and pea plants

Growth of *R.leguminosarum* PRE and pea plants, and inoculation of pea plants with bacterial strains was as described (5). Strains of *E.coli* used for cloning and sequencing purposes are listed in chapter 2 of this thesis.

5.3.2. Protein isolation

Isolation of *R.leguminosarum* PRE bacteroids, membrane extractions and partial purification of DH1 subunits were done as described (5) with the following modifications: nodules from approx. 1,200 pea plants were harvested at 21 days after inoculation. Bacteroid membranes (normal yield 50-100 mg of total membrane protein per 1,200 plants) were suspended in 50 mM TES-KOH, 5mM $MgSO_4$(pH 7.5) at a protein concentration of 20 mg/ml.
Membrane proteins were extracted with Triton X-100 and purified in four separate FPLC-runs as described (5). Samples from each run were analyzed by SDS.PAGE as described (5) and fractions enriched in the 35K polypeptide were pooled. The 35K subunit was further purified by preparative SDS.PAGE: approx. 100-200 µg of protein were loaded in a 10 cm slot on a 15% (w/v) SDS.PAA gel. Electrophoresis of the proteins through the 4% stacking gel was carried out at 6 V/cm, and separation in the 15% separating gel at 15 V/cm. After electrophoresis, proteins were electroblotted onto a PVDF membrane (Immobilon Transfer, Millipore) in ethylmorpholine buffer (25 mM 4-ethylmorpholine-formate, pH 8.3) for 18 h at 40 V. The PVDF membrane was soaked in methanol and subsequently equilibrated for 10 min in blotting buffer before use. After electroblotting, the filter was stained in 0.2% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol, 10% (v/v) acetic acid for 2 min and destained in 45% (v/v) methanol, 7% (v/v) acetic acid for 5-10 min. The 35K polypeptide band was cut from the blot with a razor blade, air-dried and stored at -20°C. The estimated yield of the 35K polypeptide was 10-20 µg/1,200 plants.

5.3.3. Amino acid sequence analysis

About 10 µg (280 pmol) of 35K polypeptide was blotted onto PVDF membrane and sequenced according to Matsudaira (8). For sequencing, the Applied Biosystems 470A Sequencer was used in combination with the Applied Biosystems 120A On-Line PTH Analyzer. Sequencing was done independently by Dr. Tim Hayes, Texas A & M University, USA and by Dr. Jan Hofsteenge, Friedrich Miescher Institute, Basel, Switzerland.

5.3.4. Oligodeoxynucleotide synthesis and labeling

Oligodeoxynucleotides (oligos) were synthesized using β-cyanoethyl phosphoramidites in a Cyclone DNA synthesizer (Bioresearch Inc.), dissolved in TE (10 mM Tris.HCl, 1 mM EDTA, pH 8.0) at a concentration of 0.5 mg/ml and stored at -20°C.
Labeling of oligos was carried out in a 25 µl reaction mixture containing 50 ng of oligo, 5 µl (50 µCi) γ-^{32}P-ATP, 2 units T4-polynucleotide kinase, 50 mM Tris.HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine and 0.1 mM EDTA at 37°C for 1 h. Oligos were separated from unincorporated label by centrifugation through 3 ml Sephadex G-200 fine columns at 1,000 rpm for 1 min in a Heraeus desktop centrifuge. The oligos were eluted from the columns by repeatedly washing with 50 µl TE at 1,000 rpm for 1 min. In a typical labeling experiment, the bulk of the labeled oligos was eluted from the column after seven washes. Before use, labeled oligos were heated at 100°C for 1 min to disrupt any DNA-DNA complexes.

5.3.5. Colony filter screening

For colony filter screening, cosmid library replicas were prepared as described in chapter 2 with the following modification: colonies were grown for 8 h at 37°C on NZY plates containing 100 µg/ml ampicillin, followed by a 14 h incubation at 37°C on NZY plates containing 100 µg/ml ampicillin and 10 µg/ml chloramphenicol in order to increase the cosmid copy number. Replica filters were pre-hybridized in 5 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 10 x DH (Denhardt's Solution, 10 x DH = 2 gr Ficoll, 2 gr polyvinylpyrrolidone, 2 gr BSA/l), 10 mg/ml sonicated single stranded salmon sperm DNA at 65°C for 6 h. The prehybridization solution, minus the salmon sperm DNA, was filtered through a 0.22 m filter prior to use.

Hybridization conditions were determined according to Davis *et al.* (2): hybridization of the filters was carried out in 3 ml/filter of 5 x SSC, 10 x DH (filtered through a 0.22 m filter prior to use), containing approx. 2 x 10^5 cpm of labeled oligos. The filters were incubated at a temperature gradient of 65-35°C (oligo 1) or 65-40°C (oligos 2+3) for approx. 4 h followed by an incubation at 35°C (oligo 1) or 40°C (oligos 2+3) for 14 h. Temperature gradients were applied by gradually cooling-down a 65°C water bath in approx. 4 h to the desired temperature. After hybridization, filters were washed once in 5 x SSC for 15 min at 35°C (oligo 1) or 40°C (oligos 2+3), followed by three washes in 5 x SSC for 10 min at 25°C (oligo 1) or 30°C (oligos 2+3). After drying, hybridizing spots were visualized by autoradiography using Kodak XAR film. In a typical experiment, films were exposed at -80°C for 2 to 4 days using an intensifying screen.

5.3.6. DNA methodology

Hybridization of Southern blots containing cosmid DNA or subcloned cosmid fragments with oligo probes was done as described for colony filters (see paragraph 5.3.5.). Hybridization of total genomic DNA was carried out in a temperature gradient from 65°C to 25°C during 4 h, followed by 14 h at 25°C. Blots were washed at 25°C, and after autoradiography more stringent washes (30, 35 and 45°C) followed by autoradiography were used to study the stability of the observed DNA-DNA hybrids. All further techniques, like nucleotide sequence analysis, cosmid library construction and hybridization with DNA probes, were done as described in chapters 2 and 3. Analysis of DNA sequences was performed on a microVax/VMS computer using the Staden DNA sequence analysis programs (10). Identified amino acid sequences were compared with sequences stored in the NBRF Protein Library using the CAMMSA computer facility of the Catholic University of Nijmegen, the Netherlands.

5.4. Results and Conclusions

5.4.1. N-terminal amino acid analyses and oligo synthesis

In order to isolate the gene coding for the 35K DH1 subunit, the 35K polypeptide was purified from Triton X-100 treated bacteroid membranes by anion exchange FPLC followed by preparative SDS.PAGE. The polypeptide was electroblotted onto PVDF membrane and the N-terminal amino acid sequence was determined in two independent experiments using a Gas Phase Sequenator. This resulted in the identification of the first 20 amino acids of the 35K protein (see Fig. 1). The amino acid at position 1 could not be assigned unambiguously, but probably is alanine, or alternatively either serine or glycine. The amino acid at position 7 could not be determined, as it gave a "blank" chromatogram, which can either result from an amino acid that has undergone post-translational modification, or from oxidation of the amino acid. Since blanks in amino acid sequencing often are due to a cysteine residue (J. Hofsteenge, pers. comm.), we have assumed a cys-residue at position 7.

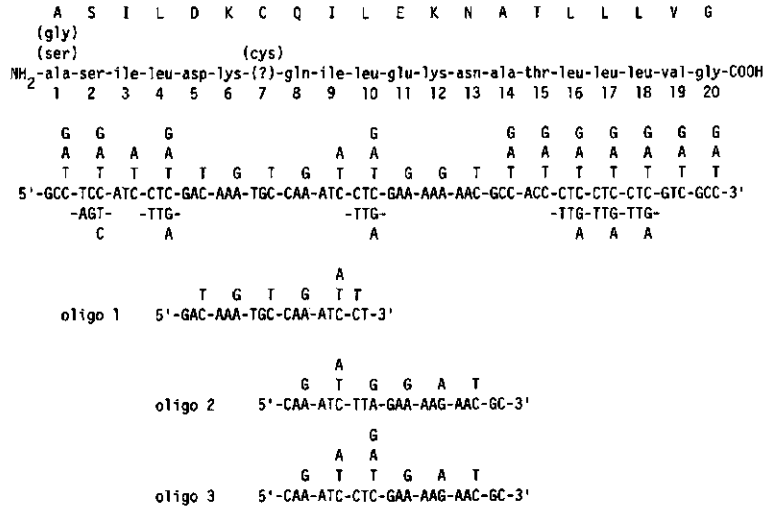

Figure 1. N-terminal amino acid sequence of the 35 K DH1 subunit.

The first 20 amino acids at the N-terminus of the 35K polypeptide are shown, as well as all the possible nucleotide sequences matching this amino acid sequence. Also shown are the nucleotide sequences of the oligodeoxynucleotide probes 1, 2 and 3.

Based on known examples of successful isolations of genes using oligodeoxynucleotide probes (4,6,9 and ref. therein), and taking into account the redundancy of the genetic code, oligos were synthesized corresponding to parts of the deduced nucleotide sequence as shown in Fig. 1. The equimolar mixture of all 96 different oligodeoxynucleotides starting at the first nucleotide of the asparagine codon at position 6 and ending at the second nucleotide of the leucine codon at position 10, is referred to as "oligo 1". These 17-mers contain the cysteine codon (position 7) which is not present with certainty in the 35K gene (see above). Since the presence of a "wrong" triplet in the oligos might prevent hybridization with the 35K-gene, two further oligodeoxynucleotide mixtures were synthesized, oligos 2 and 3, which together constitute the 288 possible oligodeoxynucleotides running from the first nucleotide of the glutamine codon at position 8 up to the second nucleotide of the alanine codon at position 14. These oligos are 20 nucleotides long and lack the cysteine codon.

5.4.2. Cosmid library screening and nucleotide sequence analysis of DNA regions hybridizing with the 35K-oligos

Oligo 1 and the equimolar mixture of oligos 2 and 3 (referred to as "oligo 2+3") were labeled with ^{32}P and used to screen a *R.leguminosarum* PRE cosmid library. This library contains 40-50 kb DNA fragments cloned in cosmid vector pJB8 and consists of approx. 2,000 *E.coli* 1046 colonies, statistically representing 20 times the total *R.leguminosarum* genome. Four clones present in the library hybridized, albeit at a very low level, with oligo 1. These clones were designated 11, 121, D21 and D31. Only the latter two also hybridized with oligo 2+3. Screening the library with oligos 2+3 revealed four clones hybridizing at a low level: the already mentioned clones D21 and D31, and clones 232 and 234, which hybridized solely with oligo 2+3.
Cosmids from the hybridizing clones were isolated (designated p11, p121, pD21, pD31, p232 and p234) and analyzed by restriction enzyme digestion and agarose gel electrophoresis (not shown). The restriction fragment patterns of cosmids p232 and p234 showed that these two cosmids contain overlapping insert fragments. This was also the case for cosmids pD31 and p121. None of the other cosmids showed any similarity in restriction fragment patterns and therefore probably all contain inserts derived from different parts of the *R.leguminosarum* genome. Hybridization of Southern blots containing EcoRI digested cosmid DNA with the oligo probes gave a somewhat different result compared to the colony filter hybridization (see table 1). None of the cosmids, which seemed to react with both oligo 1 and oligo 2+3 on the colony filter, reacted with both probes on a Southern blot. Cosmid pD21 hybridized only with

oligo 1 and pD31 only with oligo 2+3. Instead, cosmid p11 hybridized on Southern blots with both oligo 1 and oligo 2+3 at a similar level. The fact that the results obtained in the colony filter hybridization differ from those obtained with Southern blots is probably caused by the high background hybridization on the colony filters, which hampers an unambiguous interpretation of the experiment.

