IDENTIFICATION AND CHARACTERIZATION OF SYMBIOTIC GENES ON THE *RHIZOBIUM LEGUMINOSARUM* PRE *SYM*-PLASMID

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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Contents

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STELLINGEN

1. *De voorstellen van Earl en Ausubel om het vermogen tot stikstofbinding te introduceren in cultuurgewassen worden door de auteurs zelf terecht beschouwd als een wetenschappelijke uitdaging; hun beoordeling van de uitvoerbaarheid van deze voorstellen is echter in aanzienlijke mate gebaseerd op "wishful thinking".*

   - Earl C.D. and Ausubel F.M. (1983). Nutrition Reviews 41, 1-6.

2. *De bewering van Heumann et al. dat door bestraling met ultraviolet licht en door stress-omstandigheden op reproduceerbare wijze verschillende Rhizobium soorten en Rhizobium en Agrobacterium soorten in elkaar omgezet kunnen worden is zeer aanvechtbaar.*

   - Heumann W., Rösch A., Springer R., Wagner E. and Winkler K.P. (1984). Mol. Gen. Genet. 197, 425-436.

3. *In de experimenten die Okker et al. gedaan hebben om een door planten gecodeerde factor die de expressie van virulentiegenen in Agrobacterium tumefaciens kan induceren te karakteriseren, is onvoldoende rekening gehouden met de pH-afhankelijkheid van deze factor.*

   - Okker R.J.H., Spaink H., Hille J., van Brussel T.A.N., Lugtenberg B. and Schilperoort R.A. (1984). Nature 312, 564-566.
   - Stachel S.E., Nester E.W. and Zambryski P.C. (1986). Proc. Natl. Acad. Sci. U.S.A. 83, 379-383.

4. *De suggestie dat het virE genprodukt van Agrobacterium tumefaciens integrase activiteit bezit die de incorporatie van T-DNA in het plantegenoom bewerkstelligt is onvoldoende gegrond, omdat het verschil tussen T-DNA overdracht die resulteert in tumorinductie enerzijds en systemische virusinfectie anderzijds niet in aanmerking wordt genomen.*

   - Gardner R.C. and Knauf V.C. (1986). Science 231, 725-727.

5. *De Block et al. hebben geen overtuigend bewijs geleverd voor transformatie van chloroplasten met behulp van Agrobacterium Ti-plasmide vectoren, omdat een essentieel controle-experiment als clonering en analyse met restrictie-enzymen van het geïntroduceerde chimere Pnos-cat gen met de flankerende sequenties ontbreekt.*

   - De Block M., Schell J. and Van Montagu M. (1985). EMBO Journal 4, 1367-1372.

6. *Bij DNA transformaties van planteprotoplasten verdient het aanbeveling om gebruik te maken van door Ca^{++} gestimuleerde opname van DNA onder condities zoals die zijn beschreven voor het transformeren van dierlijke cellen.*

- *Graham F.L. and Van der Eb A.J. (1973). Virology 52, 456-467.*
- *Krens F.A., Molendijk L., Wullems G.J. and Schilperoort R.A. (1982). Nature 296, 72-74.*
- *Hain R., Stabel P., Czernilofsky A.P., Steinbiss H.H., Herrera-Estrella L. and Schell J. (1985). Mol. Gen. Genet. 199, 161-168.*

7. *Het lijkt ongerijmd te proberen om het relatief beperkte gastheerbereik van baculovirussen, dat een in oecologisch opzicht uiterst gunstig gegeven is, middels genetische manipulatie uit te breiden teneinde de commerciële toepasbaarheid van deze virussen als biologisch bestrijdingsmiddel tegen insecten te verhogen.*

- *Miller L.K., Lingg A.J. and Bulla Jr. L.A. (1983). Science 219, 715-721.*

8. *Een nucleaire catastrofe met internationale consequenties was helaas nodig om werkelijk een brede maatschappelijke discussie over kernenergie in gang te zetten.*

9. *De diepgang van de onderlinge communicatie in relaties is omgekeerd evenredig met het gedeelte van de vrije tijd dat besteed wordt aan televisie kijken.*

Stellingen bij het proefschrift "Identification and characterization of symbiotic genes on the Rhizobium leguminosarum PRE sym-plasmid".

Resie Schetgens

Wageningen, 28 mei 1986.

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Introduction

The conversion of atmospheric nitrogen to metabolically usable compounds by micro-organisms is an important link in the nitrogen cycle. The ability to reduce nitrogen is confined to certain classes of bacteria and blue-green algae. These nitrogen-fixing organisms can be subdivided into two major groups i.e. freeliving species (e.g. Klebsiella pneumoniae, Azotobacter vinelandii, Clostridium pasteurianum, Rhodospirilum rubrum and cyanobacteria like Anabaena cylindrica) and prokaryotes which depend on more or less intimate association (e.g. Azospirillum brasilense, Anabaena azollae) or intracellular symbiosis (actinomycetes like Frankia and the genus Rhizobium) with particular plants for the expression of nitrogen-fixing capacities (for reviews see: Vincent, 1974; Döbereiner and Boddey, 1981; Jordan, 1981; Kennedy et al. 1981; Postgate, 1981; Verma and Long, 1983). The interaction of Rhizobium and legume plant host results in the development of nodules, which are specialized structures that usually form on the roots. Inside these nodules the rhizobia inhabit plant cells and differentiate into bacteroids, which reduce dinitrogen (N_2) to ammonia, that is used by the leguminous host as a nutrient source.
The ability among members of the family of Leguminosae to grow without nitrogenous fertilizer is agriculturally of great interest since many important crop plants belong to this family. It is generally agreed that the contribution made by legume nodule N_2 fixation is fundamental to the global production of food, either for maintenance of soil fertility or directly as grain or green material. It is however difficult to present accurate estimates with respect to the quantitative importance of biological N_2 fixation in the earth's nitrogen economy. Frequently quoted is the calculation by Burns and Hardy (1975) suggesting that at least 25% of terrestrial nitrogen fixation (139×10^6 metric tonnes/annum) could be accounted for by legume crops.
Formation of nitrogen-fixing legume nodules is a complex multistep process requiring genetic input from both symbiotic partners. Because bacteroids are intracellular, rhizobia can be considered as facultative endosymbiotic or even organelle-like systems (Verma and Long, 1983). In the next paragraphs the role and contribution of Rhizobium in the establishment of

symbiotic nitrogen fixation will be dealt with more specifically.

SCOPE OF THE PRESENT INVESTIGATION

The general purpose of the experiments described in this thesis was the mapping and characterization of genes on the R.leguminosarum PRE sym plasmid essential for the onset and persistence of symbiotic nitrogen fixation. In chapter I an overview of the developmental genetics of the Rhizobium/legume symbiosis is presented. The identification of symbiotic genes in R.leguminosarum was based on the search for DNA homology with K.pneumoniae nif genes and on the selection of areas specifically transcribed during effective symbiosis. Subsequently by molecular genetic techniques as recombinant DNA cloning and site-directed Tn5 mutagenesis of selected fragments several symbiotic genes have been localized. Expression in E.coli minicells was employed to elucidate coding sequences of genes isolated by the above described methods. Interspecies hybridization experiments using specific K.pneumoniae or R.meliloti derived nif and nod probes led to the identification and functional analysis of the nifHDK operon (encoding nitrogenase polypeptides; see chapter II), nifA (encoding a regulatory protein for nif gene expression) as well as nifB (involved in FeMo-cofactor synthesis) loci (see chapters III and IV) and a nodulation gene cluster (see chapter V). Hybridization studies on sym plasmid DNA using bacteroid RNA as a probe revealed the existence of several fix genes adjacent to nifHDK and to nifA and nifB. These have been defined by their active transcription within nodules and by Fix^- mutant phenotypes after Tn5 mutagenesis without the synthesis of nifHDK proteins being affected (see chapters III and V). For some of these fix genes molecular weights of the encoded polypeptides were deduced from E.coli minicell expression, as for nifHDK and nifA (see chapters II and IV). The physical organization of sym-plasmids of different rhizobial species with respect to nod, nif and fix loci is compared.

Chapter I
The genetic analysis of symbiotic properties of *Rhizobium* - an overview

This overview will focus on the molecular biology and genetics of nitrogen fixation of rhizobia in symbiosis with their legume hosts, with a short survey of Klebsiella genetics pertaining to studies described in this thesis.

1. NODULE FORMATION BY FAST-GROWING RHIZOBIUM SPECIES.

1.1. Host range and cross-inoculation groups.

The symbiotic interaction between rhizobia and their hosts is specific i.e. Rhizobium bacteria induce root nodules only on a very limited range of host legumes. Leguminous species mutually susceptible to nodulation by a particular kind of rhizobia constitute a "cross-inoculation group". This host range specificity has for long formed the basis of Rhizobium classification. The six Rhizobium species established under this now obsolete system and the corresponding genera of plants that each nodulates are given in Table 1, part 1 (adopted from the Pocket manual on legume inoculants and their use by the Food and Agriculture Organization).
The "cross-inoculation" concept for grouping legumes and establishing Rhizobium species was questioned after numerous ineffective bacteria-plant responses were discovered.
A system of classifying Rhizobium species based on numerical taxonomy has been developed (Bergey, 1983), as shown in part 2 of Table 1.
Rhizobium species are divided into two main categories, the fast- and slow-growers (see Table 1), differing in several characteristics. The fast-growing species have a mean generation time of 2-4 hours, are perithrichously flagellated and produce acid, whereas slow growing bacteria have a mean generation time of 6-8 hours and (sub)polar flagella and are alkali-producing on yeast-mannitol agar (Elkan, 1981). A few of the slow-growing rhizobia have been shown to fix nitrogen ex planta (Kurz and LaRue,

TABLE 1.

Taxonomy of Rhizobium species.

Bacteria	Genera of host plants
	Part 1
R.meliloti	Medicago, Melilotus and Trigonella
R.trifolii	Trifolium spp.
R.leguminosarum	Pisum, Vicia, Lathyrus, Lens
R.phaseoli	Phaseolus vulgaris, P. multifloris
R.lupini	Lupinus and Ornithopus
R.japonicum	Glycine max
R.Cowpea (type)	Vigna, many other genera and species
	Part 2
Fast growers	
R.meliloti	Medicago, Melilotus and Trigonella
R.leguminosarum	
biovar. -trifolii	Trifolium spp.
biovar. -phaseoli	Phaseolus vulgaris, P. multifloris
biovar. -viciae	Pisum, Lathyrus, Lens, Vicia
R.loti	Lupinus, Lotus, Anthyllis, Ornithopus
Slow growers	
Bradyrhizobium japonicum	Glycine max
Bradyrhizobium spp.	
Bradyrhizobium sp.(Vigna)	Vigna - numerous other genera and species
Bradyrhizobium sp.(Lupinus)	Lotus pedunculatus, Lupinus sp.

1975; McComb et al., 1975; Pagan et al., 1975; Bergersen and Turner, 1978; Dreyfus et al., 1983). Because of such biochemical and genetic features it has been generally accepted now (as indicated in part 2 of Table 1) to classify the slow growing rhizobia as a distinct genus, Bradyrhizobium (Jordan, 1982). The fast growing Rhizobium species were found to be closely related to the genus Agrobacterium with respect to chromosomal background. Symbiotic versus phytopathogenic properties as well as host specificity are exclusively determined by respective plasmid contents of these bacteria (see paragraph 3.3) and therefore transferable, which led to the proposal to group fast growing rhizobia and agrobacteria in a single genus, Rhizobium (Hooykaas, 1979; Prakash, 1981). Furthermore DNA homology analyses indicate a very close taxonomic relationship between R.leguminosarum, R.trifolii and R.phaseoli, which therefore were merged into one species R.leguminosarum including different biovarieties; R.meliloti appeared to be slightly more distant (Johnston and Beringer, 1977; Jarvis et al., 1980; see Table 1, part 2). In this survey the former species names for different fast-growing rhizobia will still be used conform the classification given in original references.
Because the fast growing species are more easy to manipulate in the laboratory, they have been the object of most genetic studies of Rhizobium.

1.2. Nodule development

The infection of the roots of leguminous plants by Rhizobium bacteria results in the development of characteristic root nodules. The process of nodule morphogenesis has been extensively reviewed by Dart (1977), Vincent (1980), Newcomb (1981), Bergersen (1982) and Verma & Long (1983). In the development of a root nodule different stages can be distinguished. These are listed in Table 2 together with the phenotypic code used to indicate these different stages according to Vincent (1980).

TABLE 2.

Stages in symbiotic nodule development.

Stage	Phenotypic code
I Preinfection	
1. Multiplication in root area, colonization	Roc
2. Attachment to root surface	Roa
3. Root hair curling	Hac
II Infection and nodule formation	
4. Infection thread formation	Inf
5. Nodule initiation and development	Noi
6. Release of bacteria	Bar
7. Bacteroid differentiation	Bad
III Nodule function	
8. Nitrogen fixation	Nif
9. Complementary functions	Cof
10. Persistence of nodule function	Nop

Adapted from Vincent (1980) and Long (1984).

The interaction of rhizobia and their leguminous hosts begins with the colonization by the bacteria of the legume rhizosphere. This is followed by root hair attachment, which is considered a crucial step in host range determination. A current hypothesis is that binding is achieved by recognition of specific polysaccharide components on the cell surface of the compatible Rhizobium species by a host plant lectin; these rhizobial lectin receptors are encoded by sym-plasmid borne nodulation genes (Bauer and Bhuvaneswari, 1980; Dazzo et al., 1985). The first response observed in the plant is a characteristic deformation of the epidermal root hairs, sometimes described as "shepherds crook" curling. At this stage the actual infection occurs and the bacteria penetrate into the root tissue through a host-produced "infection thread". As the bacteria invade by this extracellular pathway, host cells within the root cortex are induced to dedifferentiate, divide and form a meristem. The infection thread branches and spreads through the nodule tissue and the bacteria are released from infection threads into nodule cells. This leads to the outgrowth of a nodule from the root surface. Inside the infected nodule cells the rhizobia differentiate morphologically as well as biochemically into nitrogen-fixing bacteroids. The bacteroids are generally larger than the bacteria and often have branched structures which are no longer able to divide; they are surrounded by a host-derived membrane, the peribacteroid membrane, which separates the bacteroid from the plant cytoplasm. An as yet unanswered question about bacteroid differentiation is, whether it is directed by a temporally ordered developmental program like sporulation in Bacillus (Losick and Pero, 1981) or is achieved by independent activation of a group of symbiotic operons (Ausubel, 1984a). Both in the bacteroid (Prakash, 1981; Krol et al., 1982) and in the plant (Legocki and Verma, 1980; Bisseling et al., 1983; Verma and Long, 1983; Govers et al., 1985) a number of genes are specifically expressed during nodule formation and in the mature nodule. The two partner organisms now actively cooperate in order to establish nodule functions. The bacteroids synthesize the nitrogenase enzyme complex (10-12% of total bacteroid protein) which reduces nitrogen to ammonia; moreover special cytochromes and new surface antigens are synthesized. The plant cell creates an optimal environment for the nitrogen-fixing bacteroids. It utilizes the ammonia while providing photosynthate as energy source and producing leghemoglobin, which

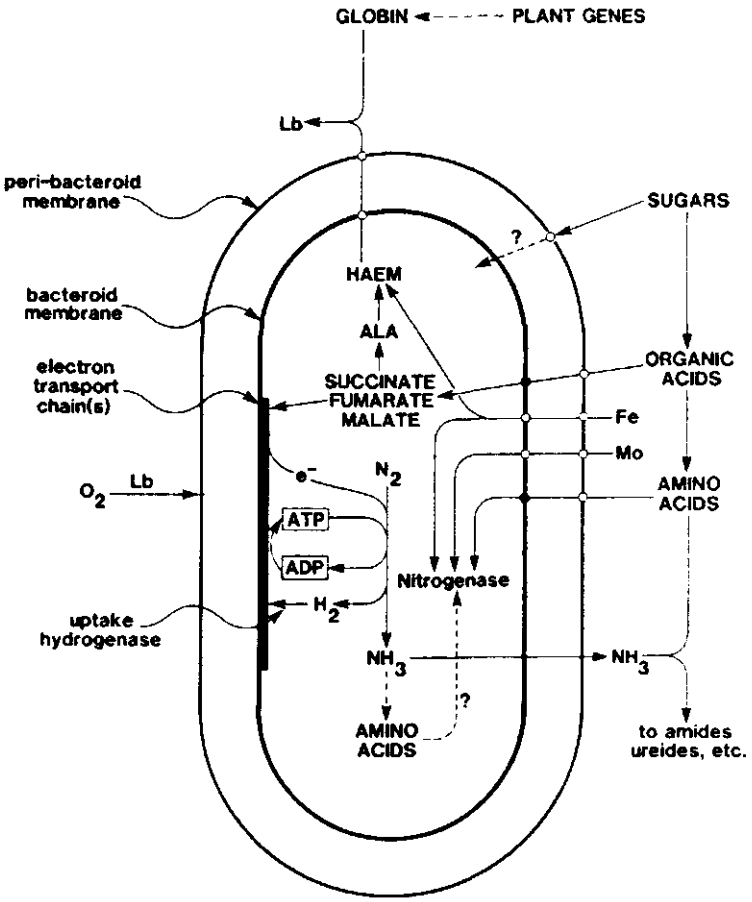
facilitates diffusion of oxygen to the bacteroids for respiration without allowing O_2 to inactivate the extremely oxygen sensitive rhizobial nitrogenase. Remarkably the haem moiety of this oxygen carrier is provided by the bacterial partner, whereas the host plant is responsible for the globin component. It has been proven now that leghemoglobin is exclusively present in the plant cell cytoplasm (Robertson et al., 1984) and not in the peribacteroid space, as has been proposed in former publications. Besides these leghemoglobin loci, a series of plant genes encoding other nodule-specific host proteins ("nodulins") is specifically derepressed during symbiosis, indicating that plant cells also undergo a characteristic differentiation process. The major metabolic pathways which support N_2-fixation in legume root nodules are presented in Fig. 1 (from Dilworth and Glenn, 1984). Not all issues presented in this figure are discussed in detail.

2. IDENTIFICATION OF RHIZOBIUM SYMBIOTIC GENES.

2.1. Rhizobium sym plasmids.

The genetic information of Rhizobium for symbiotic interaction with host plants is encoded on a large plasmid which therefore is referred to as sym plasmid or pSym. In addition to the sym plasmid Rhizobium bacteria may carry one or more high molecular weight plasmids, which are not required for symbiosis and the function of which is cryptic. The sym-plasmids carry the information for host specificity, nodulation (nod) and nitrogen fixation (fix). Several lines of evidence have led to this conclusion. One was the frequency with which symbiotic effectiveness was lost, and the stimulation of this loss by treatments known in other systems to promote plasmid instability, such as high temperature or acridine dyes (Zurkowski, 1982). Furthermore, Johnston et al. (1978) demonstrated that host range specificity of R.leguminosarum could be transferred to R.trifolii or to R.phaseoli by conjugal mobilization of the R.leguminosarum sym-plasmid. In addition to their well-documented ability to restore nodulation functions to heterologous species, sym plasmids were also shown to transfer a Fix^+ phenotype to Fix^- mutant recipients within their own species (Beynon et

Fig. 1. Schematic diagram of metabolic exchanges between plant and bacterial cells in a N_2-fixing nodule.



Known transport systems are represented by ● and potential ones by O.
Lb = leghaemoglobin and ALA = 5-amino levulinic acid.

al., 1980; Brewin et al., 1980; Hooykaas et al., 1981; Djordjevic et al., 1982 and 1983; Lamb et al., 1982; Morrison et al., 1984).

Direct evidence that Rhizobium sym genes are megaplasmid-borne was first

obtained by Nuti et al. (1979) who showed that purified sym plasmid DNA from R.leguminosarum hybridized to the previously cloned structural nitrogenase genes from K.pneumoniae (Cannon et al., 1979). By hybridizing the DNA from different rhizobial species with the K.pneumoniae nif genes it was shown, that the homologous genes in fast-growing rhizobia without exception are localized on large plasmids or megaplasmids (Krol et al., 1980; Banfalvi et al., 1981; Hombrecher et al., 1981; Rosenberg et al., 1981).