To identify the DNA sequences in the cosmids causing the hybridization with the oligo probes, the following strategy was used: Cosmids were digested with EcoRI and analyzed by Southern hybridization. Hybridizing EcoRI fragments were subcloned in pTZ19 or pJB8 and the resulting plasmids were digested with either a restriction enzyme recognizing a four basepair sequence (4-cutters), or subjected to a double digestion with two 6-cutters. By Southern hybridization of the digested plasmids with the oligos, hybridizing DNA fragments were selected with sizes between approx. 200 and 500 bp. These fragments were isolated and cloned in the sequencing vectors pTZ18 or pTZ19, and the nucleotide sequence of the entire fragment was determined. Alternatively, the sequence was determined up to a region showing a high degree of homology with the hybridizing oligo. The recombinant plasmids constructed in this way and their characteristics are listed in table 1.

Hybridization of cosmid DNA digested with various restriction enzymes showed that cosmids p121, p232 and pD21 contain only one single region reacting with one of the oligos. These regions were cloned in the plasmids pT6, pH15 and pDE4H4 respectively (see table 1). Cosmid p11, which was shown to hybridize with both oligos, contains several regions homologous to the oligos: hybridization of a blot containing EcoRI-digested cosmid p11 DNA showed that the sequences homologous to both oligo 1 and oligo 2+3 are located within the same 5.7 kb EcoRI-fragment. This fragment was subcloned in plasmid p11E4. Further analysis showed that within the 5.7 kb EcoRI- fragment, three regions are homologous to one of the oligo probes. A 330 bp HaeIII fragment, cloned in plasmid p11E4H10, hybridized only with oligo 1. Two other fragments, cloned respectively in p11E4S-II(R) and p11E4Sal2Δsp, hybridized only with oligo 2+3 and not with oligo 1. The localization of these fragments within the 5.7 kb EcoRI-fragment is not known.

Table 1. Pedigree of cloned DNA-fragments reacting with 35K oligo probes

cosmid	reacting mixture of oligos*	hybridizing EcoRI-fragment subcloned in:	sequenced fragment of the EcoRI-subclones
p121	oligo 1	5.0 kb EcoRI-fragment in pJB8 = p121/1	350 bp TaqI-fragment in pTZ19R = pH15
p232	oligo 2+3	4.3 kb EcoRI-fragment in pTZ19R = p232/2A	340 bp HaeIII-fragment in pTZ19R = pH15
p11	oligo 1 oligo 2+3	5.7 kb EcoRI-fragment in pTZ19R = p11E4	330 bp HaeIII-fragment in pTZ19R, reacts with oligo 1 = p11E4H10 570 bp Sau3AI-fragment in pTZ19R, reacts with oligo 2+3 = p11E4S-II 570 bp Sau3AI-fragment in pTZ18R, reacts with oligo 2+3 = p11E4S-IIR 440 bp SphI-SalI-fragment in pTZ19R, reacts with oligo 2+3 = p11E4SalΔsp
pD21	oligo 2+3	Approx. 10 kb EcoRI-fragment in pTZ19R = pDE4	Approx. 400 bp HaeIII-fragment in pTZ19R = pDE4H4

*The equimolar mixture of all 96 different 17-mers, explained in Fig. 1, is indicated as "oligo 1", whereas the equimolar mixture of all 288 different 20-mers is indicated as "oligo 2+3".

In Fig. 2 the nucleotide sequences of parts of the cloned fragments homologous to the oligo probes are shown. None of the isolated cosmids contains a nucleotide sequence which can encode the first 20 amino acids of the 35K polypeptide. Most of the fragments show a high homology to the 35K sequence contained within the oligos. For example, part of the DNA fragment cloned in pT6 is 77 % homologous to one of the 48 oligos present in the oligo 1 mixture, whereas part of the DNA fragment cloned in p11E4Sal2Δsp shows 80 % homology to one of the 288 different oligos of the mixture of oligo 2+3. In plasmid pH15, two regions were detected which are partly homologous to the corresponding oligo. Outside the regions homologous to the oligos the homology between the cloned fragments and the deduced 35K-nucleotide sequence is very low (see Fig. 2).

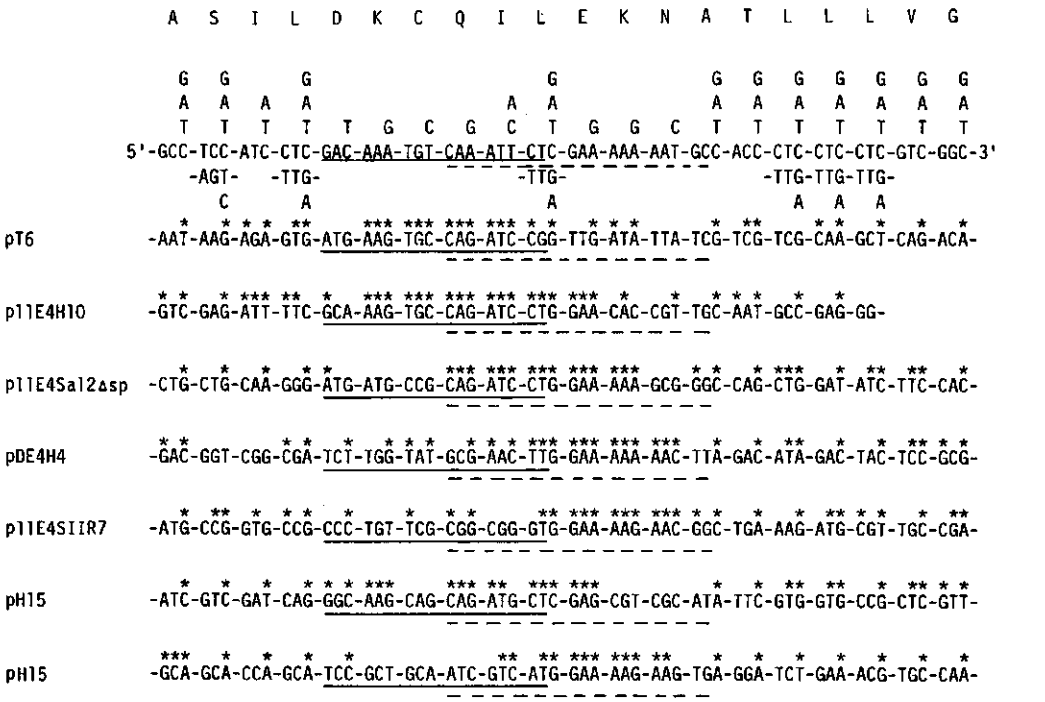

Figure 2. Comparison of cloned nucleotide sequences with the nucleotide sequence deduced for the 35K gene.

The comparison between the deduced 35K gene nucleotide sequence and the cosmid regions hybridizing with the oligodeoxynucleotide probes is shown. Nucleotides matching the deduced sequence are indicated by asterisks. The position of oligo 1 is indicated as (—), the position of oligo 2+3 as (----). For plasmid pH15, both regions identified to be homologous to oligo 2+3 are shown.

To investigate whether the cloned DNA fragments are part of genes coding for known proteins, the corresponding amino acid sequences deduced from the nucleotide sequences of the subclones were compared with amino acid sequences stored in the National Biomedical Research Foundation (NBRF) protein library. Only for the fragment cloned in p11E4Sal2Δsp a relevant homology was found. Analysis showed that the polypeptide theoretically encoded by this subclone is partly homologous to the *rbt*D gene product (ribitol dehydrogenase) of *Klebsiella aerogenes* (3,7), see Fig.3. The region from residue 15 to 33 shows a moderate homology with the ribitol dehydrogenase sequence. In this region the amino acid sequence QILEK (Fig. 3, underlined), encoded by oligo 2+3, is located (Fig. 3, underlined). Starting from residue 16, the p11E4Sal2Δsp amino acid sequence is highly homologous to the ribitol dehydrogenase. This homology continues for 27 amino acids to the extreme right hand part of the known sequence.

```
                53                                                  112
      rbtD      KLVAELGENAFALQVDLMQADQVDNLLQGILQLTGRLDIFHANAGAYIGGPVAEGDPDVW
                X   .   .XX ... X..... XX. .X.XXXXXX.X.XXXX X .XXXX.X
      p11E4Sal2Δsp FESALPRRSLEGNLIDLTDPKSVEGMMPQILEKAGQLDIFHANVGSYIGGEVLGGDPDAW
                1                                                   60
```

Figure 3. Homology between clone 11E4Sal2Δsp and *K.aerogenes* ribitol dehydrogenase.

The homology between the amino acid sequence encoded by clone 11E4Sal2Δsp and part of the amino acid sequence (residues 53 to 112) of *K.aerogenes* ribitol dehydrogenase is shown. Identical amino acids are indicated by "x", analogous amino acid substitutions are indicated by ".". Underlined: position of oligo 2+3.

5.4.3. Total genomic DNA analysis

Since the gene encoding the 35K polypeptide was not detected by screening the cosmid library with the oligodeoxynucleotide probes, it was of interest to know whether further sequences in *R.leguminosarum* are homologous to the oligos, which were not identified in a screening of the library. Therefore, total genomic *R.leguminosarum* DNA was digested with various restriction enzymes, blotted onto nitrocellulose and hybridized with oligo 1 and oligo 2+3 under low stringent conditions (hybridization in 5 x SSC in a 65-25°C gradient, washing at 25°C , 5 x SSC). As can be seen in Fig. 4, many hybridizing fragments are evident, especially when oligo 2+3 is used. The most prominent EcoRI-bands hybridizing with oligo 1 correspond to fragments of ~10, 5.5, 4.3, 2.5 and 2.2 kb (Fig. 1 lane d). The fragment of 5.5 kb may correspond to the 5.7 kb fragment cloned in plasmid p11E4, whilst the other fragments were not identified before. The reason why the 5.0 kb fragment cloned in plasmid p121/1 is not visible on the chromosomal blot is unknown. Oligo 2+3 reacts most strongly with fragments of ~10, ~8, 6.4, 5.5 and 2.5 kb (Fig. 1 lane h). The fragments of 10 and 5.5 kb might be identical to those cloned in pDE4 and p11E4 respectively. All other fragments have not been observed before. The 4.3 kb EcoRI insert of p232/2A probably is visible only as a weakly hybridizing band, possibly due to the rather low level of homology between oligo 2+3 and this DNA fragment (see Fig. 2, pH15). Also at more stringent conditions (washes in 5xSSC at 30 and 35°C) the described hybridizing fragments were visible, albeit at a lower intensity (not shown).

Only three EcoRI-fragments seem to react with both oligo 1 and oligo 2+3; the 2.5 kb fragment, the 5.5 kb fragment and the 10 kb fragment, although for the latter it is difficult to compare exactly the length of the ~10 kb DNA fragment hybridizing with oligo 1 and the ~10 kb fragment hybridizing with oligo 2+3 in the experimental conditions used. Since the 35K gene is supposed to react with both oligos, and the 5.5 kb fragment has probably already been

cloned in p11E4, the 2.5 kb fragment in the first place and next the 10 kb fragment may be considered to be candidates to encode the gene for the 35K protein.

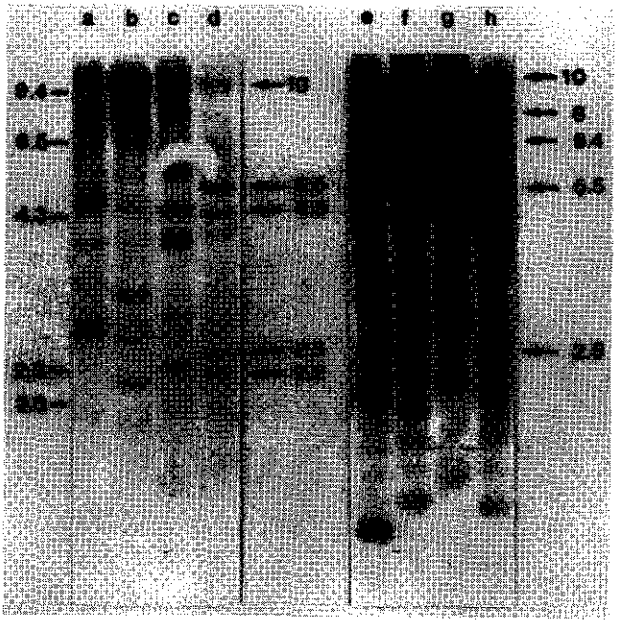

Figure 4. Hybridization of *R.leguminosarum* genomic DNA with 35K-oligodeoxynucleotide probes.

R.leguminosarum genomic DNA was digested with XhoI (lanes 1 and 6), PstI (lanes 2 and 7), HindIII (lanes 3 and 8), EcoRI (lanes 4 and 9) and BamHI (lanes 5 and 10), separated on a 1.5 % agarose gel, transferred to a nitrocellulose filter and hybridized with oligo 1 (lanes 1 to 5) and oligo 2+3 (lanes 6 to 10). The resulting autoradiogram is shown. Hybridizing EcoRI fragments are indicated by arrows. Fragment length is in kb.

5.5. Discussion

One of the possible strategies to isolate a gene coding for a known protein, is the determination of (parts of) the amino acid sequence and the subsequent screening of gene libraries with oligodeoxynucleotide probes deduced from the amino acid sequence. This approach has led already to the identification and isolation of several eukaryotic and prokaryotic genes (for examples, see 9 and 11 and references therein). The power of this strategy has significantly increased by the development of techniques for sequencing picomole quantities of protein blotted onto glass fiber sheets or polyvinylidene difluoride (PVDF) membranes (8), which allows the amino acid analysis of very small amounts of protein. Even protein quantities, for example, recovered after two dimensional gel electrophoresis have been shown sufficient for a successful amino acid analysis (1).

In this chapter an attempt is described to isolate the gene encoding the 35K DH1 subunit, using the above mentioned method. The 35K polypeptide was isolated from nitrogen fixing *R.leguminosarum* bacteroids and blotted onto PVDF membrane. Sequencing the N-terminus

of the protein resulted in the identification of the first 20 amino acids of the polypeptide. Based on this sequence two partly overlapping degenerated oligodeoxynucleotides, oligo 1 and oligo 2+3, were synthesized and used to screen a *R.leguminosarum* cosmid library. Three cosmids were identified which hybridize only with one of the used oligos, whereas one, cosmid p11, was found reacting with both oligos. This cosmid, however, was shown to contain separate regions reacting with oligo 1 and oligo 2+3. Sequence analysis of the cloned regions hybridizing with the oligos, however, showed that none of the cosmids encodes the 35K polypeptide.

To investigate whether the identified *R.leguminosarum* DNA fragments are parts of genes encoding known proteins, the deduced amino acid sequences were compared with sequences present in the NBRF Protein library. Also the N-terminal amino acid sequence of the 35K protein was included in this comparison. No significant homology was detected using this 20 amino acid sequence. Comparison of the sequenced cosmid regions only revealed a significant homology of subclone 11E4Sal2Δsp, derived from cosmid p11, with ribitol dehydrogenase of *K.aerogenes*. Since this dehydrogenase uses NADH as a cofactor, the sequence identified in cosmid 11 probably encodes a rhizobial NADH dehydrogenase. As this dehydrogenase was identified with oligo probes deduced from the 35K dehydrogenase subunit, it may tentatively be suggested that the 35K subunit posseses NADH dehydrogenase activity in complex DH1. Some support for this suggestion may be found in the occurrence of a 35 kDa protein in *B.japonicum* that is only present in bacteroids and not in free-living bacteria and probably has NADH dehydrogenase activity (G. Stacey, pers. comm.). Whether the *Rhizobium* 35K polypeptide, however, is homologous to the *Bradyrhizobium* protein is not known presently.

The experiments discussed in this chapter show that the followed procedure can lead, in principle, to the isolation of the 35K gene: the sequences isolated by screening the cosmid library show a homology varying from 55% to 80% with the oligos used, indicating that under the conditions applied hybrids between the 35K gene and the oligos will probably be formed.

The lack of success in isolating the 35K gene in a first screening of the gene library may be sought in the rather poor signal-background ratio on the obtained autoradiograms after colony filter screening. This made it very difficult to discriminate proper hybridization signals from background and therefore weak but significant signals may easily have been missed. Rescreening the library and further isolation of (weakly) hybridizing cosmids should result in the cloning of the gene. The availability of oligo probes corresponding to other parts of the polypeptide could be very helpful in this. Although the oligos used have a statistical probability of occurring only once in every 2×10^8 (oligo 1) or 1.5×10^9 (oligo 2+3) base pairs, and the genome size of *R.leguminosarum* is approx. 4×10^6 base pairs, several regions

are present in *R.leguminosarum* that are highly homologous to these oligos. This shows that (part of) the determined 35K amino acid sequence probably represents a functional protein domain present in several *Rhizobial* polypeptides. Oligos derived from less conserved parts of the protein may help to reduce the number of cosmids that have to be investigated.
Hybridization of a blot containing EcoRI-digested total genomic *Rhizobium* DNA showed the presence of at least two fragments which had not been identified in the cosmid library screening and which show a hybridization with both oligos. These fragments are good candidates for containing the 35K gene. Instead of screening the cosmic library, purification of these fragments from an agarose gel containing digested total genomic DNA might be a good alternative.

5.6. Acknowledgment

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5.7. Literature cited

1. Bauw, G., de Loose, M,. Inzé, D., van Montagu, M. and Vanderkerckhove, J. (1987). Alterations in the phenotype of plant cells studied by NH_2- terminal amino acid-sequence analysis of proteins electroblotted from two-dimensional gel-separated total extracts. Proc. Natl. Acad. Sci. USA *84*, p.4806.
2. Davis, G.D., Dibnes, M.D. and Battey, J.F. (1986). Basic methods in molecular biology, Elsevier, Amsterdam, the Netherlands.
3. Dothie, J.M., Giglio, J.R., Moore, C.B., Taylor, S.S. and Hartley, B.S. (1985). Ribitol dehydrogenase of *Klebsiella aerogenes*. Sequence and properties of wild-type and mutant strains. Biochem. J. *230*, p.569.
4. Itakura, K., Rossi, J.J. and Wallace, R.B. (1984). Synthesis and use of synthetic oligodeoxynucleotides. Ann. Rev. Biochem. *53*, p.323.
5. Klein Lankhorst, R.M., Katinakis, P. Van Kammen, A. and Van den Bos, R.C. (1988). Identification and characterization of a bacteroid- specific dehydrogenase complex in *Rhizobium leguminosarum* PRE. Appl. Environ. Microbiol. *54*, (12), p.3008.
6. Lathe, R. (1985). Synthetic oligodeoxynucleotide probes deduced from amino acid sequence data. Theoretical and practical considerations. J. Mol. Biol. *183*, p.1.
7. Loviny, T., Norton, P.M. and Hartley, B.S. (1985). Ribitol dehydrogenase of *Klebsiella aerogenes*. Sequence of the structural gene. Biochem. J. *230*, p.579.
8. Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. *262*, (21), p.10035.
9. Reyes, A.A. and Wallace, R.B. (1984). Use of synthetic oligodeoxynucleotide hybridization probes for the characterization and isolation of cloned DNAs. In Setlow and Hollaender (eds.) Genetic Engineering *6*, p.157.
10. Staden, R. (1982). Automation of the computer handling of gel reading data produced by the shotgun method of DNA sequencing. Nucleic Acids Res. *10*, p.4731.
11. Voordouw, G., (1988). Cloning of genes encoding redox proteins of known amino acid seqeunce from a library of the *Desulfovibrio vulgaris* (Hildenborough) genome. Gene *69*, p.75.

Chapter 6

Bacteroid-encoded proteins are secreted into the peribacteroid gene by *Rhizobium leguminosarum*

(Plant Molecular Biology)

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Bacteroid-encoded proteins are secreted into the peribacteroid space by *Rhizobium leguminosarum*

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Abstract

Bacteroids of *Rhizobium leguminosarum* in root nodules of *Pisum sativum* are enclosed by a plant-derived peribacteroid membrane (PBM). The contents of the interstitial peribacteroid space (PBS) between bacteroid membrane and PBM were isolated by a controlled osmotic shock of PBM-enclosed bacteroids and analysed by two-dimensional gel electrophoresis. Silver staining revealed approximately 40 PBS polypeptides. *Ex planta* ^{35}S-methionine labeling of PBM-enclosed bacteroids revealed that about 90% of the PBS proteins are synthesized by the bacteroid. Approximately 30% of the PBS polypeptides are common between the PBS and the periplasmic space of free-living bacteria; one (38 kDa) PBS protein is also excreted by free-living bacteria in the bacterial culture medium. At least four bacteroid-encoded PBS polypeptides were clearly identified as symbiosis-specific.

Introduction

Bacteria of the genus *Rhizobium* are capable of infecting plants of the family Leguminosae and induce nitrogen-fixing root nodules [17]. In their symbiotic, so-called bacteroid form, the rhizobia reside in the infected nodule cell cytoplasm enclosed by a membrane envelope, the peribacteroid membrane (PBM) [6, 15, 22]. This membrane is derived from the plant plasma membrane [15, 16, 21], and there is evidence suggesting that the endoplasmic reticulum and Golgi bodies play a role in the further synthesis of the PBM [6, 12, 16]. Some of the protein components of the PBM have been found to be nodule-specific [8, 9, 11] and may perform essential functions imposed by the symbiosis. The function(s) of the compounds in the peribacteroid space (PBS) between the envelope and the bacteroids are still largely unknown. High levels of α-mannosidase activity have been detected in the PBS of *R. japonicum* [13]. It has been proposed that the PBS contains factors that have a role in the transport of various compounds to (amino acids, carbon compounds, oxygen) and from (heme and micronutrients) the bacteroids [21]. We show that the PBS contains a discrete set of proteins most of which are synthesized by *Rhizobium*. At least 30% of the PBS proteins are common with proteins present in the periplasmic space (PS) or in the culture medium of free-living rhizobia. Approximately 10% are symbiosis-specific and thus are to be considered as bacteroidins [20].

Materials and methods

Polyvinyl pyrollidone and sodium metaperiodate were obtained from Merck. All other chemicals were purchased from Sigma. *Staphylococcus aureus* cells were obtained from Pharmacia Fine Chemicals and Aurodye from Janssen. Miracloth was from Calbiochem, nitrocellulose filter (B 85) from Schleicher and Schuell, and X-ray films (XAR-5) were from Kodak. ^{35}S-methionine (1000 Ci/mmol) was obtained from Amersham and Na^{125}I from New England Nuclear.

Cultivation of plants

Peas (*Pisum sativum* cv. Rondo) were grown as described by Bisseling *et al.* [13]. *Rhizobium leguminosarum* PRE was used for inoculation; nodules were harvested at 17 days after inoculation and used immediately.

Isolation of PBM-enclosed bacteroids, PBS and PS fractions

Bacteroids enclosed by the peribacteroid membrane were isolated as described by Brewin *et al.* [6] with the following modifications. Nodules from 50 plants were homogenized by grinding in 2 ml isolation buffer [50 mM (2N-morpholino)-ethane sulfonic acid (MES)-KOH, pH 7.3, 2.5 mM $MgCl_2$, 0.6 M sucrose and 4% (w/v) polyvinyl pyrollidone (PVP)]. All procedures were carried out at 4 °C.