Slow-growing Rhizobium strains have not been shown to carry megaplasmids; probably the nif and nod genes are integrated in the chromosome in these cases (Haugland and Verma, 1981). Deletion mapping studies (Banfalvi et al., 1981; Rosenberg et al., 1981), recombinant cloning experiments (Long et al., 1982; Downie et al., 1983b; Kondorosi et al., 1983a; Rolfe et al., 1983; Chapter V of this thesis) and analysis of sym-plasmid derivatives (Huguet et al., 1983) suggested that among fast-growers a (more or less) close linkage exists between nod and nif loci and other sym genes. The relative order of the genes in such symbiotic clusters as well as the physical organization of different sym-plasmids however may vary substantially from one species to another (see chapters III and V of this thesis). This phenomenon possibly is correlated with differences in sym-plasmid sizes, ranging from approximately 200 kb in R.leguminosarum 248 (Downie et al., 1983b) to about 350 kb in R.leguminosarum PRE (Krol et al., 1982) and more than 500 kb in R.meliloti (Banfalvi et al., 1981) respectively.

The existence of such large sym plasmids raises the question of what else they may code for, since the amount of DNA exceeds by far that which is likely needed for the symbiotic genes. Whether the observed clustering of symbiotic genes is involved in the regulation of their expression and/or whether selective pressure maintains the linkage is an as yet unanswered question. Another intriguing problem in this context is the evolutionary pathway of the Rhizobium-legume symbiosis. One theory holds that nitrogen-fixing soil bacteria acquired the ability to invade a host plant (Dilworth and Parker, 1969). The reverse idea hypothesizes that a plant pathogen developed the gene complex required for nitrogen fixation. In any case the bacteria must be able to suppress host defense reactions during recognition and following infection steps (Vance and Johnson, 1981).

The phenotypes of rhizobial mutants, blocked in different symbiotic stages, are classified as follows:

- Nod$^-$ mutants fail to elicit nodule development; these mutations refer to nodulation (nod) genes, which are essential for the initial host recognition steps up to the stimulation of host cell division producing the macroscopic nodule (stages Roa through Noi or possibly Bar in Table 2).
- Nif$^-$ mutants carry mutations in nif genes, which are specifically involved in the biochemical reduction of nitrogen and are therefore presumably analogous with well-characterized K.pneumoniae nitrogen fixation gene functions (see below under 2.4); they generally elicit the formation of nodules that appear almost normal in ultrastructural analysis (stage Nif in Table 2).
- Fix$^-$ mutants are mutated in genes essential for nitrogen fixation (fix) but for which no homologous gene in K.pneumoniae has (yet) been detected (stages Bad, Cof and Nop in Table 2).

  The localization of these different types of symbiotic genes on large-sized plasmids allowed the application of a number of molecular genetic techniques which will be dealt with in the next paragraph.

2.2. Molecular genetic techniques

The genetic analysis of Rhizobium has not been developed to the same extent as that of Gram-negative bacteria like E.coli or the free-living nitrogen fixer K.pneumoniae. However, several types of genetic manipulation which can be carried out on Rhizobium are available now.

2.2.1. DNA transfer methods.

The study of Rhizobium genes involved in symbiotic nitrogen fixation has been approached by various techniques:

- transformation has been reported for several rhizobial species but at frequencies considered suboptimal for clone-bank analyses (Kiss and Kalman, 1982);

- transduction also is extensively used but restricted to systems with efficiently transducing phages (Buchanan-Wollaston, 1979; Martin and Long, 1984);
- conjugation has proven to be the most widely applicable method so far; it is generally used in chromosomal mapping studies and for transfer of symbiotic genes into different Rhizobium species (Beringer et al., 1980).

This latter technique also has been used in order to distinguish between the roles of bacterial and legume host genes at various stages of the differentiation process. By conjugal transfer of specific Rhizobium genes into other Rhizobium species and into the related species Agrobacterium or even a bacterium of a genus that does not naturally infect plants such as E.coli, it can be elucidated whether a symbiotic-like host response is caused by transferred Rhizobium genes on the sym-plasmid and to which extent it is influenced by the chromosomal background of the bacterium and the plant host gene activity (see paragraph 2.3 for an example).

2.2.2. Transposon mutagenesis and recombinant cloning vectors.

Molecular genetic analysis of Rhizobium symbiotic genes is complicated by the fact that free-living bacteria do not differentiate to bacteroids or express symbiotic genes. To study if in symbiotically defective rhizobia the defect is due to a mutation in symbiotic genes, potential mutants must be individually screened by inoculation on plants.
At first standard techniques for random mutagenesis such as chemical or radiation treatments and penicillin selections have been employed to map metabolic and drug resistance loci in Rhizobium (see review by Beringer et al., 1980). But since the development of transposon Tn5 mutagenesis systems for Rhizobium by Beringer et al. (1978), transposon Tn5 is widely used to generate mutants, because it transposes at reasonably high frequencies with relatively little insertion-site specificity and creates polar mutations. Moreover Tn5 confers kanamycin resistance, which is expressed in most Gram-negative bacteria and serves as a genetic marker (Berg and Berg, 1983).

2.2.2.1. Random Tn5 mutagenesis.

Random transposon mutagenesis is accomplished by introducing Tn5 into the

Rhizobium genome by transferring a so called suicide plasmid from E.coli, into Rhizobium. A suicide plasmid carries Tn5 and prophage Mu and cannot stably replicate in Rhizobium (Beringer et al., 1978). Since each kanamycin-resistant Rhizobium clone tested contains an independent single transposition event, the number of plant tests required is reduced to a manageable amount.
By this method random symbiotic mutants have been obtained at frequencies ranging from 0.1% to 1.0% (for examples see Buchanan-Wollaston et al., 1980; Ma et al., 1982; Meade et al., 1982; Scott et al., 1982; Forrai et al., 1983; Noel et al., 1984; Rostas et al., 1984). An alternative method (Simon et al. 1983) utilizes a different type of suicide plasmid i.e. derivatives of E.coli cloning vectors pBR325 or pACYC184 provided with Tn5 and the mobilization site of broad-host-range plasmid RP4. These so-called class I pSUP plasmids are mobilizable from E.coli to Rhizobium with the help of RP4 transfer functions integrated in the E.coli chromosome. The transconjugants can be screened for Tn5 transposition into Rhizobium DNA because the original vector cannot stably replicate in Rhizobium.
A symbiotic gene can, if detected by insertion of Tn5, be cloned directly in E.coli by selection for kanamycin resistance. Subsequently the wild type counterpart can be isolated from a gene library by using the mutated gene as a hybridization probe.

2.2.2.2. Shuttle vectors and site-directed Tn5 mutagenesis.

A second strategy for the cloning and identification of symbiotic genes is based on site-directed Tn5 mutagenesis of Rhizobium genes cloned in E.coli and the analysis of the functions of these genes in Rhizobium. For that conjugative vectors are needed that can replicate in both E.coli and Rhizobium. The most widely and successfully used shuttle vector to date is pRK290 constructed by Ditta et al. (1980). It contains single EcoRI and BglII sites suitable for cloning, confers tetracycline resistance, and contains the mobilization site of plasmid RK2 required for conjugal transfer. RK2 transfer genes must then be supplied in trans by a helper plasmid pRK2013 (Figurski and Helinski, 1979) in a triparental mating. Helpful derivatives of pRK290 are cosmid pLAFR1 containing the λ cos site (Friedman et al., 1982) and pRK404, which is smaller and has a

multilinker site (Ditta et al., 1985). Other shuttle plasmids also are found and being searched for among other incompatibility groups than RK2 (Tait et al., 1982; class II vectors, Simon et al., 1983). It is important to have more than one vector system available since complementation and gene regulation assays require the transfer of more than one cloned gene at a time into a cell. Shuttle vectors are now commonly used for constructing clone-banks and for cloning individual fragments, to be used in complementation and/or gene replacement experiments (Cantrell et al., 1982; Long et al., 1982; Downie et al., 1983a).

A method for site-directed Tn5-mutagenesis, developed by Ruvkun and Ausubel (1981), utilizes the above mentioned conjugative broad host range vector pRK290. Cloned rhizobial DNA segments of interest are mutated by Tn5, while maintained in E.coli. Subsequently the mutated sequences are recloned in the conjugative vector pRK290 and transferred to wild type Rhizobium cells by conjugation; substitution of the wild type by the mutated fragment occurs as the result of two homologous recombination events on either side of the Tn5 insertion. Cells in which this gene replacement has taken place can be isolated then by introduction into this Rhizobium strain of a second P-group plasmid that is incompatable with pRK290, such as pR751 or pPH1JI (Beringer et al., 1978). Simultaneous selection for kanamycin (retention of Tn5) and gentamycin resistance (conferred by pPH1JI) selects transconjugants in which Tn5 has recombined into the sym-plasmid. The second conjugation step thus selects for cells in which double recombination has taken place and concommittantly removes the wild type DNA sequence which has recombined into pRK290 by this event. This technique is very useful for analysing possible symbiotic functions of specific DNA segments in Rhizobium (Corbin et al., 1982 and 1983; Ruvkun et al., 1982; Buikema et al., 1983; Zimmerman et al., 1983; Hahn et al., 1984; Jagadish and Szalay, 1984; Kondorosi et al., 1984; Schetgens et al., 1984 and 1985). Such cloned Rhizobium DNA fragments carrying Tn5 insertions can, while propagated in E.coli and after religation into vectors with a strong constitutive promoter, also be used to determine the location and transcriptional direction of rhizobial protein coding units by expression in E.coli minicells (Fuhrmann and Hennecke, 1982 and 1984; Pühler et al., 1983 and 1984; Schetgens et al., 1984 and in preparation; Schmidt et al., 1984; Weber et al., 1985).

2.3. Nodulation genes.

It is generally assumed now that relatively few sym genes are required for the early stages of nodule formation. At first this was concluded from the observation that random mutagenesis of Rhizobium yields substantially more mutants with a $Nod^{+}Fix^{-}$ phenotype than Nod^{-} mutants (Maier and Brill, 1976; Beringer et al., 1977 and 1980; Buchanan-Wollaston et al., 1980; Long et al., 1981; Paau et al., 1981; Rolfe et al., 1981; Meade et al., 1982; Forrai et al., 1983). Now, this assumption has appeared to be in agreement with the finding that relatively small sym plasmid DNA fragments from R.meliloti (8.7 kb; Long et al., 1982; Kondorosi et al., 1984), R.trifolii (14 kb; Watson et al., 1983; Schofield et al., 1984; Djordjevic et al., 1985a) and R.leguminosarum (10 kb; Downie et al., 1983c) appeared sufficient to complement intraspecies as well as heterologous Nod^{-} mutations. Moreover transfer of the cloned R.meliloti nodulation gene cluster (minimally 3.5 kb from recombinant plasmid pRmSL26; Long et al., 1982) to Agrobacterium tumefaciens or to E.coli consistently elicits the development of Fix^{-} nodules on alfalfa (Hooykaas et al., 1981 and 1982; Kondorosi et al., 1982; Wong et al., 1983; Hirsch et al., 1984; Truchet et al., 1984). A.tumefaciens carrying pRmSL26 induced nodules containing characteristic meristems and sometimes infection threads; however the bacteria were not released from these infection threads and thus remained extracellular. Nodule-like structures elicited by E.coli carrying pRmSL26 were completely devoid of bacteria and without infection threads (Hirsch et al., 1984). So it might be that nodulation requires some additional chromosomal genes, which can be supplied better by the related plant pathogen Agrobacterium than by E.coli.

The overall suggestion from these experiments is that a limited number of Rhizobium genes is involved in nodule inducement. The bacterium accomplishes many of its effects by stimulating specific gene functions in the legume host at an early stage of infection. Root hair curling and actual penetration of bacteria via infection threads are no prerequisites for an obvious host reaction like nodule meristem formation in alfalfa (Hirsch et al., 1984; Finan et al., 1985). From the complementation data

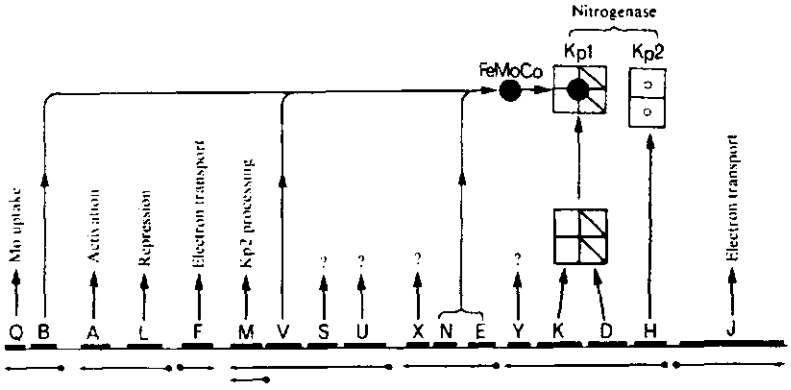
described above it can be concluded that certain nodulation genes have been highly conserved among Rhizobium species. These have been designated by Kondorosi et al. (1984) as common nod genes and are involved in triggering the root hair curling response (hac). The functional homology of several common nod loci has been confirmed by interspecies DNA hybridization studies and nucleotide sequencing (Kondorosi et al., 1984; Schmidt et al., 1984; Török et al., 1984; Rossen et al., 1984a; Lamb et al., 1985; Djordjevic et al., 1985a; Rolfe et al., 1985a; Marvel et al., 1985; Chapter V of this thesis).

The common nodulation gene cluster has been reported recently for several Rhizobium species to consist of a nodABC operon and an adjoining nodD gene, in correspondance with different classes of mutant phenotypes (Török et al., 1984; Kondorosi, 1985; Long et al., 1985; Egelhoff and Long, 1985; Fisher et al., 1985a and b; Mulligan and Long, 1985; Rossen et al., 1984a; Downie et al., 1985a and b; Djordjevic et al., 1985b; Rolfe et al., 1985b; Schofield et al., 1985; Scott et al., 1985). These studies suggest that induction of nodABC depends on active expression of nodD and the presence of an unknown factor present in plant cell exudates. Some other nodulation genes however must be species-specific to account for host range differences. Such clusters of nod genes have also been identified and are designated as hsn genes, determining host specificity of nodulation (Kondorosi et al., 1984 and 1985; Downie et al., 1985 a and b). This hsn region appears to harbor at least three genes, nodE, F and G (Kondorosi, 1985; Downie et al., 1985b). Preliminary investigations further indicate that nod gene areas are not or very weakly expressed in free-living bacteria, but are activated during the earliest stages of nodule development (see Long, 1984). An interesting finding in this respect is the identification of psi, a plasmid-linked and symbiotically essential R.phaseoli gene, that inhibits the expression of genes for exopolysaccharide synthesis which are normally expressed in a free-living culture (Borthakur et al., 1985). Nod-gene encoded proteins have been overproduced in E.coli and used to elicit specific antibodies which may be used for cytological detection of these proteins and the elucidation of the regulation of nod gene expression during nodule formation (Long et al., 1985).

2.4. Nitrogen fixation genes.

As stated in paragraph 2.1, the rhizobial nif-genes have been defined on the basis of analogy with nif-genes in the free-living nitrogen-fixing species K.pneumoniae. Therefore a short survey on Klebsiella nif genetics will be given here (for reviews see Roberts and Brill, 1981; Ausubel et al., 1982; Ausubel, 1984a and b; Dixon, 1984).
K.pneumoniae reduces dinitrogen only under anaerobic and nitrogen-limiting conditions. A combination of genetic mapping, complementation analysis, cloning and sequencing studies has revealed that 17 contiguous nif genes are clustered close to the his operon, occupying 23 kb of the genome and grouped into seven or eight operons. A physical and functional map of this nif-gene cluster is presented in Fig. 2 (from Dixon, 1984).

Fig. 2. Map of the nif gene cluster in K.pneumoniae, indicating the transcriptional organization and function of genes.



The roles of gene products are indicated schematically by the vertical arrows above the map. The horizontal arrows indicate the extent and direction of each transcript, black dots representing the location of nif promoters.

In different species of nitrogen-fixing bacteria (Eady and Postgate, 1974) the nitrogenase enzyme consists of two components; component I, CI, con-

sists of two copies of each of the two subunits α and β, and component II, CII, is composed of two identical subunits. Component I contains the site of substrate binding and the catalytic centre for N_2 reduction. For that an iron-molybdenum cofactor (FeMo-co) as a part of component I is also required. The role of component II is to reduce component I (Roberts and Brill, 1981). In K.pneumoniae the three structural polypeptides of nitrogenase are encoded by genes nifH (CII), nifD (CIα) and nifK (CIβ). Although mutations in these three structural nif genes clearly affect the synthesis of nitrogenase proteins, other mutations in the nif region were observed to impair the nitrogenase function as well, without altering electrophoretic mobility of the polypeptides of component I and II. NifM mutants produce inactive CII and the nifM gene product may therefore be required for processing of CII. The nifS and nifU proteins may also be involved in CII modification. Three genes nifB, nifN and nifE are now known to be required for FeMo-co synthesis, which is essential for CI activity; nifV determines substrate specificity of CI; the nifQ product probably sequesters molybdate for FeMo-co synthesis. NifF and nifJ both encode electron transport factors. The nifX and nifY gene products were identified as polypeptides synthesized from cloned nif fragments in E.coli minicells; their function has not yet been determined. NifL and nifA genes regulate transcription of each of the other nif operons and their role will be discussed in more detail below (see Dixon, 1984 for references).
The expression of K.pneumoniae nif genes appears to be subject to two levels of positive control, in response to the ammonia and oxygen supply (Ausubel et al., 1982). The first level is nif-specific and mediated by the products of the nifLA operon: the nifA protein is a transcriptional activator required for the expression of all other nif operons (Buchanan-Wollaston et al., 1981), whereas the nifL gene product bears repressor activity and antagonizes the nifA protein in the presence of oxygen (Hill et al., 1981). The second level of nif regulation consists in a centralized regulatory control mechanism for nitrogen assimilation in enteric bacteria, the ntr system. Two genes ntrC (glnG) and ntrA (glnF) activate, under conditions of ammonia starvation, various operons involved in nitrogen assimilation (a.o. hut for histidine utilization and put for proline utilization; see Magasanik, 1982) as well as the nifLA regulatory operon (De Bruijn and Ausubel, 1983; Drummond et al., 1983; Merrick, 1983;

Ow and Ausubel, 1983).

Interspecies homology of the structural nifHDK genes was tested by using cloned K.pneumoniae nif DNA fragments (Cannon et al., 1979) as hybridization probes (Ruvkun and Ausubel, 1980). Hybridization was detectable with the DNA of all nitrogen-fixing species tested indicating a strong evolutionary conservation of the structural nitrogenase genes. This has subsequently led to the identification and cloning of the nitrogenase genes from widely divergent species, as R.meliloti (Ruvkun and Ausubel, 1980, 1981; Banfalvi et al., 1981; Rosenberg et al., 1981; Corbin et al., 1982), R.leguminosarum (Nuti et al., 1979; Krol et al., 1982; Schetgens et al., 1984; Ma et al., 1982), R.trifolii (Scott et al., 1983a), R.phaseoli (Quinto et al., 1982), B.japonicum (Hennecke, 1981; Adams et al., 1984), Parasponia Rhizobium (Scott et al., 1983b), cowpea Rhizobium (Hadley et al., 1983), Anabaena 7120 (Rice et al., 1982), Azospirillum brasilense (Quiviger et al., 1982), Frankia, Azotobacter vinelandii, Rhodopseudomonas capsulata and Rhodospirillum (Ruvkun and Ausubel, 1980). This interspecies conservation of nifHDK gene sequences, however, does not necessarily include a preservation of the operon structure. In K.pneumoniae and in several fast-growing Rhizobium species the structural nitrogenase genes comprise one operon, which is transcribed in the order nifH - nifD - nifK (Ruvkun et al., 1982; Corbin et al., 1983; Pühler et al., 1983; Schetgens et al., 1984; Scott et al., 1983a). Two separate transcription units however, nifHD and nifK, have been identified in Anabaena 7120 (Rice et al., 1982). Among slow-growing Bradyrhizobium species a genetic organization consisting of distinct nifH and nifDK operons has been assessed (Fuhrmann and Hennecke, 1982; Kaluza et al., 1983; Scott et al., 1983b; Adams et al., 1984; Fischer and Hennecke, 1984; Yun and Szalay, 1984).