The homogenate was filtered through two layers of Miracloth, transferred to 1.5 ml microfuge tubes and centrifuged at 3000 *g* for 5 min. The pellet was gently resuspended in 1 ml isolation buffer; 0.5 ml portions were layered over 0.6 ml of a 1.2 M sucrose cushion in isolation buffer and centrifuged at 10000 *g* for 30 s. The interphase and the 0.6 M sucrose layer were removed and centrifuged for 5 min at 3000 *g*. The pellet containing PBM-enclosed bacteroids was gently resuspended in isolation buffer without PVP at a density corresponding to $OD_{600}=20$. Thereupon the PBM-enclosed bacteroids were osmotically shocked by the addition of 50 mM Tris-HCl, pH 7.3 to give a final sucrose concentration of 0.12 M, left on ice for 2 min and centrifuged at 10000 *g* for 10 min. The bacteroid pellet resulting after the osmotic shock was resuspended in isolation buffer without PVP and constitutes "PBS-depleted" bacteroids. The supernatant was centrifuged at 10000 *g* for 30 min to remove residual bacteroids and the supernatant was retained as the PBS fraction. PS proteins were isolated from free-living bacteria grown in TY medium (5 g tryptone, 3 g yeast extract, 1.3 g $CaCl_2$ per liter, supplemented with 1 mg/l uracil) as described by De Maagd and Lugtenberg (1987).

Protein labeling of PBM-enclosed bacteroids

Proteins of PBM-enclosed bacteroids were labeled with ^{35}S-methionine as described by Van den Bos *et al.* [19] with the following modifications. Incubations were carried out at 29 °C for 30 min in a mixture (0.5 ml) containing 0.6 M sucrose, 50 mM sodium succinate, 50 mM MES-KOH, 2.5 mM $MgCl_2$, pH 7.3, 0.05 ml PBM-enclosed bacteroids and 50 μCi ^{35}S-methionine. Bacteroids and PBS-fraction were prepared as described above. Incorporation of radioactivity into TCA-precipitable material was measured as previously described [19].

Polyacrylamide gel electrophoresis, Western blotting and immunological protein detection, immunoprecipitations

Polypeptides were separated by electrophoresis on 15% (w/v) denaturing polyacrylamide slab gels or by two-dimensional (2D) gel electrophoresis as described by Govers *et al.* [10]. Proteins were transferred to nitrocellulose filters (Western blots) and immunological detection of proteins was performed as described by Bisseling *et al.* [4]. Western blots of the 2D gel electrophoresis were stained with Aurodye and then the ^{35}S-labeled proteins were visualized by autoradiography. ^{35}S-methionine-labeled proteins were immunoprecipitated with antiserum specific against the PBS fraction raised in rabbits [10]. Glyco-groups were removed from blotted proteins

by treatment of the blot with sodium metaperiodate [6].

Results

Characterization of PBS proteins by 2D electrophoresis

Bacteroids enclosed in peribacteroid membranes were isolated in a buffer containing 0.6 M sucrose as described in Materials and Methods. By lowering the osmolarity of the medium the PBM was ruptured and the contents of the PBS were liberated. Two-dimensional gel electrophoresis revealed that the PBS fraction contained a discrete set of proteins shown in Fig. 1a and b. The patterns shown in Fig. 1 represent the proteins from the PBS fraction of 17-day-old pea root nodules, which have maximum nitrogen-fixing activity. Upon incubation of PBM-enclosed bacteroids for 30 min with ^{35}S-methionine the PBS proteins became rapidly labeled indicating that they are actively synthesized and excreted into the PBS. The rate of the total incorporation of ^{35}S-

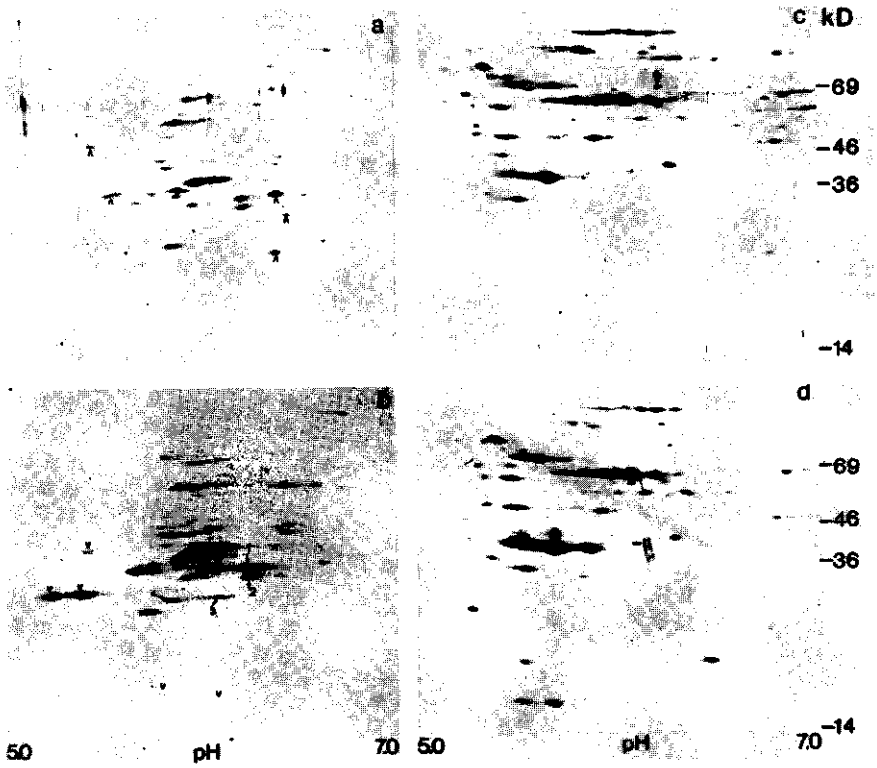

Fig. 1. Analysis of the *ex planta* synthesized and *in vivo* PBS proteins by two-dimensional gel electrophoresis. Intact PBM-enveloped bacteroids were incubated with ^{35}S-methionine and bacteroids and PBS fraction were prepared. PBS proteins (a, b) (20000 cpm) and bacteriod proteins (c, d) (95000 cpm) were analyzed on two-dimensional gels, blotted on nitrocellulose filters, stained with Aurodye (a, c) and autoradiographed (b, d). Labeled spots in (b) which are not found in the stained pattern (a) are pointed out as (V), stained spots in (a) which were not found in the labeled pattern (b) are pointed out as (Λ); spots present in the labeled and stained patterns (a, b) but absent from the PS of free-living bacteria (bacteroidins) are indicated by numbered arrows.

methionine was critically dependent on the presence of an intact PBM. If bacteroids enclosed in PBM were subjected to the osmotic shock to rupture the PBM before labeling, the total incorporation of ^{35}S into proteins decreased approximately tenfold. Since the osmotic shock had such a drastic effect on the activity of the bacteroids we examined if the proteins detected in the PBS fraction belonged to the bacteroids and were released from the bacteroids by the osmotic shock. Labeled PBS proteins were separated on a 2D-gel and transferred to a nitrocellulose filter by electroblotting. The proteins on the filter were stained with Aurodye and thereby 40–50 major spots were shown (Fig. 1a). For comparison total proteins of bacteroids isolated after the osmotic shock in the same experiment were also analysed (Fig. 1c). As the number of proteins in bacteroids is very much larger than in the PBS, the bacteroid preparation used for the 2D gel electrophoresis was taken to contain five times as much incorporated ^{35}S label as the corresponding PBS fraction in order to ensure that minor proteins were also detectable. Figure 1 shows that the PBS fraction contains a discrete set of proteins. Even the most prominent bacteroid proteins are not detectable in the PBS fraction; *vice versa* the pattern of bacteroid proteins is free of PBS polypeptides. To investigate if the PBS fraction contained proteins derived from the PBM upon rupture by the osmotic shock, the PBS fraction was examined for the presence of PBM fragments. The PBS fraction was centrifuged at 80000 *g* for 60 min and the putative pellet subsequently analyzed by SDS gel electrophoresis for the presence of PBM proteins. No silver-stained bands were visible indicating that the PBS fraction was essentially devoid of PBM fragments.

By superimposing the autoradiogram of the 2D pattern of the labeled PBS proteins (Fig. 1b) on the corresponding blot stained with Aurodye (Fig. 1a) it appeared that the majority of the stained spots coincide with spots on the autoradiogram with only few exceptions as pointed out in Fig. 1a. This indicates that most of the stable PBS proteins are synthesized and excreted in the *ex planta* conditions employed. With a few exceptions (as indicated in Fig. 1b), all labeled PBS products are also detectable on the stained blot (Fig. 1a). The stained and labeled bacteroid protein patterns (Fig. 1c and d) also appear to be very similar although much more complex than those of the PBS.

If the labeling with ^{35}S-methionine was performed with 150 μg/ml cycloheximide in the incubation medium to inhibit synthesis of proteins by plant 80S ribosomes there was no reduction of the level of ^{35}S incorporation and the patterns of stained or labeled proteins was unaltered. On the other hand the addition of 50 μg/ml chloramphenicol to the incubation medium completely inhibited the incorporation of ^{35}S-methionine into protein (results not shown). The proteins characteristic of the PBS appear therefore to be synthesized by *Rhizobium* and subsequently secreted into the PBS.

To ascertain if some of the proteins found in the PBS were synthesized by *Rhizobium* only under symbiotic conditions and might be called bacteroidins [20] a comparison was made with proteins from free-living *Rhizobium* bacteria separated on a 2D gel under the same conditons (Fig. 2a). Superimposition of this gel on the 2D pattern of silver-stained PBS proteins showed that approximately 30% of the most abundant PBS proteins coincide with bacterial proteins. The corresponding proteins appeared to be localized in the free-living *Rhizobium* bacteria in the periplasmic space (PS). This was shown by analyzing the bacterial PS fraction isolated by EDTA treatment (Fig. 2b). Comparison of this 2D gel pattern of the PS proteins with that of the PBS proteins (Fig. 1a) proved that most of the PBS proteins that are common with bacterial proteins correspond to PS proteins. Three of the most prominent of these are indicated in Fig. 2b. We estimate that a total of 10–15 of the PBS proteins coincide with PS proteins. A number of PBS proteins, labeled *ex planta* and bacteroid derived do not comigrate with total bacterial or PS proteins; the most abundant four are indicated by numbered arrows in Fig. 1b. These polypeptides are obviously symbiosis-specific *Rhizobium*-encoded proteins or bacteroidins [20].

A 38 kDa polypeptide is detectable in the PBS fraction and also in the culture medium of free-living Rhizobium leguminosarum

The results thus far show that the majority of the

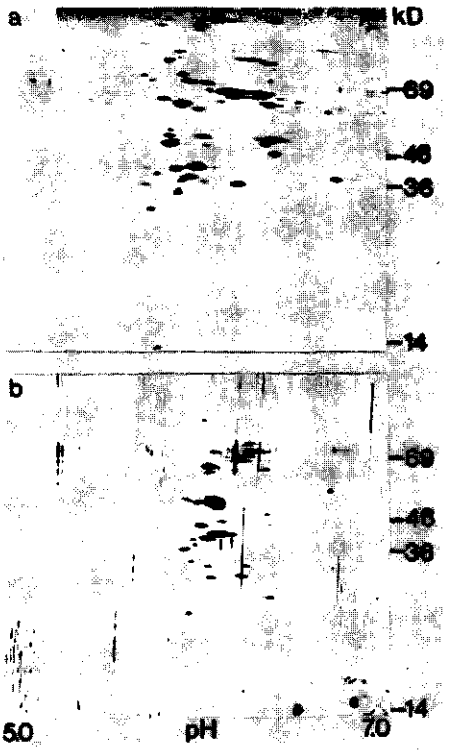

Fig. 2. Analysis of total and periplasmic-space (PS) proteins of *R. leguminosarum*. Total (a) and PS (b) proteins, 5 μg and 2 μg respectively, were resolved by two-dimensional gel electrophoresis and silver-stained. Spots of the PS fraction also detected in the PBS protein pattern by superposition of silver-stained gels are indicated by arrows.

PBS proteins are synthesized and excreted by the bacteroids into the PBS. Furthermore some of these proteins correspond to bacterial PS proteins. To test if any of the PBS proteins are also excreted by free-living bacteria, *R. leguminosarum* was grown in TY medium and cells were spun down at 12000 *g* for 30 min. Proteins present in the supernatant were separated on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter, which was probed with antiserum specific against the total PBS fraction. For a comparison, proteins from the PBS fraction, PBS-depleted bacteroids and free-living bacteria were also analyzed. These results (in Fig. 3a, lane 1, arrow) show that a 38 kDa protein which reacts with the antiserum is also present in the culture medium from free-living bacteria. The PBS contains many proteins strongly reacting with this antiserum (Fig. 3a, lane 2) while only after very long

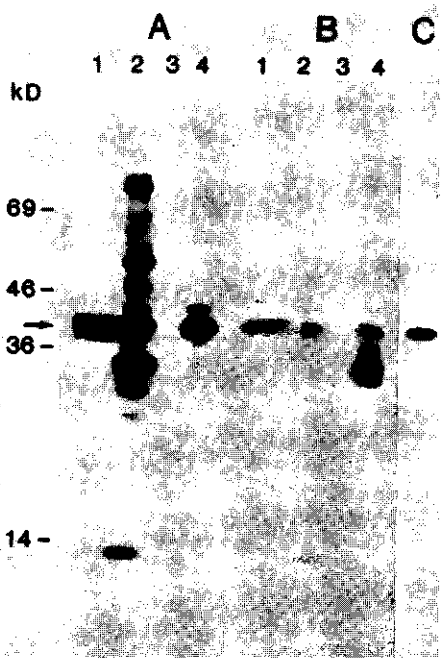

Fig. 3. Immunoreaction of proteins from PBS, bacteroids, bacteria and culture supernatant with antiserum specific against PBS proteins. Panel A: lane 1, 20 μl culture supernatant of *R. leguminosarum* grown in TY; lane 2, proteins form PBS (3 μg); lane 3, bacteroids (10 μg); lane 4, bacteria (10 μg) were separated by polyacrylamide gel electrophoresis; proteins were transferred to nitrocellulose filters by electroblotting and incubated with antiserum specific against PBS proteins. Immuno-complexes were visualized by incubation with ^{125}I-protein A. Panel B: an identical blot as in panel A was treated with sodium metaperiodate to remove glycosidic groups before incubation with antiserum. The positions of molecular weight markers are indicated. Panel C: ^{35}S-methionine-labeled PBS proteins (50000 dpm) were incubated with antiserum specific against the 38 kDa excretion product and precipitated with *Staphylococcus aureus* cells. Immuno-complexes were separated by SDS polyacrylamide gel electrophoresis, blotted onto nitrocellulose and visualized by autoradiography. Arrow indicates the 38 kDa excretion product (see text).

exposure any signal was detectable in bacteroids (Fig. 3a, lane 3); this indicates that the PBS-depleted bacteroids are essentially devoid of PBS proteins. Bacteria contain only a few proteins reactive with the antiserum among which also a 38 kDa band (Fig. 3a, lane 4).

Recently, it has been reported that monoclonal antibodies raised against proteins of the PBS fraction of *R. leguminosarum* strain 3841 recognized mainly the carbohydrate parts of glycoproteins [6]. To analyse to what extent the immune reactions in Fig. 3a are due to glycosidic groups, an identical blot was first treated with sodium metaperiodate to remove glycosidic groups and then probed with antiserum. By this treatment most of the bands (Fig. 3b) disappeared. This indicates that most bands on the untreated blot are indeed due to antibodies reacting with glycosidic groups. One bacterial protein of about 33 kDa (Fig. 3b, lane 4) on the contrary showed up much more prominently after the sodium metaperiodate treatment suggesting that in this protein certain antigenic determinants are exposed by this treatment. The 38 kDa protein excreted by bacteria as welll as some PBS specific bands were still detectable after this treatment, so the immunoreactions with these proteins are not due to glycosidic groups. For further analysis, the 38 kDa protein was purified from the medium of a *R. leguminosarum* culture and used to raise specific antibodies. This antiserum precipitates a 38 kDa protein present in the PBS fraction isolated from *ex planta* ^{35}S-methionine labeled bacteroids (Fig 3c). These results show that the 38 kDa protein is also synthesized and excreted by bacteroids into the PBS under *ex planta* conditions.

Discussion

In this paper we show that the PBS of *R. leguminosarum* bacteroids in pea root nodules contains at least forty different proteins. These proteins represent a specific group distinct from the proteins found in bacteroids as antiserum raised against the PBS fraction showed no reaction with bacteroid proteins. The PBS proteins can be released by a controlled osmotic shock. The conditions for the osmotic shock are critical for the bacteroids are damaged if the osmolarity is decreased to values lower than 0.12 M sucrose and then the PBS fraction was found to be contaminated with bacteroid proteins (results not shown).

The isolated PBM-enclosed bacteroids were able to synthesize and excrete polypeptides to a large extent similar to those found *in vivo*. This indicates that for the synthesis of most of the PBS proteins the conditions in the root nodule required for an effective symbiosis, such as the controlled oxygen concentration and exchange of nutrients between plant cells and bacteroids, are not essential. At least five PBS polypeptides found *in vivo* were not detected as ^{35}S-labeled proteins in the *ex planta* protein synthesis experiments. This may be due to the absence of methionine in these proteins but an other possibility is that these proteins represent bacteroid proteins of which the synthesis is specifically controlled and which are therefore not synthesized under the *ex planta* conditions used in the experiments. A more likely possibility is that these PBS proteins are plant-encoded. Evidence for the occurrence of plant-encoded nodule-specific proteins, *i.e.* nodulins, in the PBS has been reported by Fortin *et al.* [9], who have analyzed the PBS fraction of *Glycine max*.

In the 2D pattern of *ex planta* synthesized PBS polypeptides a number of spots was found which do not occur in the unlabelled, stained pattern of *in vivo* PBS proteins. These polypeptides may represent proteins which are present in amounts below the level of detection by staining and/or intermediates which are further processed to functional proteins.

At least four PBS proteins *ex planta* synthesized and excreted by the bacteroids and coinciding with PBS proteins found *in vivo* could not be detected in free-living bacteria (Figs. 1 and 2). These proteins may be considered as bacteroidins [20] in the true sense and represent *Rhizobium* proteins which are synthesized only in the symbiosis with the plant roots. We are aware that so far the proteins in question were absent in free-living *Rhizobium* bacteria grown in a complete medium but that we cannot exclude the possibility that they may be synthesized under different growth conditions. Biosynthetically active bacteroid preparations were earlier described by Van den Bos *et al.* [19] and Shaw [18] although

in these cases the incorporation of ^{35}S-methionine was considerably lower than that observed in this study. The difference may be due to the fact that the bacteroid preparations previously used were damaged. Breakage of the PBM will result in a changed ionic composition of the PBS causing a lower rate of protein synthesis.

Our observations that a discrete set of proteins is released from PBM-enclosed bacteroids after a controlled osmotic shock confirm the conclusions drawn by others that the isolation procedure used results in preparations of PBM-enclosed bacteroids with intact peribacteroid membranes. The very gentle osmotic shock used in this procedure disrupts the PBM and produces the soluble PBS proteins free of contaminating bacteroid proteins and also free of detectable contamination with PBM fragments. Several of the proteins in the PBS fraction were also detectable in the periplasmic space (PS) of free-living *Rhizobium* bacteria. This suggests that the osmotic shock ruptures not only the PBM but the bacteroid outer membrane as well. As a similar osmotic shock does not release the PS proteins of free-living bacteria this suggests that the outer membrane has undergone changes in the conversion of bacteria into bacteroids. A similar conclusion has been drawn from electron microscopy observations which suggest that during differentiation of *Bradyrhizobium japonicum* bacteroids the outer membrane is peeled off and appears to be replaced by a new outer membrane. Further it has been shown by electron microscopy that the PBM in pea root nodules with *R. leguminosarum* is physically associated with the outer bacteroid membrane. The present results thus do not allow the conclusion that in the nodule the bacteroid outer membrane is fully closed implying that PBS and bacteroid PS are separate compartments.

Our observation that after the osmotic shock the protein synthesis capacity of the bacteroids is greatly reduced indicates that the bacteroids are affected by a treatment which leaves free-living rhizobia unaffected. A bacteroid-encoded PBS protein with a molecular mass of 38 kDa was also found in the culture medium of free-living *Rhizobium* bacteria as demonstrated immunochemically (Fig. 3A and 3C). This protein is constitutively synthesized by free-living *Rhizobium* bacteria as well as by bacteroids but its synthesis is strongly stimulated during early stages of infection (R. M. Klein Lankhorst *et al.*, in preparation). Many of the PBS proteins cross-reacting on a Western blot with an antiserum specifc against the total PBS fraction appear to react with glycoproteins or (lipo)polysaccharides since pretreatment of the blot with sodium metaperiodate resulted in an almost complete loss of signal. Whether the glyco-groups in PBS proteins are of plant or bacterial origin is not clear. The bacterial PS proteins that are common with PBS proteins are not glycosylated to a large extent as the anti-PBS antiserum shows only very limited reaction with bacterial proteins in general (Fig. 3A, lane 4). We therefore suggest that the approximately seven prominent glycosylated PBS proteins (Fig. 3a, lane 2) are of plant origin. The most prominent band is the excreted 38 kDa protein which is still detectable after deglycosylation and therefore carries no glycosidic groups involved in the antigenic determinant.

The system of isolated PBM-enclosed bacteroids is active in synthesizing symbiosis-specific proteins and will supposedly also be useful in the study of symbiotic functions associated with the PBM and PBS.

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References

1. Bal AK, Wong PP: Infection process and sloughing off of rhizobial outer membrane in effective nodules of lima bean. Can J Microbiol 26: 1096–1103 (1982).