In contrast to earlier data implying that only the Klebsiella nif sequences encoding nitrogenase proteins had been conserved among other nitrogen-fixing species (Ruvkun and Ausubel, 1980), Anabaena DNA was found to hybridize with a nifV/nifS probe in addition (Rice et al., 1982). Moreover homology was detected recently with the genomes of different Rhizobium species using the K.pneumoniae nifA (Szeto et al., 1984; Buikema et al., 1985; Ausubel et al., 1985; Weber et al., 1985; Kim et al., 1985; Downie et al., 1983a; Rossen et al., 1984b; Schetgens et al., 1985; Adams et al., 1984; Fischer et al., 1985; Jagadish et al., 1985; Weinman and Scott, 1985) or

nifB gene (Buikema et al., 1985; Ausubel et al., 1985; Rossen et al., 1984; Schetgens et al., 1985; Fischer et al., 1985) as a probe. The Rhizobium DNA sequences hybridizing to K.pneumoniae nifA nifB were found to encode amino acid sequences showing appreciable homology to the corresponding K.pneumoniae counterparts (Rossen et al., 1984; Buikema et al., 1985). Furthermore a functional correspondance was concluded actually to exist for the rhizobial nifA like gene, from the observation that this gene is indeed required for in vivo expression of the nifHDK genes (Szeto et al., 1984; Schetgens et al., 1985). The regulation of Rhizobium symbiotic genes will be discussed in more detail in paragraph 2.6.
It seems plausible that besides nifHDK, nifB and nifA more nif genes known in Klebsiella to be involved in the assembly of an active nitrogenase enzyme complex, have counterparts in Rhizobium.

2.5. Other symbiotic genes.

In principle two methods have been employed to detect additional fix genes. The first is to search for mutants obtained after random Tn5 mutagenesis, which are affected in the nitrogen fixation capacity and carry a Tn5 insertion located outside the structural nifHDK locus (see e.g. Ma et al., 1982; Downie et al., 1983a; Noel et al., 1984). Alternatively the immediate vicinity of known symbiotic clusters can be screened for additional sym genes by site-directed Tn5 mutagenesis (see e.g. Ruvkun et al., 1982; Corbin et al., 1983; Zimmerman et al., 1983; Pühler et al., 1984). This approach is based upon the evidence for close linkage of nifHDK, fix and nod genes on the sym plasmids of fast-growing species (see paragraph 2.1). A second and different approach was derived from the observation that megaplasmid-borne symbiotic genes are exclusively expressed during nodule development and not to any significant extent in free-living bacteria (Krol et al., 1980; Chapter V of this thesis). Screening of a sym plasmid clone bank by hybridization with bacteroid mRNA as a probe and Tn5 mutagenesis of the selected sym-plasmid fragments indicated a strict correlation between regions which are actively transcribed in nitrogen-fixing nodules and areas where symbiotic fix genes have been localized by DNA homology or by Fix^- phenotypes in Tn5 mutants (Kondorosi et al., 1983b; Schetgens et al., 1984

and 1985; Hontelez et al., manuscript in preparation- see chapter V of this thesis; David et al., 1984).

2.6 Regulation of bacteroid gene expression.

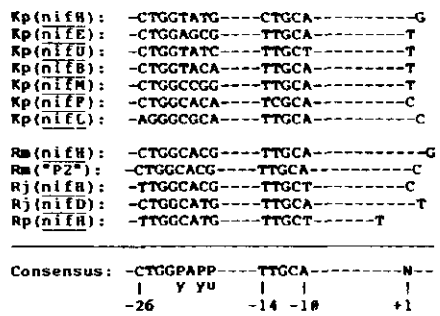
At least in one respect Rhizobium nif gene regulation is similar to that in Klebsiella. Rhizobial nif genes are just as in K.pneumoniae under direct control of a positive transcriptional activator, the nifA gene product. The existence of a structural and functional homologue of the K.pneumoniae nifA gene in several fast- and slow-growing Rhizobium species was concluded from homology studies (see paragraph 2.4 for references). This conclusion is supported by evidence based on nucleotide sequences of nifA regulated promoters. K.pneumoniae nif gene promoters are characterized by a particular consensus sequence, which also appears to be conserved in various nif promoter sequences from Rhizobium species (see Fig. 3 legend for references).

The remarkable similarity between various Rhizobium and Klebsiella nif promoters suggests a common regulatory mechanism for transcriptional control i.e. by the nifA protein, the more so as the nif promoter consensus sequences exhibit poor correspondence to promoter structures known to be typical for E.coli (see Hawley and McClure, 1983).

Furthermore the actual activation of Rhizobium nif promoters by the K.pneumoniae nifA gene product could be detected in vitro by means of nif-lacZ fusion experiments, allowing to monitor expression from a certain nif-promoter under the influence of constitutively produced K.pneumoniae nifA protein (Pühler et al., 1983; Sundaresan et al., 1983a and 1983b).

An intriguing result in this context was obtained by Better et al. (1985), who showed that an entire 160 bp conserved promoter sequence of R.meliloti nifH (P1) is necessary for heterologous activation by K.pneumoniae nifA protein in E.coli, whereas expression from this and another nif promoter (P2) in 5 week steady state root nodules requires only a 35 bp promoter fragment (-35 to 0 comprising the nif consensus region) for continued transcription. This data suggests that the complete 160 bp promoter-regulatory region may be essential only for the initial activation of nif-

Fig. 3. Sequences of various nif promoters.



The base at the 3' end of each promoter sequence is the start point of transcription. Abbreviations and references:
Kp: K.pneumoniae (Beynon et al., 1983); Rm: R.meliloti (Better et al., 1983; Sundaresan et al., 1983a); Bj: B.japonicum (Adams and Chelm, 1984; Fuhrmann and Hennecke, 1984; Kaluza and Hennecke, 1984); Rp: R.parasponiae (Scott et al., 1983b). Not included but also shown recently to meet the nif consensus sequence are the following promoters: R.parasponiae nifD (Weinman et al., 1984), cowpea Rhizobium nifH and nifD (Yun and Szalay, 1984), R.trifolii nifH (Scott et al., 1983a), R.phaseoli nifH (Quinto et al., 1985) and R.leguminosarum 248 nodA (Rossen et al., 1984a). Adapted from Ausubel 1984b.

specific transcription processes in R.meliloti.
Another important observation is the recent finding, based on sequence comparisons of nif promoters in R.meliloti and R.trifolii, that domains of inter- as well as intraspecific conservation are present within these promoter regions (Schofield and Watson, 1985). Except the above-mentioned nif-specific consensus sequence (0 to -35), one species-specific promoter element (-45 to -118) and a second stretch of nif-specific nucleotides (-122 to -165) were detected. Both nif-specific promoter elements which have been identified also in Klebsiella (Drummond et al., 1983), may be involved in the coordinated activation of nif genes by nifA and

ntrA/ntrC. The authors suggest that the species-specific regulatory component of nif promoter sequences might be required for their exclusive expression during association with appropriate plant hosts.
Several hypotheses exist with respect to the actual mode of action of the regulatory nifA protein. It has been proposed that it might modify the RNA polymerase as a novel σ-factor analogous to gene regulation in Bacillus (Losick and Pero, 1981), in order to allow recognition of nif-specific sequences. Alternatively direct binding of the regulatory product to the DNA double helix in order to create a new RNA polymerase binding site analogous to the lambda cII protein (Ho et al., 1983) has been suggested. A key developmental question that now remains to be answered is, whether or not the rhizobial nifA gene activation itself depends on a centralized ammonia control system as in Klebsiella. One alternative is that the Rhizobium nifA locus might be activated by a "centralized symbiotic regulatory system", that is responsible for activating not only nifA but also other symbiotic genes, for example those required for early steps in nodulation or for bacteroid differentiation and nodule maintenance. Thus the nifA gene, which regulates the expression of specific nif operons, would be only one of several targets for this symbiotic regulatory system (Ausubel, 1984a and 1984b).

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Chapter II
Molecular cloning and functional characterization of *Rhizobium leguminosarum* structural *nif* genes by site-directed transposon mutagenesis and expression in *Escherichia coli* minicells

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Summary: In order to study the structural organization and regulation of the expression of the nitrogenase gene cluster in *Rhizobium leguminosarum* PRE we selected relevant subfragments of the *sym*-plasmid from clone banks by homology with *R. meliloti nif*-genes. Site-directed Tn5 mutagenesis was applied to a *nif* DH-specific clone and subsequently the transposon insertions were transferred back into the wild-type rhizobial genome by homologous recombination. Phenotypic effects of Tn5 mutations in the region of the structural *nif*-genes were determined by measuring acetylene reduction in nodulated plants and by immunological analysis of bacteroid-specific proteins. The localization of Tn5 insertion sites was in accordance with observed consequences: two genotypically different Tn5-induced mutations within *nif* D caused repression of CIα and β synthesis and a strong reduction of CII production, thus resulting in a Fix$^-$ phenotype. Expression of different cloned *Rhizobium* DNA inserts, bearing *nif* K, *nif* D, *nif* H, or *nif* DH, was achieved in *Escherichia coli* minicells dependent upon the presence of a strong upstream vector promoter sequence. Gene products were identified by immunoprecipitation with specific antisera. Endogenous rhizobial transcriptional start signals in one case (*nif* H) seemed to be recognized at a low rate by the *E. coli* system; in contrast, *Rhizobium* ribosome binding sites for all three structural *nif*-genes functioned normally in minicells. The approximate location of the coding regions for *nif* KDH genes was determined and found to be contiguous. **Key Words:** *Rhizobium leguminosarum*—*nif* KDH—Transposon Tn5 mutagenesis—Minicell expression—Nitrogenase-specific antisera.

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Biological nitrogen fixation in root-nodule symbioses of leguminous plants and bacteria of the genus *Rhizobium* is effected by a complex series of interactions between bacteria and plants. During root nodule development the in-

fecting rhizobia differentiate into bacteroids and in the course of this process the bacterial genes for the production of active nitrogenase are induced (1,2). In addition, a number of nodule-specific host proteins (nodulins), in particular leghemoglobin, are synthesized in the root cells invaded by rhizobia, indicating differentiation of plant cells as well (3,4).

In the fast-growing species of *Rhizobium* the genes encoding host recognition, nodulation (*nod*), and nitrogen fixation (*nif*) are located on a single indigenous plasmid of high molecular weight referred to as a *sym*-plasmid (5–9). The *sym*-plasmid genes are strongly expressed in bacteroids during effective endosymbiosis, and only weakly or not at all in free-living rhizobia (10). The enzyme nitrogenase in all nitrogen-fixing organisms studied so far is a complex of two components: component I (CI), an FeMo protein, composed of two α and two β-subunits encoded by genes D and K respectively, and component II (CII), an Fe protein, composed of two identical subunits encoded by gene H. In the free-living nitrogen-fixing bacterium *Klebsiella pneumoniae*, for which the organization of the genes involved in nitrogen fixation has been studied in great detail (11), the genes for nitrogenase are in the order H, D, and K and comprise an operon. Likewise, in *Rhizobium meliloti* the structural genes for nitrogenase seem to constitute one transcription unit (12–14). This appears also to be the case in *Azospirillum brasilense* (15). Among other symbiotic nitrogen-fixing organisms a considerable variation in the organization of *nif* H, D, and K genes is found. In *R. phaseoli*, reiterated *nif*-gene sequences were identified (16). In the slow-growing species *R. japonicum*, *nif* H is not adjacent to *nif* D and K (17). In *Anabaena 7120*, on the other hand, *nif* K is separated by at least 11 kb from *nif* D and H and possesses independent transcription initiation signals (18,19). Such differences in the organization of structural genes for nitrogenase may affect the regulation of expression of these genes on the transcriptional and/or translational level. Studies on the regulation of the synthesis of the nitrogenase components I and II of *R. leguminosarum* PRE in pea root nodules (20) indeed indicated that synthesis of these components is not strictly coordinated.

In this paper the molecular cloning and analysis of the *nif*-genes in *R. leguminosarum* PRE are described. The effects of transposon Tn5 mutations within the structural *nif*-gene cluster of *R. leguminosarum* were determined by measuring N_2-fixing capacity of the root nodules induced by the mutants and by immunological analysis of the synthesis of bacteroid-specific proteins. The positions of *nif* H, D, and K were determined by expression of selected, mapped DNA fragments in the *Escherichia coli* minicell system. The results indicate that the *nif* H, D, and K genes map closely together and that polar transposon mutations in the gene coding for CIα strongly reduce the expression of the CII protein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phages

The strains of *R. leguminosarum*, *E. coli*, λ phages, and plasmids used are listed in Table 1

Media and Growth Conditions

E. coli was grown at 37°C under continuous aeration in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) or on media solidified with 1.5% (wt/vol) agar. *R. leguminosarum* strains were cultured at 29°C in TY (5 g tryptone, 3 g yeast extract, 1 g $CaCl_2 \cdot 2H_2O$ per liter). All ingredients for culture media were supplied by Difco, Detroit, MI.

For selective purposes antibiotics at the following concentrations (μg/ml) were added: ampicillin 50, gentamycin 25, kanamycin 20, neomycin 20, streptomycin 250 (all purchased from Sigma Chemical Corp., St. Louis, MO); chloramphenicol 25 (Boehringer Mannheim GmbH); tetracycline 10 (Nutritional Biochemicals Corp., Cleveland, OH).

DNA Methodology

Isolation

Total genomic DNA was prepared from *R. leguminosarum* as follows: after prewashing

TABLE 1. *Bacterial strains, plasmids, and bacteriophages*

Designation	Relevant character/genotype	Reference/source
Bacterial strains		
R. leguminosarum		
PRE	Wild type	37
PRE Str^r	Streptomycin and acriflavin resistance	37
E. coli		
HB101	F$^-$ *leuB6 proA2 thi-1 lacZ4 supE44 tonA21 rpsL hsdR hsdM recA13* λ$^-$	28
MM294	*endoI hsdR hsdM pro*	28
294	*pro thi* r$^-$m$^+$ *recA* λ$^-$	36
DS410	*thi minA minB ara lacY xyl malA mtl tonA rpsL Azi^r* λ$^-$ (minicell-producing)	32
Q358	R_k^- M_k^+ Su_{II}^+ 80 R	38
Q359	R_k^- M_k^+ Su_{II}^+ 80 R P2	38
Bacteriophage		
λ467	λb211*rex*::Tn5 cI857 $O_{am29}P_{am80}$	28
λEMBL3	λ1059-derived cloning vector (Q358 and Q359 hosts)	38
Plasmids		
pBR322	Ap^{r}Tc^r	39
pACYC184	Cm^{r}Tc^r	40
pSUP201	Ap^{r}Cm^{r}Tc^s; pBR325. mob (RP4 mobilisation site)	36
pLAFR1	Tc^r; cosmid vector derived from the conjugative broad host range plasmid pRK290	33,41
pRK2013	Nm^r; complements *tra* genes of pRK290 in conjugations	42
pPH1JI	Gm^r, incompatible with pRK290	43
pSA30	*K. pneumoniae nif* HKDY genes cloned in pACYC184	44
pRmR2	*R. meliloti nif* HD genes cloned in pACYC184	34

the cell pellet (derived from a 5 ml saturated culture) in 1 *M* NaCl, lysis was performed in 4 ml 10 m*M* Tris-HCl, 25 m*M* ethylenediaminetetraacetate (EDTA), pH 8.0, by addition of 1 mg lysozyme and 0.6 ml 10% (wt/vol) Sarkosyl (Ciba Geigy AG, Basel), 1 mg/ml proteinase K and incubation at 37°C for 1 h; the lysate was extracted twice with phenol and then with chloroform; DNA was precipitated with an equal volume of isopropanol, collected with a glass rod, and dissolved in H_2O at a concentration of about 0.1 mg/ml. Large plasmid DNA was isolated from *R. leguminosarum* as described by Krol et al. (5).

For bacterial plasmid preparation from *E. coli* the rapid boiling method of Holmes and Quigley (21), slightly modified as described by De Bruijn and Ausubel (22), was used; in the case of large-scale isolations this lysis procedure was followed by cesium chloride equilibrium density gradient centrifugation (23).

Endonuclease Digestion and Gel Electrophoresis

Restriction enzymes BglII, EcoRI, HindIII, and SalI (from Boehringer Mannheim GmbH), and BamHI and XhoI (from Bethesda Research Laboratories GmbH, Neu-Isenburg) were used according to the manufacturers' instructions.

Restriction endonuclease digests of DNA were analysed by horizontal 0.8% agarose gel electrophoresis in 40 m*M* Tris-HCl, 1 m*M* EDTA, 5 m*M* sodium acetate, pH 7.8. Restric-

tion fragments were eluted from agarose by freeze-squeezing (10).

Hybridization

Radioactively labeled DNA probes were produced by nick-translation (24) under the following conditions. The reaction mixture (33 μl) contained 50 m*M* Tris-HCl, pH 7.8, 5 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, 0.004 m*M* dCTP, dGTP, and dTTP, 30 μCi α-^{32}P dATP (Radiochemical Centre Amersham), 0.025 μg/ml DNase I (Boehringer Mannheim GmbH), 5 units DNA polymerase I (Boehringer Mannheim GmbH), and 0.1–1 μg DNA, and was incubated for 3–4 h at 15°C; free nucleoside triphosphates were removed by passing the mixture through a Sephadex G50 column and eluting with 0.1 × SSC–0.1% (wt/vol) sodium dodecyl sulfate (SDS). (20 × SSC is 3.0 *M* NaCl, 0.3 *M* NaCitrate, pH 7.0.)

For blotting DNA fragments (25) from agarose gels onto nitrocellulose (Schleicher and Schüll, Dassel, F.R.G.), DNA was first depurinated in 0.25 *M* HCl. Preincubation of Southern blots was carried out in 5 × Denhardt's and 3–6 × SSC for 2–5 h at 65°C; hybridization was done in the same solution with 0.5% (wt/vol) SDS, 0.1 mg/ml calf thymus DNA, and denatured labeled DNA probe at 65°C overnight.

Ligation and Transformation

Ligation reactions were performed as recommended by Maniatis et al. (26) and the *E. coli* transformation procedure of Kushner (27) was used.

Isolation and Labeling of Bacteroid RNA

Total bacteroid RNA isolated from 17-day-old nodules was 5'-end labeled *in vitro* with T4 polynucleotide kinase (5) and subsequently hybridized to Southern blots.

Transposon Mutagenesis

Transposon Tn5 was introduced into a *nif*-specific cosmid (33) clone pRB10 (Tc^r) by λcI857::Tn5 infection of HB101/pRB10 under repressing conditions (30°C); subsequent screening for pRB10::Tn5 insertions was done as described by Ruvkun and Ausubel (28). Different Tn5 insertions within the *Rhizobium* fragment cloned in pRB10 in *E. coli* were exchanged against corresponding parental wild-type DNA in *Rhizobium* by means of a double homologous recombination achieved by the following bacterial mating procedure. *E. coli* MM294/pRB10::Tn5 (Km^rTc^r) was conjugated with wild-type *R. leguminosarum* PRE (Str^r) and MM294/pRK2013 (functioning as a helper plasmid complementing *tra* genes of the pRB10 vector plasmid pLAFR1) leading to the introduction of a Tn5 mutagenized pRB10 plasmid into *Rhizobium*; subsequently marker exchange was forced by conjugation of PRE/pRB10::Tn5 with MM294/pPH1J1 (Gm^r; incompatible with pLAFR1) selecting for Str^r Km^r Gm^r Tc^s, thereby effecting actual transfer of Tn5 into the *Rhizobium* genome.