2. Bal AK, Sen D, Weaver RW: Cell wall (outer membrane) of bacteroids in nitrogen-fixing peanut nodules. Curr Microbiol 12: 353–356 (1985).
3. Bisseling T, Moen LL, Van den Bos RC, Van Kammen A: The sequence of appearance of leghaemoglobin and nitrogenase components I and II in root nodules of *Pisum sativum*. J Gen Microbiol 118: 377–381 (1980).
4. Bisseling T, Been C, Klugkist J, Van Kammen A, Nadler K: Nodule-specific host proteins in effective and ineffective root nodules of *Pisum sativum*. EMBO J 2: 961–966 (1983).
5. Bradley DJ, Butcher GW, Galfre G, Wood EA, Brewin NJ: Physical association between the peribacteroid membrane and lipopolysaccharides from the bacteroid outer membrane in *Rhizobium*-infected pea root nodule cells. J Cell Biol 85: 47–82 (1986).
6. Brewin NJ, Robertson JG, Wood EA, Wells B, Larkins AP, Galfre G, Butcher GW: Monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of pea cross-react with plasma membranes of Golgi bodies. EMBO J 4: 605–611 (1985).
7. De Maagd RA, Lugtenberg B: Fractionation of *Rhizobium leguminosarum* cells into outer membrane, cytoplasmic membrane, periplasmic and cytoplasmic components. J Bacteriol 167: 1083–1085 (1987).
8. Fortin MG, Morrison NA, Verma DPS: Nodulin-26, a peribacteroid membrane protein is expressed independently of the development of the peribacteroid compartment. Nucl Acids Res 15: 813–824 (1987).
9. Fortin MG, Zelechowska M, Verma DPS: Specific targeting of membrane nodulins to the bacteroid-enclosing compartment in soybean nodules. EMBO J 4: 3041–3046 (1985).
10. Govers F, Gloudemans T, Moerman M, Van Kammen A, Bisseling T: Expression of plant genes during the development of pea root nodules. EMBO J 4: 861–867 (1985).
11. Katinakis P, Verma DPS: Nodulin-24 of soybean codes for a peptide of the peribacteroid membrane and was generated by tandem duplication of a sequence resembling an insertion element. Proc Natl Acad Sci USA 82: 4157–4161 (1985).
12. Mellor RB, Dittrich W, Werner D: Soybean root response to infection by *Rhizobium japonicum*: Mannoconjugate turnover in effective and ineffective nodules. Physiol Plant Pathol 24: 61–70 (1984).
13. Mellor RB, Mörschel E, Werner D: Legume root response to symbiotic infection: Enzymes of the peribacteroid space. Z Naturforsch 39c: 123–125 (1984).
14. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685 (1970).
15. Robertson JF, Lyttleton P: Division of peribacteroid membranes in root nodules of white clover. J Cell Sci 69: 147–157 (1984).
16. Robertson JF, Lyttleton P, Bullivant S, Grayston GF: Membranes of lupin root nodules: 1. The role of Golgi bodies in the biogenesis of infection threads and peribacteroid membranes. J Cell Sci 30: 151–174 (1978).
17. Vincent JM: Factors controlling the legume-*Rhizobium* symbiosis. In: Newton WE, Orme-Johnson WH (eds) Nitrogen Fixation II, pp. 103–124. University Park Press, Baltimore (1980).
18. Shaw BD: Non-coordinate regulation of *Rhizobium* nitrogenase synthesis by oxygen: studies with bacteroids from nodulated *Lupinus angustifolius*. J Gen Microbiol 129: 849–857 (1983).
19. Van den Bos RC, Schots A, Hontelez J, Van Kammen A: Constitutive nitrogenase synthesis from *de novo* transcribed mRNA in isolated *Rhizobium leguminosarum* bacteroids. Biochim Biophys Acta 740: 313–322 (1983).
20. Van Kammen A: Suggested nomenclature for plant genes involved in nodulation and symbiosis. Plant Mol Biol Rep 2: 43–45 (1984).
21. Verma DPS, Fortin MG, Stanley J, Mauro VP, Purohit S, Morrison N: Nodulins and nodulin genes of *Glycine max*. Plant Mol Biol 7: 51–61 (1986).
22. Verma DPS, Kazazian V, Zogbi V, Bal AK: Isolation and characterization of the membrane envelope enclosing the bacteroids in soybean root nodules. J Cell Biol 78: 919–936 (1978).

Chapter 7
Concluding remarks

CHAPTER 7: Concluding Remarks

The aim of the studies described in this thesis was the identification and characterization of genes and gene products in *R.leguminosarum* PRE involved in symbiotic nitrogen fixation. For that purpose two approaches have been taken. At the one hand the physical map of the *sym* plasmid of *R.leguminosarum* PRE was extended (chapter 2), a novel *fix* gene (*fix*W) was identified (chapter 3) and the expression of *fix*W and the adjacent *fix*ABC operon was analyzed. At the other hand we have searched for the specific occurrence of *Rhizobium* proteins in bacteroids with a possible function in the symbiosis (chapter 4, 5 and 6). In that context we have given much effort to the identification of a 35 kD subunit of a bacteroid specific NADH dehydrogenase complex.

In a previous study (8) two regions containing *nif* and *fix* genes were identified on the *sym* plasmid: one region contains the *nif*HDK operon, *nif*E and a *fix* gene cluster immediately downstream of *nif*E where several Tn*5*-insertions resulted in a Fix$^-$ phenotype; the second region contains *nif*A, *nif*B, the *fix*ABC operon, *fix*X (7) and a *nod* gene cluster. The positions of the two regions on the *sym* plasmid relative to each other was not known. Chapter 2 describes how two extended physical maps of *sym* plasmid regions containing either *nif*HDK or *nif*A were constructed. Although these regions cover together more than 70% of the total *sym* plasmid DNA, no overlap between them was found. From the data it was deduced that the distance between the *nif*HDK operon in the one region and *nif*A in the other is somewhere between 60 and 175 kb. In this respect *R.leguminosarum* PRE differs for example from the closely related *R.leguminosarum* strain 248 (pRL1JI) where the distance between *nif*HDK and *nif*A is approx. 30 kb (2), or from *R.meliloti* strain 1021 where *nif*HDK and *nif*A are approx. 5 kb apart (9).

By nucleotide sequence analyses the DNA region upstream of *fix*A was studied in more detail and a novel *fix* gene, designated *fix*W, was identified immediately upstream of *fix*A. This gene is transcribed in the same direction as the *fix*ABC operon, has a *nif*-consensus promoter and encodes a 25 kD polypeptide of unknown function. Upstream of *fix*W two further open reading frames, ORF 71 and ORF 79, were found which form a single transcriptional unit. The transcription of this operon is directed by a *nif*-consensus promoter in front of ORF 71 and reads towards *fix*W. Since the *fix*W promoter is located within ORF 79, the transcription of *fix*W might be directed by the ORF 71 promoter as well as by its own promoter.

The expression of *fix*W and *fix*ABC was studied by expressing cloned DNA fragments in *E.coli* and by DNA-RNA hybridizations using RNA isolated from *R.leguminosarum nif*A,

*fix*A and *fix*W mutants. From the expression experiments in *E.coli* and nucleotide sequence analysis of the *fix*A-*fix*W intergenic region it was deduced that *fix*W forms a transcriptional unit with *fix*ABC, and that the expression of the *fix*ABC operon is dependent on the expression of *fix*W. The DNA-RNA hybridization studies, however, showed that in a *Rhizobium fix*W mutant the transcription of the *fix*ABC operon is directed by a *nif*-consensus promoter upstream of *fix*A, independently of the expression of *fix*W. It is not known at present whether the expression of the *fix*ABC operon in wild type *R.leguminosarum* bacteroids is regulated by the *fix*A promoter, by the *fix*W promoter or by the ORF 71 promoter, or by a combination of these three possibilities.

For *R.meliloti* it was shown (4) that part of the *fix*ABC transcripts read through into the downstream situated *nif*A gene. If the same is true for *R.leguminosarum* then also (part of) the *nif*A expression in *R.leguminosarum* bacteroids might be dependent on the expression of further upstream situated genes. Since *nif*B, downstream of *nif*A, probably is co-transcribed with *nif*A (7), in *R.leguminosarum* theoretically one single transcript of approx. 8 kb can be formed running from the ORF71 promoter through ORF 71, ORF 79, *fix*W, *fix*ABC, *fix*X and *nif*A into *nif*B.

The organization of genes and *nif*-consensus promoters in the *nif*A region may lead to a variety of overlapping transcripts during nitrogen fixation. The function of this complex organization is not known at present and the regulation mechanism of the genes in this *sym* plasmid region needs further research.

Southern hybridization of cloned *sym* plasmid DNA fragments with various *nif* and *fix* genes as probes showed that parts of *nif*H, *fix*A and *fix*W are reiterated on the *sym* plasmid. These reiterations do not represent functional copies of the mentioned genes, because mutants carrying Tn*5*-insertions in *nif*H, *fix*A or *fix*W exhibit a Fix$^-$ phenotype. The *nif*H reiteration forms part of a small open reading frame, designated *nif*H'.upstream of the above mentioned ORF 71. Since *nif*H' possesses a *nif* consensus promoter, this open reading frame probably will be transcribed during nitrogen fixation. ORF71 itself is highly homologous to part of *fix*A, and as already mentioned, also this open reading frame might be transcribed during nitrogen fixation. A further *fix*A reiteration, as well as a sequence homologous to *fix*W are present downstream of the *nif*HDK operon. Whether these two reiterations also are transcribed during nitrogen fixation is not known. Since all reiterations are situated within known *nif* and *fix* gene clusters, they may form part of functional *nif* or *fix* genes. Alternatively, they merely represent the result of recombinational events of *sym* plasmid sequences, in which the region containing *nif*HDK became separated from the *nif*A region.

In chapters 4 and 5 an attempt is described to identify *R.leguminosarum* genes involved in the electron transport to nitrogenase. Starting point for this was the model of Klugkist *et al.* (5) stating that the electron transport to nitrogenase in aerobic nitrogen fixing diazotrophs is mediated by a membrane bound NAD(P)H dehydrogenase. In a search for membrane linked NAD(P)H dehydrogenase activity, specifically induced during nitrogen fixation, we isolated a 550 kD NADH dehydrogenase complex from *R.leguminosarum* bacteroid membranes. This complex, which is referred to as DH1, is present in bacteroids but not in free-living bacteria, and might form part of the electron transport chain to nitrogenase. In an analysis of DH1, we showed that this complex probably contains four different subunits with molecular masses of 20, 29, 35 and 58 kD respectively. By serendipity, a further polypeptide with a molecular mass of 56 kD was identified that may also form part of DH1. Evidence is presented that the gene encoding this 56 kD polypeptide is located near the gene encoding the 58K subunit. By using antibodies directed against the isolated DH1 subunits we showed that only the 35 kD subunit (referred to as the 35K polypeptide) is bacteroid specific, whereas the other subunits can also be detected in free-living bacteria. Furthermore we showed that the expression of the gene encoding the 35K polypeptide is not regulated by the *nif* regulatory protein NifA, which excludes the possibility that this polypeptide is encoded by any of the *nif*A dependent *R.leguminosarum nif* or *fix* genes.

The function of DH1 might be assessed by studying *Rhizobium* mutants impaired in the synthesis of the 35K subunit. As a first step in constructing such mutants, an attempt was made to isolate the gene encoding this polypeptide. The N- terminal amino acid sequence of the purified 35K polypeptide was determined and *R.leguminosarum* gene libraries were screened with oligodeoxynucleotide probes deduced from this amino acid sequence. The screening resulted in the isolation of various DNA fragments theoretically encoding polypeptides partly homologous to the N-terminus of the 35K protein, but the gene encoding the 35K polypeptide itself was not found. Computer analyses showed that one of the cloned DNA fragments might encode a cytoplasmic NADH dehydrogenase, ribitol dehydrogenase.

Recently Kahn *et al.* (3) identified a cluster of *fix* genes (*fix*GHI) on the *R.meliloti sym* plasmid encoding membrane proteins. These proteins probably constitute a membrane bound complex involved in electron transfer processes, and the regulation of the *fix*GHI genes seems to be *nif*A- independent (D. Kahn, pers.comm.). These characteristics raise the possibility that one of the proteins encoded by the *R.meliloti fix*GHI operon is similar to the *R.leguminosarum* 35K protein. Since genes homologous to the *R.meliloti fix*GHI were reported to be present in *R.leguminosarum* (3), isolation and mutagenesis of these genes might result in the identification of the 35K gene. Alternatively, the isolation of this gene might be

accomplished by using further rhizobial genes encoding membrane proteins which are indispensable in nitrogen fixation as probes. Such genes were recently described by Long *et al.* (6) who identified four symbiotic loci encoding membrane proteins in the genome of *R.meliloti*. Tn*phoA* mutagenesis of these loci, one of which is located on the *sym* plasmid, resulted in mutants exhibiting a Fix$^-$ phenotype.

In our search for symbiosis specific *Rhizobium* genes and gene products, we analyzed a symbiotic compartment, the peribacteroid space (PBS), to which not much attention has been paid so far. This compartment, however, fulfills an important role in symbiotic nitrogen fixation since it forms the interface between the endosymbiont and the plant host, and might be involved in transport processes and signal transduction between the two symbiotic partners. In chapter 6 we show that the PBS contains a discrete set of proteins, most of which are synthesized by the bacteroid. About one third of these proteins were also present in the periplasmic space (PS) of free-living bacteria. Since during bacteroid development the bacterial outer membrane is sloughed off and replaced by a new one (1), an explanation for the appearance of PS proteins in the PBS is that these proteins are released into the PBS during this membrane replacement. Alternatively, these results show that in bacteroids the PBS and the periplasmic space are interconnected. Some of these PS proteins might be indispensable in nitrogen fixation. For *R.meliloti* mutants have been reported (6) which exhibit a Fix$^-$ phenotype and were found to be disturbed in the synthesis of several PS proteins.

Our analysis of the PBS showed that among the *Rhizobium* encoded PBS proteins there is at least one, which can be found in the culture supernatant of *R.leguminosarum* bacteria and hence is excreted by free-living bacteria as well as by the bacteroids. Four *Rhizobium* encoded proteins which were not detectable in free-living bacteria were shown to be present in the PBS of *R.leguminosarum* bacteroids and thus appear to be symbiosis specific. These four proteins might play an intrinsic role in nitrogen fixation and a further characterization of these might lead to the identification of novel symbiotic loci on the *R.leguminosarum* genome.

Literature cited.

1. Bal, A.K., Sen, D. and Weaver, R.W. (1985). Cell wall (outer membrane) of bacteroids in nitrogen-fixing peanut nodules. Curr.Microbiol. *12*, p.353.
2. Downie, J.A., Ma, Q.-S., Knight, C.D., Hombrecher, G. and Johnston, A.W.B. (1983). Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and a *nif*A-like gene. EMBO J. *2*, (6), p.947.
3. Kahn, D., David, M., Domergue, O., Daveran, M.-L., Ghai, J., Hirsch, P.R. and Batut, J. (1989). *Rhizobium meliloti fix*GHI sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J.Bacteriol. *171*, (2), p.929.

4. Kim, C.-H., Helinski, D.R. and Ditta, G., (1986). Overlapping transcripts of the *nif*A regulatory gene in *Rhizobium meliloti*. Gene *50*, p.141.
5. Klugkist, J. (1985). Electron transport to nitrogenase in *Azotobacter vinelandii*. PhD Thesis, Agricultural University, Wageningen, The Netherlands.
6. Long, S., McCune, S. and Walker, G.C. (1988). Symbiotic loci of *Rhizobium meliloti* identified by random Tn*pho*A
7. Roelvink, P. (1989). Characterization of the *nif*A regulatory gene of *Rhizobium leguminosarum* PRE. PhD Thesis, Agricultural University, Wageningen, The Netherlands.
8. Schetgens, T.M.P. (1985). Identification and characterization of symbiotic genes on the Rhizobium leguminosarum PRE *sym*-plasmid. PhD Thesis, Agricultural University, Wageningen, The Netherlands.
9. Szeto, W.W., Zimmerman, J.L., Sundaresan, V. and Ausubel, F.M. (1984). A *Rhizobium meliloti* symbiotic regulatory gene. Cell *36*, p.1035.

Chapter 8
Samenvatting

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Het element stikstof (N) komt voor in alle levende organismen en is een belangrijke bouwsteen voor essentiële verbindingen, zoals bijvoorbeeld eiwitten en nucleïnezuren. De stikstof die in de levende natuur voorkomt, ook wel aangeduid als "gebonden stikstof", doorloopt een cyclus: planten halen via hun wortels eenvoudige stikstofverbindingen uit de bodem en maken hieruit ingewikkelde chemische verbindingen, zoals aminozuren en eiwitten. Mensen en dieren gebruiken planten als voedsel en krijgen zo de stikstof binnen die noodzakelijk is voor allerlei belangrijke processen in het lichaam. Via uitscheidingsprocessen en ook door het afsterven van planten en dieren komen de ingewikkelde stikstofverbindingen in de bodem terecht, waar ze door bacteriën weer worden omgezet in eenvoudige verbindingen en de kringloop gesloten wordt.

Op allerlei manieren kan echter stikstof ontsnappen uit deze cyclus, en het is dus van cruciaal belang voor het voortbestaan van levende organismen dat de hoeveelheid gebonden stikstof continu wordt aangevuld. Dit aanvullen gebeurt vanuit de atmosfeer rondom de aarde. De lucht die we inademen bevat circa 80% stikstofgas, de "ongebonden stikstof", en vormt een bijna onuitputtelijke stikstofbron. Dit stikstofgas, echter, is een zeer stabiele chemische verbinding en kan door de meeste organismen niet worden benut. Voordat het stikstofgas uit de lucht kan worden gebruikt moet het worden omgezet in een bruikbare vorm. Dit omzetten van stikstofgas noemen we "stikstofbinding". De twee belangrijkste processen waarin stikstof gebonden wordt zijn de industriële en de biologische stikstofbinding. In de chemische industrie wordt jaarlijks circa 100 miljard kilo kunstmest gemaakt uit stikstofgas, ten koste van een ontzaglijke hoeveelheid fossiele brandstof. De oliecrisis in de jaren zeventig en het groeiende milieubewustzijn hebben gezorgd voor een sterk toegenomen interesse in biologische stikstofbinding. Hierin wordt het stikstofgas gebonden door bepaalde groepen bacteriën, die als enige levende organismen in staat zijn het chemisch stabiele stikstofgas om te zetten in ammonia. De stikstofbindende bacteriën kunnen in twee klassen worden verdeeld: de vrijlevende stikstofbinders en de symbiontische stikstofbinders. Bacteriën uit de laatste klasse binden alleen stikstof in samenwerking met bepaalde planten.

Economisch gezien zijn de *Rhizobium* bacteriën de belangrijkste stikstofbinders. Deze zijn in staat om vlinderbloemige planten, zoals erwten, bonen en klaver, te infecteren, waarbij knolletjes ontstaan op de wortels van de plant. In deze knolletjes zetten de *Rhizobium* bacteriën het stikstofgas om in ammonia, dat door de plant kan worden gebruikt. Van deze eigenschap is in de landbouw al eeuwen lang gebruik gemaakt: hoewel ze (waarschijnlijk) nog nooit van

bacteriën gehoord hadden, wisten de Romeinen al dat het laten groeien van bepaalde gewassen een verhoging van de bodemvruchtbaarheid met zich mee bracht, en pasten ze een systeem toe dat we tegenwoordig "groenbemesting " noemen. De bijdrage die *Rhizobium* bacteriën leveren aan de totale hoeveelheid stikstof die jaarlijks gebonden wordt ligt in dezelfde orde van grootte als die van de chemische industrie, dus ook circa 100 miljard kilo. Voor het milieu heeft groenbemesting door *Rhizobium* het grote voordeel dat alle gebonden stikstof direct aan de plant ten goede komt, terwijl bij het gebruik van kunstmest een groot deel van de stikstof weer uit de bodem spoelt en in het oppervlaktewater terecht komt.

De stikstofbinding door *Rhizobium* bacteriën is de laatste jaren onderwerp geworden van veel wetenschappelijk onderzoek. De uiteindelijke doelen die men zich daarbij voor ogen houdt zijn o.a. het vergroten van het stikstofbindend vermogen van de bacteriën, het veranderen van de *Rhizobium* bacteriën zodat ze ook niet-vlinderbloemige planten kunnen infecteren en dus van stikstof kunnen voorzien, en het overbrengen van het stikstofbindend vermogen van de bacterie naar de plant. Voordat dit soort zaken gerealiseerd kunnen worden, is er nog veel onderzoek nodig aan de *Rhizobium* bacterie zelf om te weten te komen hoe deze functioneert. In dit proefschrift is een moleculair-biologisch en biochemisch onderzoek beschreven waarin een aantal processen is bestudeerd, die zich binnen de stikstofbindende bacterie afspelen. Ook is geprobeerd de genetische achtergronden van deze processen te ontrafelen.

In hoofdstuk 2 is de kartering van een groot deel van het *sym*-plasmide van *Rhizobium leguminosarum* PRE beschreven. Op dit plasmide waren in een eerder onderzoek twee gebieden geïdentificeerd waarop genen liggen die betrokken zijn bij de stikstofbinding. Een van deze gebieden bevat het *nif*HDK operon, *nif*E en een groep niet nader gekarakteriseerde *nif* of *fix* genen. Het andere bevat de genen die betrokken zijn bij de infectie van de gastheerplant en bij de knolvorming (*nod* genen), de *nif* genen *nif*A en *nif*B, en de *fix* genen *fix*ABC. Hoe deze gebieden ten opzichte van elkaar op het *sym*-plasmide liggen was echter niet bekend. Om dit te onderzoeken is een cosmide bank gemaakt van gezuiverd *sym*-plasmide DNA, waarmee via "cosmid hopping" circa 70% van het plasmide in kaart gebracht is. Rondom het *nif*HDK operon is een gebied van 140 kb gekarteerd, en rondom *nif*A een stuk van 105 kb. Aangezien deze gebieden niet aan elkaar blijken te grenzen, is de exacte ligging van de genen in het *nif*HDK gebied ten opzichte van de genen in het *nif*A gebied nog niet bekend. De afstand tussen *nif*HDK en *nif*A moet ergens tussen de 60 en de 175 kb liggen, waarmee de organisatie van de *nif* genen op het *sym*- plasmide van *R.leguminosarum* PRE afwijkt van die in een sterk verwante stam als *R.leguminosarum* 248 of van *R.meliloti* 1021.

Het gebied op het *sym*-plasmide rondom *nif*A is aan een nauwkeurige analyse onderworpen, omdat gebleken was dat hier waarschijnlijk nog niet eerder geïdentificeerde *fix* genen liggen.

Inderdaad werd aan de 3'-kant van het *fix*ABC operon een nieuw *fix* gen gevonden (hoofdstuk 3), dat we *fix*W genoemd hebben. Verder bleken er aan de 3'-kant van *fix*W nog drie "open leesramen" (ORFs) te liggen, waarvan er één (ORF 71) een sterke homologie vertoont met een gedeelte van *fix*A en een tweede sterk homoloog is met een gedeelte van het *nif*H gen. Een duplicatie van een gedeelte van de *fix*W sequentie werd aangetroffen op het *sym*-plasmide stroomafwaarts van het *nif*HDK operon. De functie van *fix*W, van de *fix*W duplicatie en van de drie ORFs is nog niet bekend.