Growth and Inoculation of Pea Plants

Pisum sativum (Rondo) seeds were surface sterilized, inoculated with *R. leguminosarum* PRE Str^r or Tn5-mutagenized strains, and cultured in sterile vermiculite (three seeds) or in gravel for large-scale experiments (50 seeds). The inoculum consisted of a suspension of one fresh colony in 5 ml of sterile water or a 100-ml saturated culture, respectively. From 15 days after inoculation plants were examined for the occurrence of nodules. Nitrogen-fixing ability was measured with samples of whole plants at different times after infection by the acetylene reduction assay in a Pye 104 gas chromatograph (29).

Protein Analysis of Bacteroid Proteins

Bacteroid-specific proteins were isolated from pea nodules (4) and separated by electrophoresis in a 12.5% (wt/vol) SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue (30). For immunological identification, proteins were transferred by electro-

blotting to nitrocellulose (31); the filter was incubated with antisera specific against CI and CII (20) and finally with ^{125}I-labeled protein A (30) to visualize immune complexes.

Purification and Radioactive Labeling of *E. coli* Minicell-Producing Strains Containing Recombinant Plasmids

The minicell-producing strain of *E. coli* DS410 was transformed with plasmid isolated from *E. coli* 294 r^-m^+ to ensure modification of DNA. Subsequently minicells were purified from the DS410 derivatives by sucrose gradient centrifugation (32), and used to analyze the protein synthesis under direction of plasmid templates.

To 100 µl of a minicell suspension, 10 µl thiamine-HCl (0.2 mg/ml), 25 µl 20% (wt/vol) glucose, and 100 µl complete amino-acid mix (0.5 mg/ml each) except methionine were added. This solution was brought up to 998 µl with MS, a mineral salt solution [0.5% (wt/vol) NH_4Cl, 0.1% (wt/vol) NH_4NO_3, 0.01% (wt/vol) $MgSO_4 \cdot 7\ H_2O$, 0.2% (wt/vol) Na_2SO_4, 0.9% (wt/vol) K_2HPO_4, 0.3% (wt/vol) KH_2PO_4] and preincubated for 30 min at 37°C; 2 µl (10–20 µCi) ^{35}S-methionine (Radiochemical Centre Amersham) was added and this mixture was kept at 37°C for 30 min, after which cells were pelleted, washed once with MS, and suspended in 500 µl MS. From this suspension 100 µl was pelleted and resuspended in 20 µl sample buffer [10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 0.001% (wt/vol) bromophenol blue], heated at 100°C for 5 min, and used for electrophoresis.

Immunoprecipitation

A pellet derived from 200 µl ^{35}S-labeled minicell suspension was resuspended in a mixture of 22.5 µl T-TDS [10 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium desoxycholate (DOC), 0.1% (wt/vol) SDS], 10 mg/ml bovine serum albumin (BSA), and 2.5 µl 10% (wt/vol) SDS, and kept at 100°C for 5 min. After addition of 25 µl T-TDS-BSA and centrifuging at 15,000 rpm for 15 min at room temperature, the supernatant was collected; 5 µl of preimmune serum was added and the mixture was kept at 4°C for 4 h. Protein A specific complexes were precipitated by addition of 25 µl of a 10% (wt/vol) *Staphylococcus aureus* (from the Enzyme Center, Boston, MA) cell suspension in T-TDS-BSA and after incubation for 1 h at 4°C removed by centrifugation. The supernatant was then brought up to 98 µl with T-TDS-BSA and 2 µl specific antiserum was added; this mixture was incubated overnight at 4°C. Immune complexes were bound by addition of 10 µl 10% (wt/vol) *S. aureus* cells in T-TDS-BSA and incubation for 1 h at 4°C. This mixture was loaded on two sucrose layers (500 µl 1 *M* sucrose in T-TDS, 200 µl 0.5 *M* sucrose in T-TDS) and centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was washed three times with 150 µl T-TDS and suspended in 20 µl sample buffer. After heating for 5 min at 100°C and centrifugation the supernatant was used for gel electrophoresis.

Autoradiography

Standard techniques were used for autoradiographical exposure of DNA and protein blots or dried gels. Sakura A2B and Kodak Safety ARD X-ray films were employed.

Chemicals

All chemicals not specified were from Merck, Darmstadt.

RESULTS

Cloning of the *nif* Region of *R. leguminosarum* PRE

In order to construct a genomic clone bank, total DNA from *R. leguminosarum* PRE was partially digested with EcoRI, and 15–25 kb fragments of this DNA, isolated by sucrose gradient centrifugation, were ligated into the

cosmid vector pLAFR1 digested with EcoRI (33). The ligation mixture was packaged into λ phage heads *in vitro* and the resulting particles were used to infect *E. coli* HB101; this resulted in 800 Tc^r colonies which were screened for *nif*-homologous sequences by colony hybridization using the *R. meliloti* structural *nif*-genes cloned in pRmR2 as a probe. The insert of this plasmid contains sequences of *nif* D and H, but not *nif* K (34). The single positive hybridizing colony was designated clone pRB10 and the insert was physically mapped using a combination of digestions with restriction endonucleases EcoRI, BamHI, XhoI, BglII, and HindIII. The physical map of the 17.10 kb insert of pRB10 is shown in Fig. 1.

Hybridization of nick-translated pRB10 DNA to Southern blots of BamHI-, BglII- or HindIII-digested total DNA of *R. leguminosarum* PRE revealed that the pRB10 insert is a composite of at least two noncontiguous PRE DNA fragments (results not shown). The EcoRI fragments derived from another part of the genome are given as a broken line, starting from an EcoRI site between BamHI and BglII sites as deduced from the above mentioned hybridization results. The leftmost 6.0 kb EcoRI fragment of the pRB10 insert revealed homology with *R. meliloti* nitrogenase structural genes D and H by hybridization with ^{32}P-labeled pRmR2 DNA. This 6.0 kb EcoRI fragment corresponds in size to the *R. leguminosarum sym*-plasmid fragment which was shown to hybridize with the *K. pneumoniae nif* D and H genes (5). More precise determinations of the regions within pRB10 homologous with *R. meliloti nif*-genes showed that homology with *nif* H (an XhoI fragment of pRmR2) is limited to the 2.2 kb HindIII-XhoI fragment and homology with *nif* D (an EcoRI-HindIII fragment of pRmR2) to the 1.1 kb EcoRI-HindIII fragment of pRB10, as indicated in Fig. 1.

Additional *R. leguminosarum* DNA fragments bearing structural *nif*-genes and adjacent

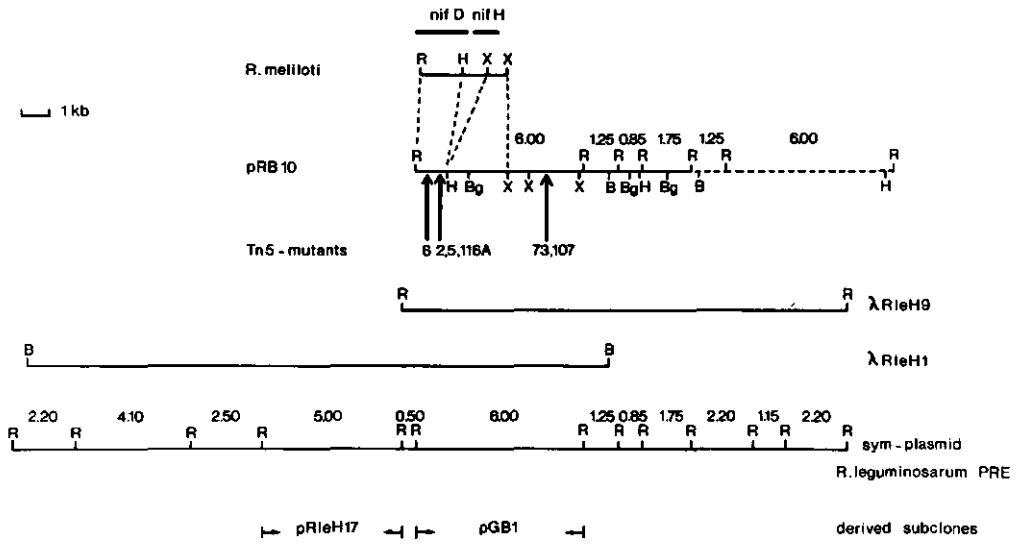

FIG. 1. Nitrogenase gene region of the *R. leguminosarum* PRE *sym*-plasmid. In the upper part the physical map of the insert of clone pRB10 (in vector pLAFR1) is shown. EcoRI restriction fragment lengths are given in kb (kilobases). The broken line refers to noncontiguous DNA fragments. *R. meliloti nif* D and H (pRmR2) homology data are marked with dotted lines. The positions of Tn5 insertions are indicated by numbered arrows. Insert maps of two overlapping clones λRleH1 and 9 (in phage λEMBL3) are also delineated. The lower line shows a contiguous part of the *R. leguminosarum sym*-plasmid bearing the structural *nif* region as well as the genomic positions of two derived subclones pRleH17 and pGB1 within it. Restriction sites are abbreviated as follows: B (BamHI), Bg (BglII), H (HindIII), R (EcoRI), S (SalI), X (XhoI).

sequences were isolated by screening clone banks of purified *R. leguminosarum* PRE *sym*-plasmid DNA, digested with BamHI or EcoRI, in phage λ EMBL3 (to be described in detail elsewhere). This analysis was done by hybridization of recombinant plaques transferred to nitrocellulose with the nick-translated *nif* DH-specific 6.0 kb EcoRI fragment derived from pRB10. The clones λ RleH1 and λ RleH9 indicated in Fig. 1 originated from these banks and both overlap the pRB10 insert. Clone λ RleH9 (15.90 kb insert) contains EcoRI fragments contiguous to both ends of the fragments cloned in pRB10.

Clone λ RleH1 (20.7 kb insert) extends 13.8 kb from the left end of the pRB10 insert. The lower line in Fig. 1 represents the EcoRI restriction map of a 29.7 kb part of the *R. leguminosarum* PRE *sym*-plasmid containing the *nif* D and H genes and, as will be shown below, also *nif* K.

Introduction of Site-Specific Tn5 Mutations into *R. leguminosarum* PRE

Transposon Tn5 (Km^r) was introduced into the *Rhizobium*-specific insert of pRB10 by λ::Tn5 infection of *E. coli* HB101/pRB10 (Tc^r) under lysogenic conditions (30°C). From Km^rTc^r *E. coli* HB101 transformants plasmid DNA was isolated and transformed into *E. coli* MM294. Selection for Km^rTc^r resulted in 137 colonies with a Tn5 insertion in pRB10. In eight of the pRB10::Tn5 plasmids the Tn5 insertion was localized by restriction enzyme analysis and hybridization with pRmR2 (results not shown) in the left 6.0 kb EcoRI fragment of pRB10 (Fig. 1) near or in the putative structural *nif*-genes. These eight pRB10::Tn5 mutant plasmids were each conjugated into *R. leguminosarum* PRE (Str^r) by the mating procedure described by Ruvkun and Ausubel (28) (see Materials and Methods), and Km^rStr^r transconjugants in which the Tn5 insertion might be transferred by homologous recombination from pRB10::Tn5 to the *R. leguminosarum* genome were analyzed by hybridizing blots of restriction fragments of total PRE::Tn5 DNA with appropriate DNA probes as illustrated in Fig. 2. Lane 1 represents the hybridization pattern of BglII-digested pRB10 using total pRB10 as a probe; sizes of hybridizing fragments are (see also the physical map in Fig. 1): 27.0 kb (comprising 8.10 kb right-hand terminal BglII-EcoRI fragment of pRB10 insert and 18.90 kb from pLAFR1); 5.75 kb (internal fragment of pRB10 insert); 3.00 kb (1.90 kb left-hand terminal BglII-EcoRI fragment of pRB10 insert and 1.10 kb from pLAFR1); 1.60 kb (vector fragment); 1.35 kb (internal fragment of the pRB10 insert).

BglII restriction fragments of parental *Rhizobium* PRE Str^r DNA were hybridized with nick-translated pRB10 DNA; the resulting autoradiogram (lane 2, Fig. 2) shows two fragments corresponding with the 5.75 kb and 1.35 kb BglII internal fragments of pRB10 (Fig. 1) and fragments of 19.00 kb and 3.20 kb which

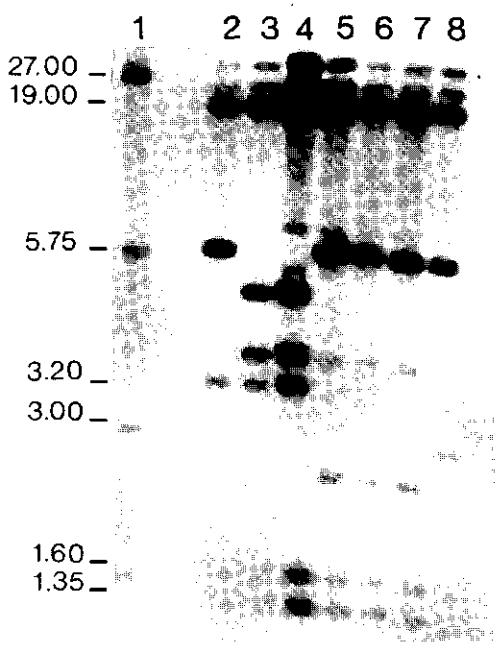

FIG. 2. Mapping of the position of Tn5 insertion sites within the chromosomal DNA of *R. leguminosarum* PRE mutants. A Southern blot of BglII restriction fragments from pRB10 (lane 1), parental PRE Str^r (2), and Tn5 derivatives 73 (3), 107 (4), 2 (5), 5 (6), 116A (7), and 6 (8) hybridized with nick-translated pRB10-probe is shown (exposure time: 111 h). Relevant restriction fragment lengths are indicated in the margin in kb.

contain respectively the 8.10 kb right-border and 1.90 kb left-border BglII-EcoRI fragments occurring in pRB10 (Fig. 1). The hybridizing 1.50 kb fragment is probably due to the non-contiguous character of the pRB10 insert. The patterns of the Tn5 mutants in lanes 3–8 of Fig. 2 show that the mutants split up into three groups. Two mutants, 73 and 107 (lanes 3 and 4, respectively), both appear to have the Tn5 inserted in the 5.75 kb genomic fragment: the 5.75 kb has disappeared from the Southern hybridization pattern and instead two fragments of 5.10 and 3.70 kb, respectively, are found. These two fragments are generated because the Tn5 inserted into the 5.75 kb rhizobial DNA fragment contains two BglII recognition sites at 1.50 kb from both ends (35). The sizes of 5.10 kb and 3.70 kb are in good correlation with similar interpretations for other restriction enzyme digestions and define the position of the Tn5 insertion in the mutants 73 and 107 as indicated in Fig. 1. Similarly, we conclude that mutants 2, 5, and 116A (lanes 5, 6, and 7) have a Tn5 insertion in the same position of the 3.20 kb BglII fragment of PRE DNA, since in these mutants the 3.20 kb BglII fragment is replaced by 3.90 and 2.25 kb fragments.

Mutant 6 (lane 8) appears to have a Tn5 insertion also in the 3.20 kb BglII fragment, but in a different position, since with this mutant two fragments, each of 2.95 kb, arise by insertion of the Tn5 transposon. The mutants 2, 5, and 116A, and mutant 6, are therefore located on the PRE *sym*-plasmid as indicated on the physical map shown in Fig. 1. All four inserts are in the region hybridizing with *nif* D DNA; the inserts in the numbers 73 and 107, on the other hand, are located outside this region.

The other two PRE Str^r::Tn5 mutants, 3 and 26, were aberrant in the sense that mutant 3 produced a Southern hybridization pattern, corresponding exactly with that of the parent PRE Str^r DNA (lane 2), and mutant 26 gave a pattern composed of a combination of bands derived from pRB10 (lane 1) and PRE Str^r (lane 2). Instead of the desired double homologous crossing over, cointegration of the pRB10::Tn5 plasmid has possibly occurred with mutant 26 and transposition of Tn5 with mutant 3.

Symbiotic Phenotypes of *R. leguminosarum* PRE Str^r::Tn5 Mutants

Upon inoculation of pea seeds all eight isolated Tn5 mutants of *R. leguminosarum* PRE Str^r showed a Nod$^+$ phenotype. Four mutants (nrs 3, 26, 73, and 107) were Nod$^+$ Fix$^+$ and indistinguishable from the parent PRE Str^r. Rose-red nodules were visible from 15 days after germination and nitrogenase activity developed just as with the PRE Str^r induced nodules. It was noted that the onset of acetylene reduction showed a delay of about three days, compared with the original wild-type *Rhizobium* (Str^s). The other four Tn5 mutants (nrs 2, 5, 116A, and 6) were Nod$^+$ Fix$^-$. The nodules which developed were relatively small and were found mostly on the lateral roots; at an early developmental stage the nodules looked pink, suggesting that leghemoglobin synthesis occurred, but they rapidly turned pale or green. The shape of the bacteroids in the nodules did not significantly differ from that of Fix$^+$ strains, but no acetylene reduction was detected.

The two phenotypes observed for these mutants corresponded to the different positions of the Tn5 insertions. The Fix$^+$ mutants 73 and 107 have Tn5 mutations outside the *nif* DH homologous region of the genome, whereas mutants 2, 5, 116A, and 6 are Fix$^-$ and possess Tn5 insertions at two respective sites either in the sequence homologous to *nif* D or in the DH intergenic region (see Fig. 1).

Synthesis of Nitrogenase Components in *R. leguminosarum* PRE Str^r::Tn5 Bacteroids

Bacteroids were isolated from root nodules induced by *R. leguminosarum* Tn5 mutants at different times after inoculation and analyzed for the occurrence of CI and CII components of nitrogenase. Bacteroid proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and the pro-

tein blots were incubated with a mixture of antisera specific against nitrogenase components I and II (20) and subsequently with ^{125}I-labeled protein A to detect immune complexes. Figure 3 shows the result of such an analysis of proteins from bacteroids of Fix$^+$ strain 107, with a phenotype not different from that of the parental PRE Str^r, and from bacteroids of Fix$^-$ mutant 2, at 16, 21, 24, and 29 days, respectively, after inoculation. Strain 107 shows a pattern of CI and CII synthesis similar to that found for parental PRE Str^r bacteroids, except that root nodule development with the PRE Str^r strain is delayed in comparison with the wild type PRE (20) and synthesis of CI and CII components starts approximately three days later: from 16 days after inoculation an increasing amount of both CI α and β-subunits and CII was observed. The lanes with proteins from bacteroids of 24- and 29-day-old nodules show a smear of proteins reacting with antisera specific against CI and CII, probably caused by aggregation of CI and CII proteins present in large amounts, and perhaps augmented by degradation of nitrogenase components in older bacteroids. Similar results to strain 107 were obtained with bacteroids of mutants 3, 26, and 73 (not shown). In contrast, bacteroids from nodules induced by Fix$^-$ mutant 2 (and also by mutants 5, 116A, and 6; not shown) did not show production of CI subunits whereas the synthesis of CII was greatly reduced. These findings are in agreement with the localization of the Tn5 insertion outside the *nif* DH region in mutants 3, 26, 73, and 107, and within *nif* D or the DH intergenic region in the Fix$^-$ mutants 2, 5, 116A, and 6. The fact that a (polar) Tn5 mutation, as in strain 2, causes the cessation of transcription of the genes coding for CIα as well as CIβ implies that these genes probably form part of one transcription unit and are thus contiguous on the genome.

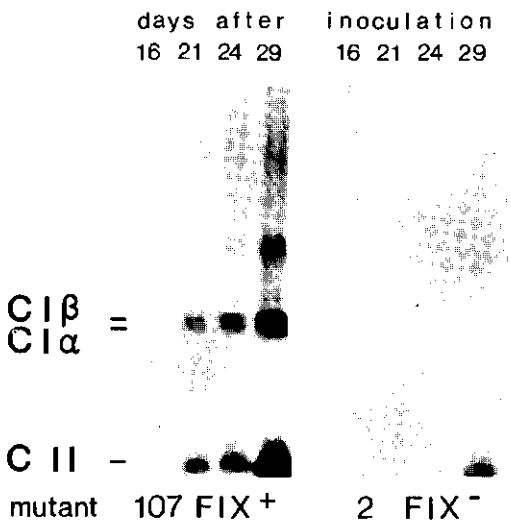

FIG. 3. Synthesis of nitrogenase components I and II by *R. leguminosarum* PRE::Tn5 mutants during symbiosis. Autoradiogram of a Western blot with bacteroid proteins isolated by nodule fractionation at the times after inoculation indicated, for a Fix$^+$ (107) and a Fix$^-$ (2) representative. The blot was incubated with a mixture of antisera specific against CI and CII and subsequently with ^{125}I-labeled protein A to visualize immune complexes. The positions of CIα and β and CII bands are marked. The amount of protein loaded per gel slot was approximately the same for all lanes.

Subcloning of *R. leguminosarum* PRE Nitrogenase Genes and Expression in *E. coli* Minicells

As shown in Fig. 1, clone λ RleH1 represents a leftward extension with respect to the 6 kb EcoRI fragment of pRB10 bearing *nif* H and *nif* D gene sequences, as was deduced from pRmR2 homology data and from the effects of Tn5 mutations within this region. These latter experiments suggested a contiguous ordering of *nif* D and *nif* K. Therefore we expected the gene K coding region to be located on the 0.5 kb and the following 5.0 kb EcoRI fragments, both cloned in λ RleH1 and directly adjacent to *nif* D.

When the 0.5, 5.0, and 6.0 kb EcoRI fragments were hybridized on Southern blots with ^{32}P-labeled bacteroid RNA from 17-day-old nodules (5), the right-hand SalI-EcoRI part of the 5 kb fragment and the 0.5 kb EcoRI fragment hybridized roughly with the same intensity as the 3.3 kb *nif* DH-specific EcoRI-XhoI fragment (Fig. 4). This result indicates that this

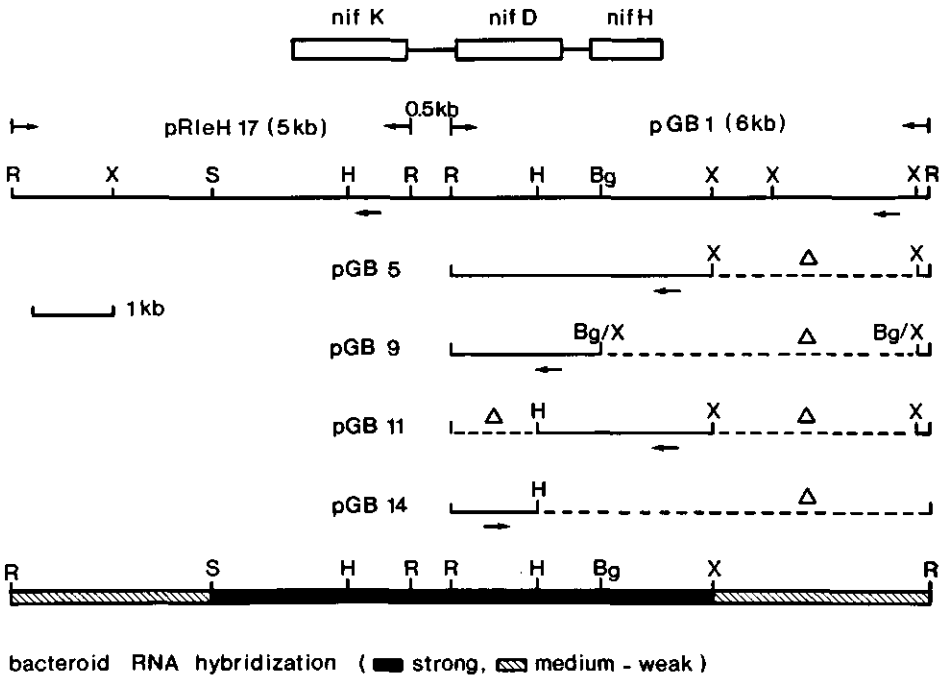

FIG. 4. Genetic organization of the structural *nif*-gene cluster of *R. leguminosarum* PRE. Restriction enzyme maps for the *nif* K-specific pRleH17 insert (5 kb EcoRI fragment in vector pACYC184) and the *nif* DH-encoding pGB1 insert (6 kb EcoRI fragment in vector pSUP201), as well as for deletion derivatives (Δ) of the last mentioned subclone (pGB5, pGB9, pGB11, pGB14), are presented. The genomic positions of pGB1 and pRleH17 are also marked in Fig. 1. Arrows indicate transcriptional directions with respect to the *E. coli* vector promoter; next higher even subclone numbers (pRleH18, pGB2, pGB6, pGB10, not marked) possess opposite insert orientations. *Nif* coding sequences are given by open bars, bacteroid ^{32}P-mRNA hybridization intensities by differentially shaded bars.

region is actively transcribed in nitrogenase-synthesizing bacteroids, and strengthens the conclusion that the 0.5 kb and/or 5 kb EcoRI fragment contains *nif* K.

In order to establish definitely the position of *nif* H, D, and K on the *sym*-plasmid DNA, the 6 kb and 5 kb EcoRI fragments were subcloned in the EcoRI site of plasmid pSUP201 (Ap^rCm^r; clone pGB1) and pACYC 184 (Cm^rTc^r; clone pRleH17), respectively. In both cases the fragment was cloned within the gene coding for chloramphenicol acetyl transferase (CAT) and thus downstream of the strong constitutive CAT promoter, which allows for expression of cloned genes if these are inserted in the right orientation. The different cloned fragments as well as deletion derivatives of the pGB1 insert are indicated in Fig. 4. Plasmids pGB5 and 6 were constructed by deletion of two XhoI fragments from pGB1 and 2, respectively. Elimination of a BglII-XhoI fragment from pGB5, filling in with DNA polymerase, and blunt end ligation gave rise to pGB9. Clone pGB11 represents a deletion of a HindIII fragment from pGB5, in which part of the vector is also deleted; this implies that no inverted orientation could be constructed in this case. The same holds for pGB14, comprising removal of the other part of the insert from pGB6.

The expression of these fragments was studied in *E. coli* DS410 minicells. Clones with odd numbers (pGB1, pGB5, pGB9, pGB11, and pRleH17) have the inserted fragment in the opposite orientation to that of the clone with the next even number. The direction of tran-

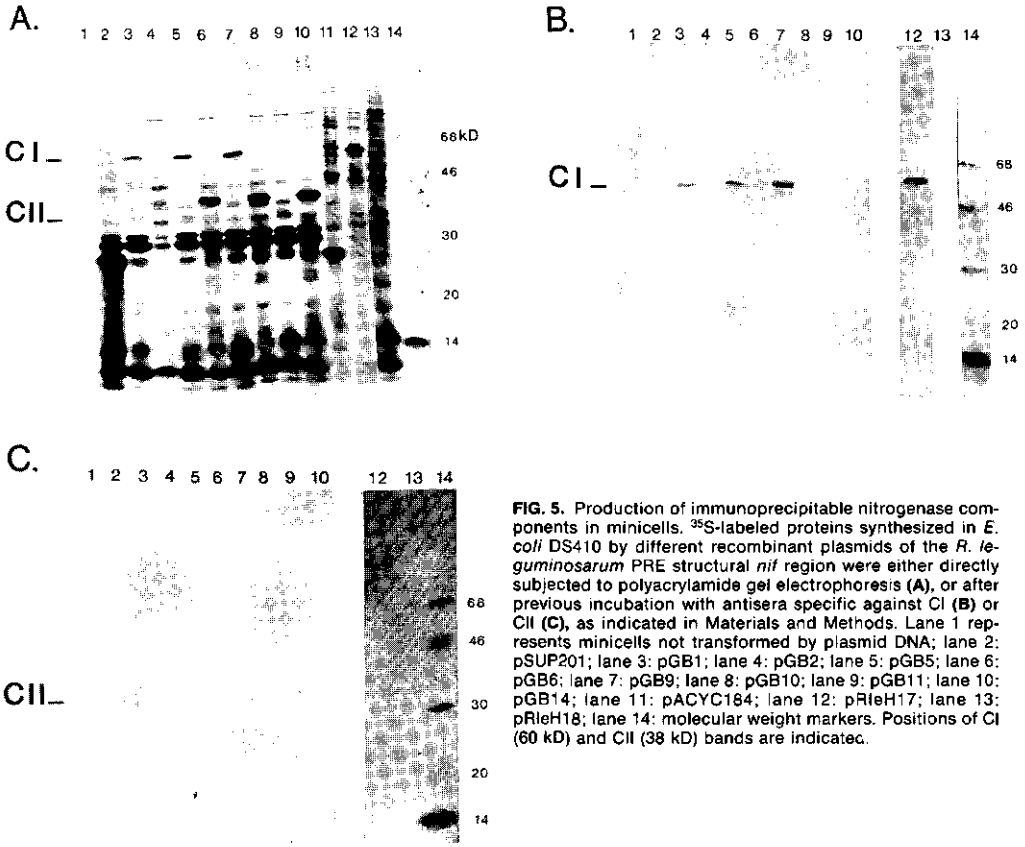

FIG. 5. Production of immunoprecipitable nitrogenase components in minicells. ^{35}S-labeled proteins synthesized in *E. coli* DS410 by different recombinant plasmids of the *R. leguminosarum* PRE structural *nif* region were either directly subjected to polyacrylamide gel electrophoresis **(A)**, or after previous incubation with antisera specific against CI **(B)** or CII **(C)**, as indicated in Materials and Methods. Lane 1 represents minicells not transformed by plasmid DNA; lane 2: pSUP201; lane 3: pGB1; lane 4: pGB2; lane 5: pGB5; lane 6: pGB6; lane 7: pGB9; lane 8: pGB10; lane 9: pGB11; lane 10: pGB14; lane 11: pACYC184; lane 12: pRleH17; lane 13: pRleH18; lane 14: molecular weight markers. Positions of CI (60 kD) and CII (38 kD) bands are indicated.

scription from the CAT promoter is indicated by arrows. ^{35}S-labeled protein lysates derived from *E. coli* DS410 minicells transformed by different recombinant plasmids were either analyzed directly by SDS-gel electrophoresis (Fig. 5A) or first treated with antiserum specific against CI or CII of nitrogenase, after which the immunoprecipitates were analyzed by gel electrophoresis (Fig. 5B and C, respectively). With clone pRB10, no synthesis of CI or CII proteins or other *Rhizobium* specific proteins could be detected in the minicells (data not shown). This suggested that transcription from possible promoters on the rhizobial plasmid DNA does not take place in *E. coli* minicells. Shorter fragments of pRB10, with either *nif* DH, *nif* H, or *nif* D homology, if inserted behind the CAT promoter of pSUP201, produced either CII (pGB11) or CIα (pGB9) or both (pGB 1 and 5) nitrogenase components (compare Figs. 4 and 5, lanes 9, 7, 3, 5). Apparently, expression of *nif* H and D can occur in minicells under the control of the strong *E. coli* CAT promoter but from a *Rhizobium* ribosome binding site on a hybrid mRNA. The results of these experiments further indicated that the direction of sense transcription is from right to left in the order HD as indicated by the arrows in Fig. 4. In the case of opposite polarities only for pGB2 and pGB6 a low rate of synthesis of exclusively CII was observed, suggesting weak activity of a *Rhizobium*-specific promoter in front of *nif* H within the *E. coli* minicell system. Plasmids with the rhizobial DNA insert in the reverse orientation with respect to the CAT promoter,

pGB2, pGB6, pGB10, and pGB14, produced a 44 kD protein (Fig. 5A, lanes 4, 6, 8, 10) which was precipitated with the antisera against CI and CII but also with preimmune serum. The nature of this protein is unclear. In view of the fact that the 44 kD protein was found with four different clones, it seems probable that it arises from the non-sense strand of the *nif* D region because the four clones share the same 1.1 kb EcoRI-Hind III fragment. We have not determined whether this protein is a *Rhizobium* gene product or a fusion protein composed of vector and insert information. From the results with the different pGB clones it can be concluded that *nif* H (CII) is located mainly on the 1.4 kb BglII-XhoI fragment and *nif* D (Clα) on the 1.1 kb EcoRI-HindIII fragment (see Fig. 4). The intergenic region probably lies on the 0.8 kb HindIII-BglII fragment overlapping clones pGB9 and pGB11.

The position of *nif* K (coding for CIβ) was established by expression of the 5 kb EcoRI fragment cloned in pRleH17 (Fig. 4) in DS410 minicells. This 5 kb fragment produced in minicells a polypeptide with the electrophoretic mobility of CIβ protein (Fig. 5A, lane 12), which reacted specifically with anti-CI serum (Fig. 5B, lane 12). Generally we observed that *nif* K was expressed stronger than *nif* D and *nif* H. We have no explanation for this. The results of Southern hybridization experiments with bacteroid RNA described in a preceding section, indicating that the expressed part of the pRleH17 insert is localized within the rightmost 2.5 kb SalI-EcoRI fragment, suggest that the position of the coding sequences of K with respect to D is as shown in Fig. 4.

However, it can not be excluded that the "*nif* K product" in minicells is actually a fusion protein with fortuitously the same molecular weight as CIβ; this would mean that a minor part of the *nif* K gene sequence lies on the 0.5 kb EcoRI fragment. Lengths of the structural nitrogenase genes given in Fig. 4 were deduced from protein molecular weights and mRNA size determinations by Krol et al. (10).

DISCUSSION

The results presented in this paper show that in *R. leguminosarum* the structural genes for nitrogenase, *nif* K, D, and H, are next to each other on the *sym*-plasmid and probably constitute a single operon. From collections of cloned fragments of *R. leguminosarum* DNA in the cosmid vector pLAFR1 and in phage λ EMBL3, clones were isolated with fragments which showed sequence homology with *R. meliloti nif* H and *nif* D genes. These fragments were the starting point for a physical map of the region of the *sym*-plasmid of *R. leguminosarum* containing *nif* H and *nif* D and their immediate environment. By selecting overlapping fragments from the clone banks, we have made a physical map of a piece of 29.7 kb on which *nif* D and *nif* H are located close to each other on a 3.3 kb piece and which extends 14.3 kb to the left and 12.1 kb to the right from the *nif* D and H homologous region.

The functions encoded on the fragments showing homology with, respectively, *nif* H and *nif* D of *R. meliloti* were established by site-directed Tn5 mutagenesis and by translation of cloned fragments in *E. coli* DS410 minicells. We have used the homogenotization procedure of Ruvkun and Ausubel (28) to introduce Tn5 mutations in the potential *nif* D gene of *R. leguminosarum*. We have compared this method with the homogenotization technique recently described by Simon et al. (36), which uses vector plasmid pSUP201 and *E. coli* SM 10 for mobilization, but in our hands this latter method gave high frequencies of cointegrate formation instead of double homologous recombination and therefore was not further used.

Four Tn5 mutants (nrs 2, 5, 116A, and 6) of *R. leguminosarum* were obtained representing two different Tn5 insertion sites in the plasmid fragment bearing sequence homology with *R. meliloti nif* D. All these mutants were Nod^+ Fix^-, indicating that the relevant gene was functionally involved in nitrogen fixation. Two other Tn5 mutations (nrs 73 and 107) localized

1.4 kb to the right of the *nif* DH region on the *sym*-plasmid, were Nod$^+$ Fix$^+$ and showed no phenotypic aberration in symbiosis. Probably this region does not carry functions essential for the process of nitrogen fixation and its regulation. With either group of mutants, Tn5 insertions had occurred, as far as detectable, at identical positions in the *sym*-plasmid DNA. Even though the number of Tn5 insertions we have analysed so far is limited, the observed preference for Tn5 insertion at certain sites indicates hot spots for Tn5 rather than random transposition.

R. leguminosarum Tn5 mutants 2, 5, 116A, and 6 were defective in synthesizing both CIα and CIβ nitrogenase subunits, as was demonstrated by analysing the proteins produced in bacteroids from nodules induced by these mutants. The fact that the synthesis of both CIα and CIβ was affected by mutations in a single gene indicates that the genes for CIα and CIβ probably form part of a single transcription unit. The synthesis of CII in these mutants was not stopped, but the amount synthesized was decreased, suggesting that the CII synthesis is not independent of CIα and CIβ and may be controlled by CI synthesis in some way. We also searched for effects of these Tn5 mutations in the *Rhizobium* genome on the synthesis of plant-encoded nodulins. These results will be published elsewhere by Bisseling et al. (in preparation).

Translation of cloned fragments in *E. coli* DS410 minicells allowed for the more accurate mapping of *nif* H (coding for CII) and *nif* D (coding for CIα) and also of *nif* K (coding for CIβ). When relevant fragments were cloned behind the strong CAT promoter of the expression vectors pSUP 201 or pACYC 184, the synthesis of CII, CIα, and CIβ could be detected by SDS-gel electrophoresis of minicell extracts and the identity of these proteins was confirmed by immunoprecipitation with specific anti-CII and anti-CI sera. For all fragments tested (Fig. 4), synthesis of CI or CII components in minicells was generally only detected with the rhizobial DNA insert in one defined orientation with respect to the CAT promoter. This proved that the symbiotic expression of *nif* H, D, and K occurs by transcription from right to left (see Fig. 4). A further indication for the overall dependence of the expression of these subclones in minicells on the presence of the CAT promoter was the following. A diminishing distance between this CAT promoter and the start of the respective coding regions had a clearly stimulating effect on the level of protein synthesis (compare the intensities of the CIα band in Fig. 5A and B for clones pGB1, pGB5, and pGB9 and of the CII band in Fig. 5A and C for clones pGB1 and pGB5).

A plausible explanation for this phenomenon is that, for example, in the case of pGB1 with a 6 kb insert, only a small fraction of the relatively long hybrid transcripts required for CII and CIα synthesis would survive degradation or premature polymerase stops caused by possible regulation signals between the CAT promoter and the start of gene H translation.

For clones pGB2 and pGB6, representing opposite insert polarities, a weak synthesis of CII was detected; production of CIα or CIβ, on the contrary, was never observed with clones possessing reversed insert orientations with respect to the CAT promoter. This might indicate that a promoter upstream of *nif* H is recognized by *E. coli* RNA polymerase with low efficiency. The mRNAs responsible for CII synthesis in pGB2 and pGB6 are apparently produced at a low level and for unknown reasons do not extend into *nif* D.

By direct electrophoresis of ^{35}S-labeled plasmid-encoded proteins synthesized in minicells, the CIα equivalent is easily discernable in pGB5 and pGB9 (Fig. 5A, lanes 5 and 7), while CII protein is hardly distinguishable from the background. This might be due to differential ^{35}S-methionine incorporation or possibly reflect a different translational efficiency from both rhizobial ribosome binding sites in *E. coli*. In conclusion, it can be said that the translation signals on the rhizobial RNA transcripts are faithfully recognized by the *E. coli* protein synthesizing machinery. The best demonstration

of this was the synthesis of both CII and CIα from one insert, as in pGB1 and pGB5. This appears to be in contrast to the results on the expression of *nif* H, D, and K from *R. meliloti* in *E. coli* minicells, as reported by Pühler et al. (14). From pRmR2, containing *nif* H and a large part of *nif* D, exclusively CII expression (a double band of 30 and 31 kD) was obtained starting from the CAT promoter; weak expression was also found when the insert had the opposite orientation. When we repeated this experiment (result not shown), essentially the same was concluded, except that on immunoprecipitation of the pRmR2 products with our *R. leguminosarum* CII antiserum only one band was visible, with a computed molecular weight value of 39 kD. For *R. meliloti*, so far, *nif* D and K expression in minicells was only observed in recombinant constructions allowing synthesis of fusion proteins. With *R. japonicum* cloned fragments, on the other hand, as we found for *R. leguminosarum*, complete *nif* D and K products can be synthesized in minicells from rhizobial ribosome binding sites on hybrid messengers (17).