De expressie van de verschillende genen in het *nif*A gebied is onderzocht door middel van RNA-DNA hybridisaties en het tot expressie brengen van genen uit het *nif*A gebied in *E.coli*. Het bleek dat er een scala van mogelijkheden bestaat waarop de genen in dit gebied tot expressie kunnen komen: *fix*W heeft een *nif*-consensus promoter en de expressie van dit gen bleek inderdaad *nif*A afhankelijk te zijn. Nucleotidensequentie analyse liet echter zien dat twee van de drie stroomopwaarts gelegen ORFs waarschijnlijk één transcriptie-eenheid (operon) vormen met *fix*W, en dat deze twee ORFs ook worden gereguleerd vanaf een *nif*A afhankelijke promoter. Verder bleek dat *fix*W een operon kan vormen met het stroomafwaarts gelegen *fix*ABC operon. Hoewel dit operon een eigen *nif*-consensus promoter bezit, kan de expressie van het *fix*ABC operon dus ook door de *fix*W promoter gereguleerd worden, of zelfs door de nog verder stroomopwaarts gelegen ORF 71-promoter. Omdat waarschijnlijk een gedeelte van de *fix*ABC transcripten doorloopt in het stroomafwaarts gelegen *nif*A gen tot in het daarnaast gelegen *nif*B gen, kan er in theorie één lang transcript gevormd worden van circa 8 kb dat start bij de ORF 71 promoter en dat stopt bij *nif*B. Het expressiepatroon van de verschillende genen in dit gebied is waarschijnlijk erg ingewikkeld door het voorkomen van verschillende *nif*-consensus promotors, die kunnen leiden tot een groot aantal overlappende transcripten. Nog niet bekend is, of al deze transcripten ook werkelijk gevormd worden in stikstofbindende *Rhizobium* bacteroïden en wat de functie van deze complexe organisatie is.

In de hoofdstukken 4 en 5 is een onderzoek beschreven naar eiwitten en genen in *Rhizobium* die mogelijk betrokken zijn bij het electronentransport naar nitrogenase. Voor de reductie van stikstofgas tot ammonia zijn in *Rhizobium* bacteriën electronen met een hoog reducerend vermogen nodig. Hoe deze electronen gegenereerd worden en welke genen daarbij betrokken zijn is nog niet opgehelderd. Voor de stikstofbindende *Azotobacter vinelandii* zijn er aanwijzingen dat een membraangebonden NAD(P)H dehydrogenase bij dit proces betrokken is, dat alleen tijdens de stikstofbinding gemaakt wordt. Hiervan uitgaande is onderzocht of een dergelijk dehydrogenase ook aanwezig is in *R.leguminosarum*. Inderdaad kon er een membraangebonden NADH dehydrogenase (DH1) in bacteroïden worden geïdentificeerd (hoofdstuk 4), dat niet aanwezig is in vrijlevende *Rhizobium* bacteriën, en dat dus mogelijk een

membraangebonden NADH dehydrogenase (DH1) in bacteroïden worden geïdentificeerd (hoofdstuk 4), dat niet aanwezig is in vrijlevende *Rhizobium* bacteriën, en dat dus mogelijk een rol speelt in het proces van de stikstofbinding. Een gedeeltelijke zuivering van DH1 liet zien dat dit complex, met een molecuulgewicht van 550 kD, waarschijnlijk opgebouwd is uit vijf verschillende subeenheden met molecuulgewichten van respectievelijk 20, 29, 35, 56 en 58 kD. Met behulp van antisera specifiek gericht tegen de afzonderlijke DH1-subeenheden kon worden vastgesteld dat alleen de 35 kD subeenheid bacteroïd specifiek is, en dat alle andere subeenheden ook aantoonbaar zijn in vrijlevende bacteriën, waarschijnlijk als onderdelen van andere dehydrogenases. Door verschillende *R.leguminosarum* mutanten te bestuderen, kon verder worden bewezen dat de expressie van het gen dat kodeert voor de 35K subeenheid niet afhankelijk is van het *nif*-regulerende *nif*A-eiwit, en dat dus geen van de *nif*A- afhankelijke *R.leguminosarum nif* of *fix* genen voor dit eiwit kodeert.

Aangezien de 35K subeenheid als enige DH1 component alleen tijdens de stikstofbinding gemaakt wordt, zou een mogelijke functie van DH1 in het electronentransport naar nitrogenase wellicht bewezen kunnen worden met behulp van een *R.leguminosarum* mutant die dit eiwit niet meer produceert. Om een dergelijke mutant te maken is geprobeerd het gen, dat kodeert voor het 35K eiwit, in handen te krijgen (hoofdstuk 5). Het eiwit is daarom geïsoleerd, waarna de aminozuursequentie van de eerste 20 aminozuren aan de N-terminale kant bepaald is. Aan de hand van deze sequentie zijn oligodeoxynucleotide probes (oligo's) gemaakt waarmee een *R.leguminosarum* cosmidebank gescreend is. Deze screening leidde tot de isolatie van zes verschillende *R.leguminosarum* DNA fragmenten, die homoloog zijn met (een gedeelte van) de gebruikte oligo's. Analyse van de nucleotidensequentie liet echter zien dat geen van deze zes fragmenten het gen voor het 35K eiwit bevat, maar dat deze fragmenten kunnen koderen voor polypeptiden die gedeeltelijk homoloog zijn met het N-terminale deel van het 35K eiwit. Een van de gevonden fragmenten bevat zeer waarschijnlijk het gen voor een cytoplasmatisch NADH dehydrogenase, namelijk ribitoldehydrogenase. Vanwege een gebrek aan tijd is de speurtocht naar het 35K gen niet voortgezet. Wel kon nog worden aangetoond dat er in het genoom van *R.leguminosarum* nog meer DNA fragmenten voorkomen die een homologie met de gebruikte oligo's vertonen. De kans is groot dat het gezochte gen op een van deze fragmenten ligt.

In hoofdstuk 6 tenslotte staat een analyse beschreven van een belangrijk symbiontisch compartiment in de plantecel, namelijk de peribacteroïd ruimte (PBS). Dit compartiment is de ruimte tussen de bacteroïde en een plantemembraan dat de bacteroïde in de plantecel omsluit, en speelt een belangrijke rol in de stikstofbinding. De PBS vormt namelijk de verbinding tussen de gastheerplant enerzijds en de endosymbiont anderzijds, en is waarschijnlijk betrokken bij

allerlei transportprocessen tussen plant en bacteroïde, en mogelijk ook bij signaaloverdracht tussen deze twee. Door middel van *in vivo* eiwitlabeling en 2-dimensionale gelelectroforese is aangetoond dat de PBS veertig tot vijftig verschillende eiwitten bevat. Het grootste deel hiervan, ongeveer 90%, blijken *Rhizobium*-eiwitten te zijn, terwijl de overige 10% waarschijnlijk door de plant gemaakt wordt. In de PBS konden een viertal bacteroïd eiwitten worden aangetoond die niet worden gemaakt door vrijlevende *R.leguminosarum* bacteriën. Deze eiwitten, bacteroïdines genaamd, kunnen mogelijkerwijs gebruikt worden om nieuwe *fix*-gebieden op het genoom van *R.leguminosarum* te localiseren.
Verrassenderwijs bleek circa een derde van de PBS eiwitten ook aantoonbaar te zijn in de periplasmatische ruimte van vrijlevende bacteriën. Deze eiwitten komen waarschijnlijk vrij in de PBS tijdens de differentiatie van de bacterie tot stikstofbindende bacteroïde in de plantecel, waarbij de bacteroïde van een nieuw buitenmembraan wordt voorzien. De functie van de periplasmatische eiwitten in de PBS is op dit moment nog niet bekend. De analyse van de PBS heeft duidelijk aangetoond dat dit compartiment de moeite van een verdere bestudering zeker waard is, omdat de eiwitten hierin tot nieuwe inzichten in het functioneren van de stikstofbindende bacteroide in de wortelknol kunnen leiden.

NAWOORD

Het is maandagavond, 17 juli 1989, en al zittend achter mijn tekstverwerker overpeins ik de gebeurtenissen van de afgelopen jaren, die hebben geleid tot de standkoming van dit proefschrift. Het is een omzien in gemengde gevoelens: trots en voldoening, omdat het karwei er bijna opzit en al die jaren onderzoek zijn uitgekristalliseerd in dit boekje, maar ook een gevoel van teleurstelling, omdat de *Rhizobium* groep (voorlopig ?) is opgeheven en het onderzoek dat in dit proefschrift beschreven staat niet wordt voorgezet. Maar gelukkig overheersen de trots en voldoening, in de wetenschap dat ik het allemaal niet voor elkaar gekregen zou hebben zonder de hulp van vele mensen, die ik daarvoor op deze plek wil bedanken.

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De dagelijkse begeleiding van het onderzoek is in handen geweest van mijn co-promotor, Rommert van den Bos. Beste Rommert, de manier waarop jij het onderzoek begeleid hebt is me altijd zeer goed bevallen. Niet alleen gaf je me de nodige vrijheid om zelf de onderzoekslijnen uit te zetten, maar je wist ook met je grenzeloze optimisme het onderzoek draaiende te houden door telkens alle resultaten van de zonnige kant te bekijken. Bovendien gaf je vaak blijk van je overtuiging dat er meer is dan de Wetenschap alleen, onder andere in de vele gesprekken die we samen gevoerd hebben over de meest uiteenlopende onderwerpen.

Als er één persoon geweest is die de afgelopen jaren een duidelijk stempel gedrukt heeft op het onderzoek binnen de *Rhizobium* groep, dan is dat Jan Hontelez wel. Jan, jongen, ik wil je hier niet in eerste instantie bedanken voor het uitvoeren van een groot aantal experimenten dat in dit proefschrift beschreven staat; je grote experimentele vaardigheden zijn bij iedereen binnen de vakgroep wel bekend. Veel meer wil ik je bedanken voor alle experimenten die je voor me hebt uitgevoerd die niet in dit proefschrift zijn opgenomen. Je bent altijd bereid geweest de proeven te doen die we vaak als laatste strohalm beschouwden. Veel van die proeven zijn inderdaad op niets uitgelopen, maar ook deze negatieve resultaten zijn veelal belangrijk geweest voor de voortgang van het onderzoek. Verder heb jij er in hoge mate toe bijgedragen dat ik me al die jaren op het lab prima thuis gevoeld heb; met jouw absurde gevoel

voor humor heb je ervoor gezorgd dat iedere normale werkdag meestal wel veranderde in een prettig gestoorde chaos !
Verder een woord van dank aan de overige, min of meer permanente leden van de *Rhizobium* groep: Peter Roelvink, die al die tijd als de culturele agenda van lab A gefunctioneerd heeft en ons vocabulair aanzienlijk verrijkt heeft met vele, vaak kernachtige uitspraken, Willie van de Greef, Jan-Dirk Jansma, Diman Lehlohonolo van Rossum, Marcel Kool, Sander Peters, Jos Buys en Michel Wissink, die tijdens hun studie enige maanden in het onderzoek hebben meegedraaid en bergen werk hebben verzet, en Panagiotis Katinakis, onze vaste zomergast: Dear Takis, it was always great fun having you around and thank you for losing our bet on the European Footbal Championship 1988 !
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klaverjassen, bridgen, jeu-de-boulen, zwemmen, muziek maken, goede b-films kijken en aan de bar hangen !

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CURRICULUM VITAE

René Klein Lankhorst werd op 29 november 1958 geboren te Enschede. In 1977 haalde hij het eindexamen Atheneum B aan het Twentsch Carmellyceum te Oldenzaal en ging scheikunde studeren aan de Rijksuniversiteit Groningen. In 1984 werd het doctoraalexamen gehaald met als hoofdvak Biochemie (Prof.dr. M. Gruber) en als bijvak Anthropogenetica (Prof.dr. C. Buys). In hetzelfde jaar begon hij zijn promotieonderzoek bij de vakgroep Moleculaire Biologie van de Landbouwuniversiteit Wageningen. De eerste 3 jaar van het onderzoek was hij in dienst van BION, waarna hij als toegevoegd onderzoeker in tijdelijke dienst van de Landbouwuniversiteit trad.

Vanaf 1 september 1989 is hij werkzaam als wetenschappelijk medewerker bij de vakgroep Moleculaire Biologie van de LUW, waar hij onderzoek doet aan de genetische organisatie van chromosoom 6 van de tomaat.