With none of the inserts we have studied have we obtained evidence for a fusion protein consisting of a part of the CAT protein and CI or CII proteins. In all cases, CIα, CIβ, and CII produced for different clones in minicells showed the same mobility upon SDS-gel electrophoresis as the purified CIα, CIβ, or CII components. Moreover, with none of the clones did we encounter truncated proteins resulting from reading frame shifts, as would be expected for fusion proteins.

The synthesis of both CII and CIα from pGB1 and pGB5 inserts indicated that these proteins are translated in minicells from a polycistronic mRNA. It appears plausible that this will also be true in *R. leguminosarum*, indicating that CII and CI are transcribed from a single operon. As stated above, this conclusion is corroborated by the effect of a Tn5 insertion in *nif* D, which resulted in the blocking of CIβ (*nif* K product) synthesis *in vivo*.

The only direct evidence for the position of *nif* K on the *sym*-plasmid DNA was from the minicell experiments. *Nif* K was definitely proven to be located on the pRleH17 insert (see Fig. 4) but its accurate position within this fragment has not yet been determined. Based on the following arguments, we assume that *nif* K is adjacent to *nif* D. The expression of *nif* K in minicells was strong when the pRleH17 insert was downstream from the CAT promoter. As the level of expression of *nif* H and *nif* D was higher when the distance between the CAT promoter and the starting point of the coding sequence was shorter, we assume that the start of *nif* K is close to the CAT promoter in pRleH17. Furthermore, RNA-DNA hybridization studies with bacteroid RNA showed that the right SalI-EcoRI fragment of pRleH17 but not the left part is expressed in bacteroids to an extent comparable to *nif* D and *nif* H. An intriguing question remains whether the 0.5 kb fragment assumed to represent the intergenic region between *nif* D and K will be essential for combined synthesis of all three nitrogenase components in minicells. In conclusion, the structural genes for nitrogenase appear to be contiguous on the *sym*-plasmid of *R. leguminosarum*. The structural organization of *nif* K, D, and H is similar to that in *K. pneumoniae* and *R. meliloti*, but differs from that of *R. japonicum*, *R. phaseoli*, and *Anabaena*. There are good reasons to assume that *nif* H, D, and K constitute a single operon. This is in agreement with our earlier conclusion based on the size of RNA isolated from bacteroids, hybridizing with *nif* probes (10). In earlier reports it has been shown that in *R. leguminosarum* bacteroids the synthesis of CI and CII varies, and that during early developmental stages of root nodule development, synthesis of CI is in excess over CII, whereas this ratio is reversed at later stages (20). From our present results we cannot yet conclude whether this differential synthesis of CI and CII *in vivo* might be controlled at the level of transcription or translation.

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Chapter III
Identification and phenotypical characterization of a cluster of *fix* genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE

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Summary. A *nif* regulatory gene in *R. leguminosarum* PRE was identified by interspecies DNA hybridization and site-directed Tn*5* mutagenesis. Significant homology was found with the *K. pneumoniae nifA* locus, a *R. meliloti* symbiotic regulatory gene and *E. coli ntrC*; Tn*5* insertions within this *nifA* gene inhibit the expression of the *nifHDK* operon, encoding synthesis of the nitrogenase polypeptides.

Specific DNA hybridization also was detected between a downstream adjacent part of the PRE *sym* plasmid and the *R. leguminosarum* 248 *fixZ* gene, a homologue of the *K. pneumoniae nifB* locus. To detect further *fix* genes we investigated a region of the *sym* plasmid which is localized within a short distance upstream from the *nifA* gene and is transcribed selectively at a high rate during symbiosis. This approach revealed the existence of a *fix* cluster in which Tn*5*-mutations cause a Fix⁻ phenotype although wild-type levels of nitrogenase synthesis were detectable. In a *sym* plasmid fragment, which is immediately upstream adjacent to the *nifA* locus and only moderately expressed in *Rhizobium* bacteroids, a second *fix* gene conferring the same symbiotic phenotype was detected.

Introduction

The genes that enable fast growing *Rhizobium* species to establish a nitrogen-fixing symbiosis with leguminous plants are located on large, low copy number plasmids (*sym* plasmids). Among such *sym* genes are those required for initiation of nodule development (*nod* genes) and genes involved in nitrogen fixation (*fix* genes); some of these (*nif* genes) have a *Klebsiella pneumoniae* homologue, like the structural genes for nitrogenase (*nifKHD*). In the symbiosis *Rhizobium* bacteria penetrate into cells of the root nodules, where they occur as bacteroids, surrounded by a host-derived membrane, and differentiate into the nitrogen-fixing state characterized by the presence of nitrogenase. The *sym* genes are not expressed in free-living bacteria, but solely in the interaction with the host plant as the root nodule develops (Krol et al. 1980; Kondorosi et al. 1984). The regulation of the expression of the *sym* genes, their temporal coordination and the signals involved are still poorly understood.

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The only *sym* genes that have been studied in great detail so far are the structural genes (*nifKDH*) for nitrogenase. Concerning the regulation of the *nif* (*KDH*) genes in *Rhizobium* much has been deduced by comparison with the well-studied regulation of *nif* genes in *Klebsiella*. In *K. pneumoniae* the *nifKDH* operon is located on the chromosome within a cluster of 17 *nif* genes organized in 7 or 8 transcription units. One of these operons, *nifLA*, codes for regulatory proteins and *nifA* is involved in the activation of all other *nif* operons (see Roberts and Brill 1981; Ausubel et al. 1982 and Ausubel 1984 for reviews). Recently it has been found that the *nifKDH* genes of *R. meliloti* are under the direct positive control of a transcriptional activator, which has both functional and structural homology with *K. pneumoniae nifA* (Zimmerman et al. 1983; Szeto et al. 1984; Buikema and Ausubel 1984; Weber et al. 1984).

Here we report on the identification and phenotypic characterization of a gene cluster on the *sym* plasmid of *R. leguminosarum* PRE. In this *Rhizobium* the *sym* genes are on a 350 kb plasmid (Krol et al. 1982). Previously we reported the functional characterization of the structural *nif* genes on this plasmid (van den Bos et al. 1983; Schetgens et al. 1984). From a *sym* plasmid library in λEMBL3 we have isolated a clone which hybridized strongly with RNA isolated from bacteroids and in addition showed DNA homology with cloned *nifLA* genes of *K. pneumoniae*. We used site-directed Tn*5* mutagenesis to identify *sym* genes and found a region containing a regulatory *nifA* gene in addition to two gene clusters producing a Fix⁻ Nif⁻ HDK⁻ phenotype upon mutation. Recently Downie et al. (1983a; Rossen et al. 1984) similarly found evidence for a *nifA* gene in *R. leguminosarum* 248.

Materials and methods

Microbiological techniques. The strains of *R. leguminosarum* and *Escherichia coli*, λ phages and plasmids used are listed in Table 1. Media, concentrations of antibiotics and growth conditions were as described by Schetgens et al. (1984).

Clone banks of purified *R. leguminosarum* PRE *sym* plasmid DNA digested with *Eco*RI or *Bam*HI in phage λEMBL3 (also described earlier by the same authors) have been screened to isolate relevant fragments.

Table 1. Bacterial strains, plasmids and bacteriophage

Designation	Relevant character/genotype	Reference/source
Bacterial strains		
R. leguminosarum PRE	Wild type	Lie et al. (1979)
PRE Str^r	Streptomycin and acriflavin resistance	Lie et al. (1979)
E. coli HB101	F$^-$ *leuB6 proA2 thi-1 lacZ4 supE44 tonA21 rpsL hsdR hsdM recA13* λ^-	Ruvkun and Ausubel (1981)
MM294	*endoI hsdR hsdM pro*	Ruvkun and Ausubel (1981)
Q358	R_k^- M_k^+ *Su*$_{II}^+$ 80 R	Karn et al. (1980)
Q359	R_k^- M_k^+ *Su*$_{II}$ 80 R P2	Karn et al. (1980)
N5387	a lysogenic strain of λb211 *rex*::Tn*5* c1857 with a chromosomal Tn*5*	from A. Krol, Amsterdam
Bacteriophage		
λEMBL3	λ1059-derived cloning vector	Karn et al. (1980)
Plasmids		
pBR322	Ap^{r}Tc^r	Bolivar et al. (1977)
pACYC184	Cm^{r}Tc^r	Chang and Cohen (1978)
pRK290	Tc^r; conjugative broad host range plasmid	Ditta et al. (1980)
pRK2013	Nm^r; complements *tra* genes of pRK290 in conjugations	Figurski and Helinski (1979)
pPH1J1	Gm^r, incompatible with pRK290	Beringer et al. (1978)
pGR102	*K. pneumoniae nif QBALFM* genes cloned in pMB9	Riedel et al. (1979)
pRmB3.8H	*R. meliloti* symbiotic regulatory gene cloned in pBR322	Szeto et al. (1984)
pGln53Y	*E. coli nrC/glnG* clone	Szeto et al. (1984)
pIJ1286	*R. leguminosarum* 248 pRL1JI *fixZ* gene cloned in pKT 230	Rossen et al. (1984)

DNA methodology. Isolation of *R. leguminosarum* genomic or *sym* plasmid and *E. coli* plasmid DNA, restriction endonuclease digestion, agarose gel electrophoresis, fragment elution, nick translation, Southern blot hybridization (routinely carried out at 65° C in 6 × SSC for 15–19 h), ligation and *E. coli* transformation have been described by Schetgens et al. (1984).

Preparation and labeling of bacteroid RNA. Total bacteroid RNA isolated from 17-day-old nodules was 5′-end labeled in vitro with T4 polynucleotide kinase (Krol et al. 1980) and subsequently used for screening the *R. leguminosarum sym* plasmid clone bank in phage EMBL3 by plaque hybridization (Karn et al. 1980; Maniatis et al. 1982) or for probing Southern blots.

Transposon mutagenesis. Sym plasmid DNA fragments cloned in vector pACYC184 (Chang and Cohen 1978) were transformed into *E. coli* Tn*5* donor strain N5387. Single colonies were inoculated independently in LB medium containing 20 µg/ml kanamycin and after 15 h incubation at 37° C these cultures were 100-fold diluted in LB containing 250 µg/ml kanamycin.

After growth to stationary phase, plasmid DNA was isolated and transformed into *E. coli* HB101. Plasmid preparations from kanamycin-resistant transformants were analysed by restriction enzyme digestions to map the positions of Tn*5* insertions. Transposon mutagenized insert fragments were religated into vector pRK290 (Ditta et al. 1980) and transformed into *E. coli* MM294. Marker exchange with *R. leguminosarum* DNA was achieved in conjugation experiments according to Ruvkun and Ausubel (1981) by double homologous recombination.

Analysis of bacteroid proteins. From pea nodules induced by *R. leguminosarum* wild type or Tn*5*-mutagenized strains, bacteroids were isolated and analyzed for the synthesis of nitrogenase polypeptides by immunological identification as described (Schetgens et al. 1984).

In vitro assay of nitrogenase activity in permeable bacteroids. For preparations of in vitro nitrogen fixing bacteroid suspensions all assay mixtures and solutions were made anaerobic before use by evacuation and flushing with argon. Nodulated main root segments (derived from fifty 17-day-old pea plants) were homogenized in 20 ml isolation buffer containing 50 mM TES (N-tris [hydroxymethyl] methyl-2-aminoethane sulfonic acid)/NaOH, pH 7.4, 50 mM *D*(+)-glucose, 440 mM sucrose, 0.15% (w/v) dithiothreitol, 4% (w/v) polyvinylpyrrolidone, 1 mg/ml glucose-oxidase (Boehringer, Mannheim, FRG grade III) and filtered through Miracloth (Calbiochem, Los Angeles, USA); bacteroids were pelleted by centrifugation (5 min, 5,000 rpm, 4° C). The pellet was washed in 10 ml washing buffer containing 50 mM TES/NaOH, pH 7.4, 50 mM *D*(+)-glucose, 440 mM sucrose, 1% (w/v) bovine serum albumin (fatty acid free) and suspended in 1.0 ml of the latter medium at 0° C in an anaerobic vial.

To measure the nitrogenase activity of intact bacteroids in an ATP-regenerating system, to 100 µl assay buffer, consisting of 50 mM HEPES (N-2 hydroxyethyl piperazine N′-2-ethane sulfonic acid)/KOH, pH 7.0, 10 mM ATP, 15 mM $MgCl_2$, 10 mM creatine phosphate, 0.3 mg/ml creatine kinase (Boehringer) and 1 mg/ml bovine serum albumin were added 50 µl of 0.2 M sodium dithionite (as an electron donor) in 25 mM Tris/HCl, pH 7.5, 5 µl cetyltrimethylammonium bromide (CTAB; to render cell walls permeable) and 50 µl of the anaerobic bacteroid suspension and the

final volume was made up to 500 µl with H_2O; volumes of CTAB and bacteroid suspensions were varied to optimize acetylene reduction.

The gas phase was made up to 20% acetylene (purified by storage over a Fieser solution; Braaksma et al. 1982) and the assay mixture was incubated at 30° C under shaking; subsequently ethylene production was measured by gas chromatography (Haaker and Wassink 1984; Braaksma et al. 1982).

Cell-free extracts were prepared by sonicating bacteroids in washing buffer under argon, followed by centrifugation (10 min 25,000 × *g*), and subsequently used under standard assay conditions to test in vitro complementation with optimum levels of separately purified *Azotobacter vinelandii* nitrogenase proteins (Haaker and Wassink 1984; Braaksma et al. 1982).

Results

Clone banks of *R. leguminosarum* PRE *sym* plasmid DNA consisting of *Eco*RI or *Bam*HI fragments in phage λEMBL3 were screened by hybridization with ^{32}P-labeled RNA isolated from bacteroids of 17-day-old nodules, to select genes that are expressed in nitrogen-fixing root nodules. Among the different recombinant phages hybridizing with the bacteroid RNA we describe here the further characterization of clone λRleH6 as the *sym* plasmid DNA contained in this phage also hybridized with cloned fragments carrying *K. pneumoniae nifLA* genes.

By hybridizing Southern blots of restriction digests of λRleH6 DNA with ^{32}P-labeled bacteroid RNA the major part of the 16.15 kb *sym* plasmid fragment showed only weak to medium hybridization (Fig. 1 A, lanes 1 and 2), definitely not representing background, but implying that this region contains gene(s) that are moderately expressed during symbiosis. A 2.20 kb *Hin*dIII-*Sal*I fragment, obtained by *Eco*RI-*Hin*dIII-*Sal*I-digestion in this case, produced a very strong hybridization signal (Fig. 1 A, lane 3) of approximately the same intensity as that observed for the *nifHDK* genes (not shown; see Schetgens et al. 1984). The differential hybridization of the restriction fragments is indicated in the map of λRleH6 in Fig. 2. Presumably much more RNA transcribed from (the) gene(s) contained in the 2.20 kb *Hin*dIII-*Sal*I fragment is accumulated in the bacteroid RNA than from those in the 13.55 kb region on the left of this fragment.

Southern blots of restriction enzyme fragments of λRleH6 were hybridized using a nick-translated 3.30 kb *K. pneumoniae Sma*I-DNA fragment from pGR102 (Riedel et al. 1979), containing *nifA* and parts of the *nifB* and *nifL* sequences, as a probe (Fig. 1 B, lanes 2–4). We were able to localize a cross hybridizing region on the *Rhizobium sym* plasmid within a 3.30 kb *Bam*HI fragment (see also Fig. 2). This region lies about 1.25 kb left of the strongly transcribed area mentioned earlier. Screening *Eco*RI or *Bam*HI-digested chromosomal and purified *sym* plasmid DNA from PRE with the same probe revealed that this *nifA* homologous region is present as one unique copy (Fig. 1 B, lanes 1 and 5). Likewise we found that the 3.30 kb *Bam*HI *sym* plasmid fragment of λRleH6 hybridized with the cloned fragment of pRmB3.8H from *R. meliloti* (Szeto et al. 1984) containing a *nif* regulatory gene (data not shown).

It has been reported that the *E. coli ntrC* gene can substitute for *K. pneumoniae nifA* in nif promoter activation (Merrick 1983; Sundaresan et al. 1983; Szeto et al. 1984). Using a 2.00 kb *Eco*RI-*Hin*dIII fragment from clone pGln53Y (Szeto et al. 1984) containing *E. coli ntrC* sequences we similarly found hybridization with the 3.30 kb *Bam*HI fragment of the *R. leguminosarum* PRE *sym* plasmid (data not shown).

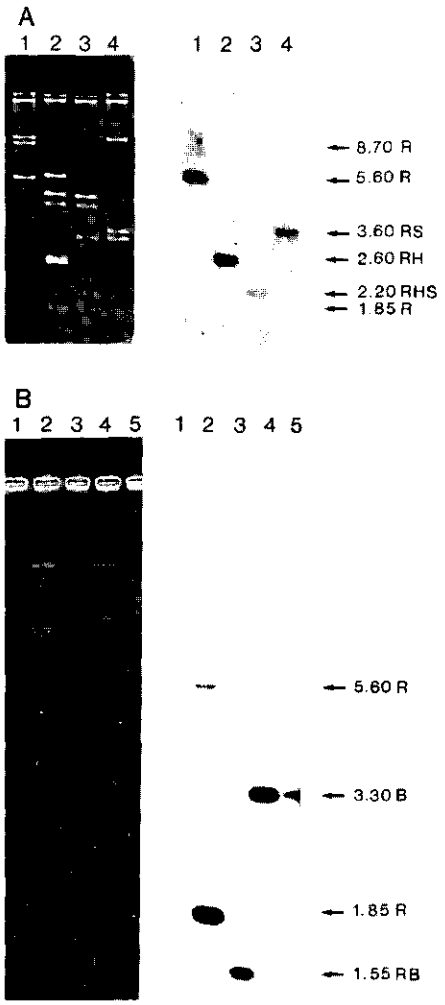

Fig. 1 A, B. Hybridization of ^{32}P-labeled bacteroid RNA (**A**) and *K. pneumoniae nifA* probe (**B**) with the *R. leguminosarum* PRE *sym* plasmid region cloned in λRleH6. Agarose gel patterns are given in the left panels and Southern blot hybridizations on the right hand. Sizes of relevant fragments are indicated in kb. **A** Hybridization of 5'-end-labeled total bacteroid RNA, isolated from 17-day-old nodules, with different restriction endonuclease digests of λRleH6 DNA. Lane 1: *Eco*RI; lane 2: *Eco*RI + *Hin*dIII; lane 3: *Eco*RI (R) + *Hin*dIII (H) + *Sal*I (S); lane 4: *Eco*RI + *Sal*I. **B** Hybridization of a nick-translated 3.30 kb *K. pneumoniae Sma*I fragment containing the *nifA* gene with digests of *sym* plasmid DNA and λRleH6. Lane 1: *sym* plasmid *Eco*RI; lane 2: λRleH6 *Eco*RI; lane 3: λRleH6 *Eco*RI(R) + *Bam*HI(B); lane 4: λRleH6 *Bam*HI; lane 5: *sym* plasmid *Bam*HI

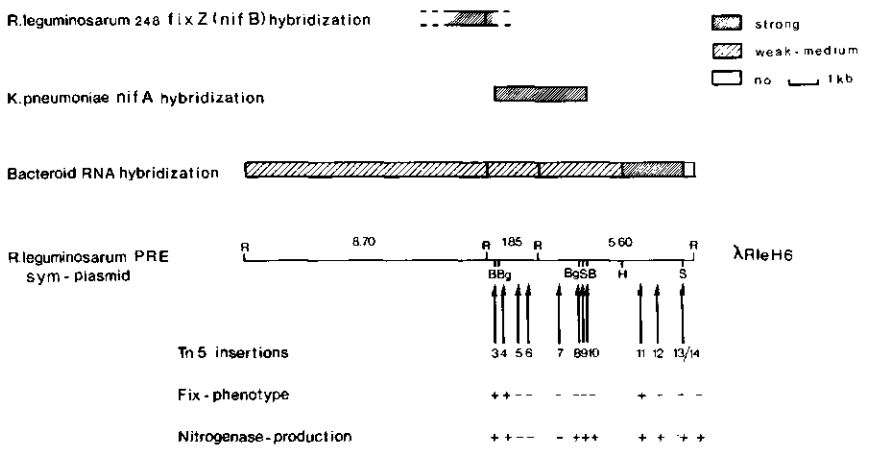

Fig. 2. Map of the *sym* plasmid region surrounding the *R. leguminosarum* PRE *nifA* gene and the symbiotic phenotypes induced by Tn*5* insertions. The physical map of the insert of clone λRleH6 is shown. Restriction sites are abbreviated as follows: B (*Bam*HI), Bg (*Bgl*I), H (*Hin*dIII), R (*Eco*RI), S (*Sal*I). *Eco*RI-restriction fragment lengths are given in kb. *R. leguminosarum* PRE bacteroid RNA, *K. pneumoniae nifA* and *R. leguminosarum* 248 *fixZ* (*nifB*) hybridization data are indicated by differentially shaded bars above the map. The positions of Tn*5* insertions are indicated by numbered arrows and corresponding Fix phenotypes and nitrogenase production profiles are given by + (wild type) or − (absent)

These observations on the DNA sequence homology between the 3.30 kb *Bam*HI fragment and *K. pneumoniae nifA*, *R. meliloti nif* regulatory gene and *E. coli ntrC* respectively strongly suggest that this piece of the *sym* plasmid of *R. leguminosarum* PRE contains *nif* regulatory genes.

Recently Rossen et al. (1984) localized a *fixZ* gene in *R. leguminosarum* 248 that is homologous to *K. pneumoniae nifB* at the nucleotide sequence level as well as at the level of the amino acid sequence of the polypeptide encoded by this gene. Hybridization of a Southern blot of an *Eco*RI restriction enzyme digest of λRleH6 DNA with a nick-translated 2.00 kb *Sal*I fragment from pIJ1286 containing the coding region of *fixZ* (Rossen et al. 1984) showed homology with both the 1.85 kb and 8.70 kb *Eco*RI fragments (result not shown). This indicates that adjacent to the left of the *nifA* homologous region on the 3.30 kb *Bam*HI fragment, overlapping the border of the 1.85 kg and 8.70 kb *Eco*RI fragments, a gene homologous to *nifB* in *K. pneumoniae* and *fixZ* in *R. leguminosarum* 248 may be found (see Fig. 2).

Site-directed Tn5 mutagenesis

For identifying possible symbiotic functions in the regions of λRleH6 characterized by the hybridizations with bacteroid RNA and different DNA probes we used site-directed Tn*5* mutagenesis of the 1.85 and 5.60 kb *Eco*RI fragments (Fig. 2). We followed essentially the procedure developed by Ruvkun and Ausubel (1981) for mutating a cloned fragment in *E. coli* and subsequently exchanging this Tn*5*-mutagenized fragment against the wild-type *R. leguminosarum* PRE gene. With two Tn*5* insertions located 0.1 kb and 0.2 kb respectively from the lefthand border of the 1.85 kb *Eco*RI fragment the substitution into the wild-type *Rhizobium* genome by double-homologous recombination was not successful, probably because the stretch of homologous sequence on one side of the transposon was not long enough. The 12 Tn*5* insertions that were finally obtained in different positions are indicated in Fig. 2. The Tn*5* insertions 13 and 14 had occurred at an identical position but with opposite orientations of the transposon.

Symbiotic genes in the λ RleH6 region of the R. leguminosarum PRE sym plasmid

All 12 PRE::Tn*5* mutants normally induced the formation of root nodules upon inoculation of pea seeds and showed therefore a Nod^+ phenotype. Measurement of acetylene reduction activities of the intact nodulated plants showed that three mutants (3, 4, and 11) were Nod^+Fix^+ whereas the remaining nine were Nod^+Fix^-.

The Fix^- mutants correspond to two different clusters of Tn*5* insertions separated by a Tn*5* insertion producing a Nod^+Fix^+ phenotype. The Nod^+Fix^- mutants induced the formation of root nodules quite comparable to those induced by the *nifD*::Tn*5* mutants described in previous papers (van den Bos et al. 1983; Schetgens et al. 1984). The nodules were relatively small and were found mostly on the lateral roots; at an early developmental stage the nodules looked pink, but they rapidly turned pale or green; the shape of the bacteroids in the nodules did not visibly differ from that of the wild type.

The Fix^- mutants were characterized further by analyzing the corresponding bacteroids for the occurrence of CI and CII components of nitrogenase. Figure 3 shows a Western blot of bacteroid proteins incubated with a mixture of antisera specific against components CI and CII and subsequently with ^{125}I-protein A to detect immune complexes (Bisseling et al. 1980). The result indicates that the bacteroids of Fix^- mutants 8, 9, 10, 12, 13 and 14 produce approximately wild-type amounts of the *nifKDH* polypeptides, whereas no *nifKDH* polypeptides were found in mu-

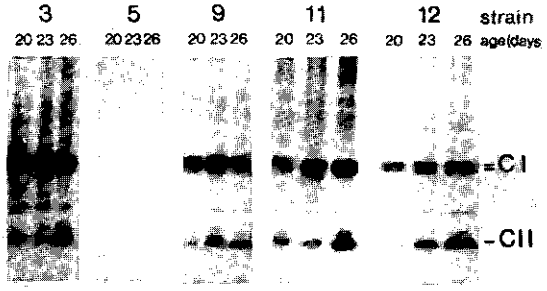

Fig. 3. Synthesis of nitrogenase components I and II by *R. leguminosarum* PRE::Tn*5* mutants during symbiosis. An autoradiogram of a Western blot with bacteroid proteins isolated by nodule fractionation at the indicated number of days after inoculation. A representative of each of the different phenotypic groups of mutants is shown (see also Fig. 2). The positions of CI and CII protein bands are marked. The amount of protein loaded per gel slot was approximately the same for all lanes

tants 5, 6 and 7 (see also Fig. 2). The second panel in Fig. 3, representing these mutants 5, 6 and 7, apart from the absence of nitrogenase components I and II, also shows no background bands (as visible in the other panels); this is not unexpected because these "background" reactions originate from the presence of aggregation/degradation products of CI and CII in our bacteroid protein preparations (see also Schetgens et al. 1984).

These results imply that there are at least three different genetic units in this 7.45 kb fragment of the *sym* plasmid. The Tn*5* insertions 5, 6 and 7 are in the region with DNA sequence homology to *nif* regulatory genes from *K. pneumoniae* and *R. meliloti* and confer a Nod$^-$Fix$^-$ Nif KDH$^-$ phenotype on *R. leguminosarum* PRE as expected for a mutation in a *nif* regulatory gene. We shall further refer to this region as the *nifA* region of PRE. Further evidence for a *nifA* gene in this part of the PRE *sym* plasmid was obtained from hybridization experiments with the *nif*-regulatory gene of *R. meliloti*.

From a plasmid containing Tn*5* insertion no 6 (see Fig. 2) a 3 kb *Bam*HI-*Eco*RI fragment consisting of the rightmost 0.35 kb of the 1.85 kb *Eco*RI fragment of λRIcH6, encompassing a central region of the PRE *nifA* gene, and 2.65 kb of the Tn*5* sequence was isolated. When this fragment was used as a probe in hybridizing a Southern blot containing an *Eco*RI-*Bam*HI digest of the *R. meliloti* pRmR3.8H clone, strong hybridization was found with the 0.9 kb fragment derived from the middle part of the coding sequence of the *R. meliloti nif* regulatory gene (Szeto et al. 1984) (data not shown).

Contiguous to the *nifA* gene on the PRE *sym* plasmid is a *fix* gene identified by the phenotype of the mutants with Tn*5* insertions 8, 9, and 10 respectively, which is moderately expressed in bacteroids. The Tn*5* insertions 12, 13 and 14 define another region with one or more *fix* genes, which are strongly expressed during symbiosis.

In vitro nitrogenase activity

The PRE mutants with Tn*5* insertions at positions 8, 9 or 10, and 12, 13 or 14 are unable to fix nitrogen but produce the CI and CII polypeptides of nitrogenase in approximately normal amounts. We made an attempt at defining the possible defects in the nitrogen fixation process by testing in vitro the nitrogenase activities of the mutant bacteroids. The nitrogenase activity (acetylene reduction) of *R. leguminosarum* PRE bacteroids was measured in the assay system described by Haaker and Wassink (1984). In this system bacteroids isolated under anaerobic conditions are rendered permeable to small molecules by treatment with CTAB. The nitrogenase activity confined to the bacteroids can then be measured in an incubation mixture with an ATP-regenerating system and dithionite as (nonphysiological) electron-donor.

The PRE mutants with Tn*5* insertions 8, 9, 10 and 12, 13, or 14 did not show nitrogenase activity under such conditions, whereas wild-type bacteroids and PRE mutants with insertions 3, 4, and 11 (all Fix$^+$ Nif KDH$^+$) showed normal nitrogen fixing activity. This result indicates that the defect in nitrogen fixation of the Tn*5* mutants is not due to the lack of an electron donor, but may be due to a defect in nitrogenase itself. Haaker (personal communication) has shown that *R. leguminosarum* nitrogenase component I can be complemented in vitro with *Azotobacter vinelandii* component II (Av2) to produce an active nitrogenase complex. Similarly nitrogenase component I of *A. vinelandii* (Av1) can be complemented with purified component II of *R. leguminosarum*.

Therefore we added to anaerobic cell-free extracts of Fix$^-$Nif HDK$^+$ bacteroids either purified Av1 or Av2 to see if complementation occurred. In neither case, however, could acetylene reduction be measured. When as a control both Av1 and Av2 were added to the bacteroid extract normal ethylene production was observed, indicating that the system did not contain substances inhibitory to the nitrogenase reaction. From these results it can be concluded that both rhizobial nitrogenase components I and II are inactive in bacteroids of Fix$^-$ Nif HDK$^+$ mutants with Tn*5* insertions in the positions 8–10 or 12–14, even though the *nifHDK* polypeptides are present.

Discussion

The results presented in this paper provide evidence for a *nif* regulatory gene in the *sym* plasmid of *R. leguminosarum* PRE analogous to *nifA* in *K. pneumoniae* (Dixon et al. 1980; Buchanan-Wollaston et al. 1981) and in *R. meliloti* (Szeto et al. 1984). A 16 kb fragment of *sym* plasmid DNA cloned in λRIeH6 contains a 3.30 kb *Bam*HI fragment that specifically hybridizes with *K. pneumoniae nifA* and a *R. meliloti* regulatory gene. By site-directed Tn*5* mutagenesis we demonstrated that Tn*5* insertions in a stretch of approximately 1.45 kb within this fragment produced mutants which formed nodules that did not accumulate detectable amounts of *nif HDK* polypeptides. Because these Tn*5* insertions are in the region with *nifA* sequence homology we refer to this *nif* regulatory gene on the *sym* plasmid as the *R. leguminosarum* PRE *nifA* gene. As in *R. meliloti* the PRE *nif-A* also showed sequence homology with the *E. coli ntrC* gene, which plays a key role in the control of nitrogen assimilation in this organism (Merrick 1983; Sundaresan et al. 1983). Recently a similar *nifA* gene has been identified on the 220 kb *sym*-plasmid of *R. leguminosarum* 248 (Downie et al. 1983b; Rossen et al. 1984).

Closely linked to the *R. leguminosarum* PRE *nifA* gene are on one side a gene with DNA sequence homology with *R. leguminosarum* 248 *fixZ*, which is similar to *K. pneumoniae nifB* in nucleotide and amino acid sequence (Rossen et al. 1984), and at the other side two other *fix* genes. These newly detected *fix* clusters of as yet unknown function result in a Fix$^-$ Nif HDK$^+$ phenotype when mutagenized. One of these *fix* genes hybridizes intensely with bacteroid RNA, which observation strengthens our hypothesis that the products encoded by highly expressed regions of the *sym* plasmid play an essential role in symbiosis.

From an anaerobic in vitro nitrogenase assay and in vitro complementation studies with purified nitrogenase components from *A. vinelandii*, we conclude that both nitrogenase components are inactive in the two types of Fix$^-$ Nif HDK$^+$ bacteroids. In *K. pneumoniae* no *nif* genes except *nifA* and *nifH* are known which, if switched off, effect the inactivation of components I and II at the same time. We still presume that one of the defined *Klebsiella nif* gene functions like modification or processing of the nitrogenase components I (*nif V, N, E, B*) or II (*nif M* and *S*) and electron transport (*nifF* and *J*) (Roberts and Brill 1981) might be assigned to these PRE *fix* genes. Inactivation of both nitrogenase components could have emerged then as a secondary effect: the impossibility of forming a functional enzyme complex might lead to a nonoptimal oxygen tension within the plant cell, resulting in damage to the nitrogenase polypeptides.

We have not yet found physical linkage between the 16 kb of PRE *sym* plasmid DNA cloned in λRIeH6, on which the *nif* regulatory *nifA* gene and at least three other *fix* genes have been identified, a 6 kb fragment involved in nodulation and a 75 kb region surrounding the *nif HDK* operon, including fragments that are expressed significantly during bacteroid development and which could be correlated with several *sym* genes (Schetgens et al., unpublished results). In *R. leguminosarum* 248, on the contrary, the *nifHDK* genes and a second *fix* cluster containing *fixZ* (*nifB*) and *fixY* (*nifA*) are only 27 kb apart with an intervening *nod* region (Ma et al. 1982; Downie et al. 1983a, b). In *R. meliloti sym* genes are also more clustered than in *R. leguminosarum* PRE; in this species two Fix$^-$ Nif HDK$^+$-clusters are located at 1.4 and 7.0 kb respectively upstream of the *nifH* promoter and the *nif* regulatory gene has an intermediate position (Zimmerman et al. 1983); the *nod* region lies within a distance of 30.0 kb leftward from the *nifK* locus (Long et al. 1982; Kondorosi et al. 1984). This implies that substantial differences exist among these organisms in the physical organization of the respective *sym* plasmids, *R. leguminosarum* PRE showing larger distances between different *sym* gene clusters.

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Chapter IV
Protein encoding properties of a *Rhizobium leguminosarum* PRE *fix* gene cluster including the regulatory *nif A* locus

SUMMARY

Proteins encoded by the R.leguminosarum PRE regulatory nifA gene and an adjacent fix gene cluster were analysed by expression of sym plasmid fragments and transposon Tn5 containing derivatives in Escherichia coli minicells. The nifA homologue encodes a 59 kD protein while the adjacent coding units produce 23, 29, 42 and 50 kD polypeptides respectively. The genetic organization of this region in PRE resembles that in R.meliloti in which the fixABC, nifA and nifB genes were identified.
Remarkably the rhizobial promoter upstream of the 23 kD gene is efficiently recognized by E.coli RNA polymerase. Possibly one or more weak endogenous promoters, which are not active in E.coli, might be preceding the coding units localized distally to the 23 kD gene. In vivo complementation of Tn5 mutagenized Rhizobium nifA by the Klebsiella pneumoniae nif A protein was not detectable.

INTRODUCTION

Nitrogen fixation in legume root nodules is the result of the concerted action of Rhizobium bacteria and a leguminous plant, in which a number of specific genes both in the bacteria and in the host plant are involved. In fast-growing rhizobia essential symbiotic (sym) genes are located on large plasmids and can be divided into different groups. The genes necessary for nodule development are in the nodulation (nod) region (Kondorosi et al. 1984); two other groups of genes are involved in the nitrogen fixation process and are distinguished as nif genes and fix genes. The nif genes comprise the nitrogenase operon and other homologues of the nif genes of Klebsiella pneumoniae (Dixon, 1984), whereas the fix genes are also involved in

the establishment of symbiotic nitrogen fixation but have no known functional equivalent in Klebsiella.

Evolutionary conservation of the structural nitrogenase genes (nif HDK) among all nitrogen-fixing organisms is well-documented (Ruvkun and Ausubel, 1980; Ausubel et al. 1982). DNA sequence homology with the K. pneumoniae regulatory nifA gene (involved in nif gene activation) and nif B (involved in FeMo-cofactor synthesis) has been shown to exist for R. meliloti 1021 (Szeto et al. 1984; Buikema et al. 1985; Weber et al. 1985), Bradyrhizobium japonicum 110 (Adams et al. 1984; Fischer et al. 1985), R. leguminosarum 248 (Rossen et al. 1984) and R. leguminosarum PRE (Schetgens et al. 1985a).

In the case of R. meliloti and R. leguminosarum PRE besides nifA homology at the DNA level also functional homology with Klebsiella nifA has been shown (Szeto et al. 1984; Schetgens et al. 1985).

Additional evidence for a nifA-like system positively regulating nif-gene expression in Rhizobium is that a consensus sequence for K.pneumoniae nif promoters (Beynon et al. 1983) appears to be conserved within the majority of rhizobial nif-promoters (Better et al. 1983; Sundaresan et al. 1983; Ausubel 1984).

Furthermore the R.meliloti nifHDK promoter and another symbiotic promoter (P2) were shown to be efficiently derepressed by the K.pneumoniae nifA protein in E.coli (Sundaresan et al., 1983, Pühler et al., 1983; Better et al., 1985) or in broth-cultured Rhizobium (Szeto et al., 1984).

Previously we have reported on the identification and phenotypical characterization of a cluster of fix genes from R.leguminosarum PRE, cloned in the recombinant phage λRleH6 (Schetgens et al., 1985a). The insert of this λRleH6 contains a regulatory nifA gene identified by homology with a K.pneumoniae nifA probe, while Tn5 insertions within this region resulted in a Fix^- $NifHDK^-$phenotype (i.e. absence of nitrogenase polypeptides). Immediately downstream from the nifA region specific hybridization was detected with the R.leguminosarum 248 fixZ gene, a homologue of the K.pneumoniae nifB locus (Rossen et al., 1984). Upstream of the nifA gene two Fix^- Nif HDK^+ clusters were found, one of which is actively transcribed during symbiosis whereas the other cluster was only moderately expressed.

In this paper we report on the mapping of protein coding regions in a

cluster of fix genes including nifA in R. leguminosarum PRE by expression of the relevant DNA fragments in E.coli minicells. Molecular weights of the encoded gene products as well as the localization and transcriptional direction of different operons have been determined. The genomic positions of the nifA gene and the adjacent nifB homologue were mapped. Upstream from nifA we located a locus probably homologous to fixABC in R.meliloti (Ausubel et al., 1985) and a novel fix gene encoding a 23 kD polypeptide. Furthermore an in vivo complementation experiment of R.leguminosarum bacteroids mutagenized in nifA with the Klebsiella regulatory protein is described.

MATERIALS AND METHODS

Microbiological techniques

The strains of R.leguminosarum and E.coli, λ phages and plasmids used are listed in Table I. Media, concentrations of antibiotics and growth conditions were as described by Schetgens et al. (1984).

Molecular cloning and transposon mutagenesis

DNA methodology, selection of Rhizobium leguminosarum PRE sym-plasmid clones and site-directed transposon Tn5 mutagenesis have been described by Schetgens et al. (1985a).

Cultivation of pea plants and analysis of bacteroid proteins

Growth and inoculation of pea plants and measurement of acetylene reducing activity were as described by Schetgens et al. (1984). Bacteroid proteins were analysed for the presence of nitrogenase polypeptides by an immunological assay (Schetgens et al. 1984) or by two-dimensional gel-electrophoresis (De Vries et al. 1982; Govers et al. 1985).

Table I. Bacterial strains, plasmids and bacteriophage.

Designation	Relevant character/ genotype	Reference/source
- Bacterial strains		
R. leguminosarum PRE	wild type	Lie et al. (1979)
PRE Str^R	streptomycin and acriflavin resistance	Lie et al. (1979)
E.coli MM 294	endoI hsdR hsdM pro	Ruvkun and Ausubel(1981)
294	pro thi r^-m^+ recA λ^-	Simon et al. (1983)
DS410	thi minA minB ara lacY xyl malA mtl tonA rpsL Azi^r λ^- (minicell-producing)	Reeve (1979)
- Bacteriophage		
λEMBL3	λ1059-derived cloning vector	Karn et al. (1980)
- Plasmids		
pBR322	Ap^rTc^r	Bolivar et al. (1977)
pACYC184	Cm^rTc^r	Chang and Cohen (1978)
pRK404	Tc^r; conjugative broad host range vector derived from pRK290	Ditta et al. (1985)
pRK2013	Nm^r; complementation of tra genes in conjugations	Figurski and Helinski (1979)
pPH1JI	Gm^r; incompatible with pRK290	Beringer et al. (1978)
pWK130	constitutively expresses K. pneumoniae nifLA genes in E.coli	Pühler et al. (1983)
pWK131	constitutively expresses the K. pneumoniae nifA gene in E.coli	Pühler et al. (1983)

Complementation of R.leguminosarum PRE nifA::Tn5 mutants by K.pneumoniae nifA protein

The inserts of recombinant clones pWK130 and pWK131 (Pühler et al. 1983) contain the K. pneumoniae nifLA and nifA genes respectively, which are under the control of a preceding pACYC184 chloramphenicol promoter and therefore constitutively expressed. The pWK130 and 131 inserts were excised with HindIII and recloned into the conjugative broad host range vector pRK404. The resulting plasmids (pRK404-130 and pRK404-131 respectively) were conjugated into R.leguminosarum PRE nifA::Tn5 mutants (Ruvkun and Ausubel, 1981).

Expression of recombinant clones and Tn5 derivatives in E.coli minicells

The minicell-producing E.coli strain DS410 was transformed with recombinant plasmids and Tn5 derivatives isolated from E.coli 294 r^-m^+ (to ensure DNA modification). Purification and ^{35}S-methionine protein-labeling of minicells were performed essentially as described earlier (Schetgens et al. 1984). Minor modifications were the following. Sucrose gradient centrifugation was performed only twice and the band of minicells (about 10 ml) was further purified by pelleting residual parental cells for 5 min. at 2,000 x g. The supernatant containing the minicells was used immediately for protein synthesis experiments (1h incubation). For SDS-polyacrylamide gel electrophoresis 50,000 cpm per lane were loaded (i.e. 1 to 20 µl from 100 µl labeled minicell portions, depending on the efficiency of incorporation of ^{35}S-methionine).

RESULTS

Subcloning of the R.leguminosarum PRE nifA gene and adjacent fix genes.

The restriction map of the region of the R.leguminosarum PRE sym plasmid cloned in λR1eH6 is presented in the upper part of Fig. 1 together with the hybridization data reported previously (Schetgens et

al., 1985a). This map shows the presence of several symbiotic genes on this sym plasmid fragment. The 5.60 and 1.85 kb EcoRI fragments of this region were each inserted in two orientations in pACYC184, resulting in pRleH24/23 and pRleH21/20 respectively. A 3.00 kb BamHI fragment with the complete nifA gene sequence was first subcloned in pBR322 (pRleH12; not shown) and subsequently this fragment was inserted in two orientations in the EcoRI site of pACYC184 using EcoRI linkers. This resulted in clones pRleH12.1 and pRleH12.2, constructed in order to achieve nifA expression from the constitutive chloramphenicol acetyl transferase (CAT) promoter.

Expression of R.leguminosarum sym plasmid fragments in E.coli minicells

To localize protein coding regions the subclones shown in Fig. 1 and also pRleH21 and pRleH24 containing Tn5 in different positions (see Fig. 1) were transformed into E.coli DS410 and the synthesis of polypeptides in minicells directed by these recombinant plasmids was studied by labeling with ^{35}S-methionine. The results of these experiments are shown in the autoradiogram presented in Fig. 2 and also in the lower part of the diagram in Fig. 1.
Fig. 2 lane 2 shows "empty" minicells and thus represents a background pattern. The vector pACYC184 encodes proteins responsible for tetracycline (46kD) and chloramphenicol (26 kD) resistance (Fig. 2, lane 3).
The main two protein bands synthesized from the 1.85 kb fragment in pRleH24 have molecular weights of about 35 kD (Fig. 2, lane 4); in addition a 55 kD polypeptide is synthesized. The opposite orientation of the insert in pRleH23 (Fig. 2, lane 5) encodes a prominent 30 kD polypeptide. Tn5 insertions 5 and 6 in pRleH24 lead to the synthesis of 28 and 20 kD truncated proteins respectively (Fig. 2, lanes 6 and 7) instead of the 35 kD polypeptide. From the sizes of the truncated polypeptides and the known positions of the Tn5 insertions the C-terminus of the coding sequence was derived (see Fig. 1). The synthesis of the 55 kD protein is not affected by Tn5 mutations 5 and 6. The Tn5-encoded polypeptides with sizes of 54 and 58 kD (transposase) and of 27 kD (neomycin resistance product; Rossetti et

Fig. 1

Physical map of the sym-plasmid region carrying the R.leguminosarum PRE nifA gene and a graphical presentation of gene products synthesized in E.coli minicells from different subfragments and Tn5-derivatives (as deduced from Fig. 2).

Intensities of hybridization with R. leguminosarum PRE bacteroid RNA, K. pneumoniae nifA and R.leguminosarum 248 fixZ (nifB) (see Schetgens et al. 1985a) are indicated by differentially shaded bars above the map.

Restriction enzyme sites are abbreviated as follows: B (BamHI), H (HindIII), R (EcoRI), S (SalI); EcoRI-fragment lengths are given in kb.

The position of the chloramphenicol promoter in vector pACYC184 with respect to the cloned insert fragments is indicated by an open circle (○) and the direction of transcription by a small arrow (→); the N-terminal (8 kD) or C-terminal (18 kD) part of the CAT-protein is given by a black box (■) or arrow (➡) respectively.

Rhizobium-encoded gene products and directions of expression are indicated by thick open arrows (⇨) with protein molecular weights of complete, truncated or fusion proteins in kD.

Tn5-insertions are shown by vertical bars as well as the corresponding symbiotic phenotypes Fix (upper + or - signal indicates the presence or absence of acetylene reducing activity) and NifHDK (lower + or - signal indicates the presence or absence of nitrogenase polypeptides; Schetgens et al. 1985a).

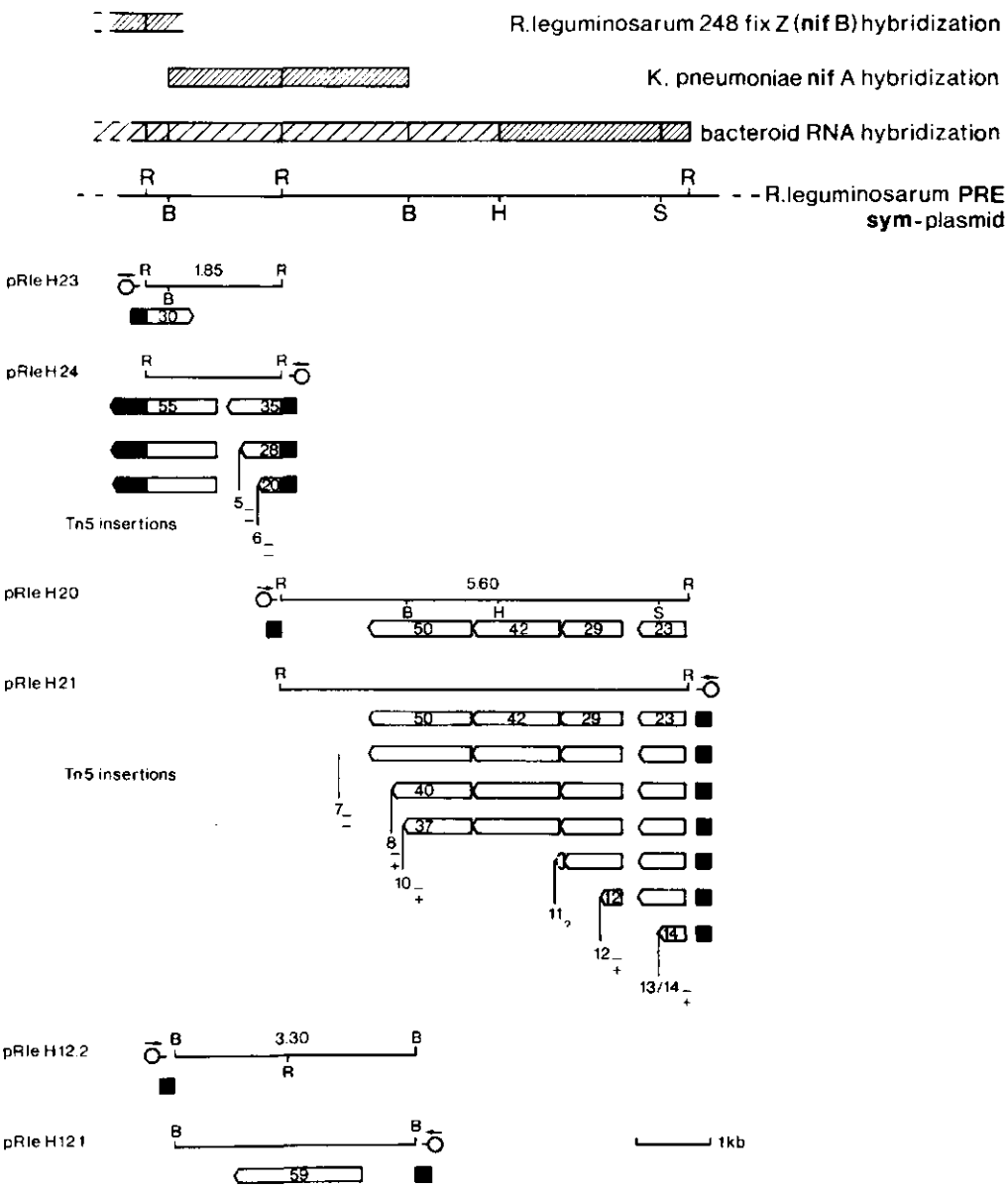
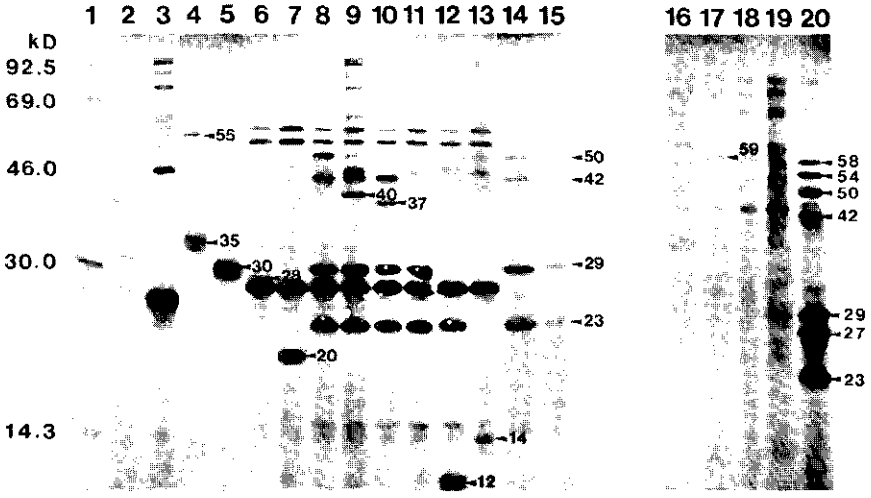

R.leguminosarum 248 fix Z (nif B) hybridization
K. pneumoniae nif A hybridization
bacteroid RNA hybridization
R
R
R
B
B
H
S
R.leguminosarum PRE sym-plasmid
pRle H23
R
1.85
R
B
30
pRle H24
R
R
55
35
28
20
5
6
Tn5 insertions
pRle H20
R
5.60
R
B
H
S
50
42
29
23
pRle H21
R
R
50
42
29
23
Tn5 insertions
40
37
7
8
10
11
12
12
13/14
pRle H12.2
B
3.30
B
R
pRle H12.1
B
B
59
1kb

Fig. 2

Synthesis of ^{35}S-labeled proteins in E.coli minicells from the cluster of fix-genes including nifA from R. leguminosarum PRE.



An autoradiogram of an SDS-polyacrylamide gel is shown.
Lane 1: ^{14}C-labeled marker proteins; lanes 2 and 16: E.coli DS410 minicells without plasmid DNA; lane 3: pACYC184; lane 4: pRleH24; lane 5: pRleH23; Lane 6: pRleH24-Tn5 insertion 5; lane 7: pRleH24-Tn5 insertion 6; lanes 8 and 20: pRleH21-Tn5 insertion 7; lane 9: pRleH21-Tn5 insertion 8; lane 10: pRleH21-Tn5 insertion 10; lane 11: pRleH21-Tn5 insertion 11; lane 12: pRleH21-Tn5 insertion 12; lane 13: pRleH21-Tn5 insertion 13/14; lane 14: pRleH21; lane 15: pRleH20; lane 17: pRleH12.2; lane 18: pRleH12.1; lane 19: pRleH12.
Rhizobium-encoded proteins and truncated products are indicated by arrows. Protein molecular weights are given in kD.

al. 1984) are also visible in lanes 6 and 7. The 35 kD polypeptide encoded by pRleH24 is a fusion protein, composed of the N-terminal part of the CAT protein (8 kD; Schröder et al. 1981) and a 27 kD C-terminal portion of a Rhizobium-encoded polypeptide. According to nifA homology data (Fig. 1) and the symbiotic phenotypes of Tn5 insertions 5 and 6 (Schetgens et al. 1985a) this polypeptide is the C-terminal part of the R.leguminosarum PRE nifA product. This contrasts our earlier conclusion (Schetgens et al. 1985b) that the 35 kD polypeptide represents the complete nifA gene product, based on an incorrect construction of subclone pRleH22. The limited coding capacity of the pRleH24 insert (maximally 65 kD) implies that the 55 kD polypeptide probably is a fusion between a 37 kD N-terminal protein portion encoded by the insert of pRleH24 and the 18 kD C-terminal part of the CAT protein. Thus a second Rhizobium gene downstream and in the same orientation with respect to nifA was mapped, which presumably coincides with nifB/fixZ-homologous sequences (see Fig. 1; Schetgens et al. 1985a). The synthesis of 26 and 16 kD truncated proteins (not shown) from Tn5 insertions 3 and 4 (Schetgens et al., 1985a) in pRleH24 confirms the location of the translational start of the nifB/fixZ fusion protein indicated in Fig. 1. The opposite insert orientation (pRleH23) results in a 30 kD polypeptide (lane 5), which probably (because of the high intensity of this band) also is a fusion between the N-terminal part of the CAT protein and an insert encoded polypeptide.

Expression of the 5.60 kb EcoRI sym plasmid fragment in either orientation with respect to the CAT promoter (pRleH21 or pRleH20, Fig. 2, lanes 14 and 15) resulted in the synthesis of 50, 42, 29 and 23 kD polypeptides. Tn5 insertion 7 in pRleH21 (Fig. 2, lane 8) has no effect on the expression of any of these polypeptides. Tn5 insertions 8 and 10 (Fig. 2, lanes 9 and 10) yield 40 and 37 kD truncated proteins respectively of the 50 kD product. This result led to the location and orientation of the 50 kD protein coding sequence as shown in Fig. 1. Tn5 insertion 11 (Fig. 2, lane 11) interrupts the synthesis of the 50 kD as well as of the 42 kD product, but no truncated protein is evident. Probably this mutation interrupts the 42 kD gene resulting in a smaller product that either coincides with other bands or is very

small and ran off the gel. Tn5 insertion 12 (Fig. 2, lane 12) leaves only the 23 kD product intact; besides a 12 kD truncated protein is visible derived from the 29 kD protein. This defined the position of the 29 kD gene (Fig. 1). Tn5 insertion 13/14 (Fig. 2, lane 13) blocks the synthesis of all four proteins, while a 14 kD truncated protein probably derived from the 23 kD polypeptide arises. These results fit the position and transcriptional orientation of the 23 kD protein encoding sequence shown in Fig. 1.

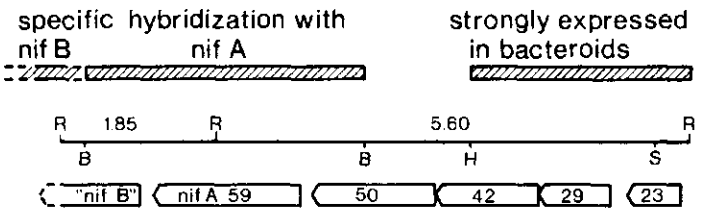
In order to define the location of the nifA coding sequence, expression in minicells of a 3.30 kb BamH1 fragment (Fig. 1) containing the complete nifA gene was studied. This fragment cloned in pBR322 however does not express Rhizobium-specific polypeptides (pRleH12; Fig. 2, lane 19), neither when it is cloned via EcoRI linkers in the EcoRI site of pACYC184 (pRleH12.2; Fig. 1 and Fig. 2, lane 17). Only from pRleH12.1 when the insert orientation is inversed respective to pRleH12.2 a 59 kD polypeptide is synthesized (pRleH12.1; Fig. 1 and Fig. 2, lane 18), as a result of translation from a CAT-Rhizobium hybrid messenger RNA. The position of the 59 kD nifA protein coding region (Fig. 1) follows from the location of the C-terminus in pRleH24. The conclusions from the minicell experiments are summarized in Fig. 3 in which protein coding sequences are related to earlier identified genetic functions. The tentatively identified translation termination site for the nifB/fixZ homologous gene in PRE is based upon an expected molecular weight of approximately 40 kD as determined for the nifB protein in K. pneumoniae (Pühler and Klipp, 1981) and in R.leguminosarum 248 (Rossen et al. 1984).

Are R.leguminosarum PRE nifA mutations in vivo complemented by K.pneumoniae nifA gene product ?

In order to test whether nifA mutations in R.leguminosarum PRE can be complemented in vivo by the K.pneumoniae nifA gene product, plasmids constitutively expressing the K.pneumoniae nifLA (pRK404-130) or the nifA operon (pRK404-131) in E.coli (Pühler et al. 1983) were constructed and conjugated into PRE strains carrying Tn5 insertions in nifA (mutants 5, 6 and 7; see Fig. 1). Inoculation of pea plants with

Fig. 3

Correlated physical and genetic map of the R. leguminosarum PRE fix gene cluster including nifA.



DNA stretches showing strong hybridization with nifB and nifA or high in vivo expression levels are indicated by shaded bars above the map. Protein coding regions and directions of transcription are shown by thick open arrows with corresponding molecular weights of synthesized polypeptide chains in kD. The partly dotted arrow indicates that the size of this complete gene product has not yet been determined and that the translation termination site was tentatively chosen (see text).

the transconjugants revealed a Fix^- Nif HDK^- phenotype indistinguishable from that of the nifA::Tn5 parent strains. No nitrogenase polypeptides were detectable on Western blots containing proteins from free-living or endosymbiotic Rhizobium upon incubation with antiserum specific against nitrogenase. Plasmid loss is not the cause for the lack of complementation as restriction analysis of plasmids isolated from the corresponding broth-cultured bacteria or bacteroids from 21 days old nodules demonstrated the presence of the original plasmids (not shown).

These data are in accord with those of Szeto et al. (1984) as for the absence of complementation during symbiosis of Rhizobium nifA

mutations by the Klebsiella nifA regulatory protein, but no "improvement" in the morphological condition of nodules and host plant as reported by these authors was observed. We also could not confirm the activation of the nifHDK operon by the K.pneumoniae nifA gene product in free-living cultures of R.leguminosarum nifA::Tn5 mutants; the only experimental difference with the approach of Szeto et al. (1984) was that we did not assay for the synthesis of nifHDK mRNA but for nitrogenase polypeptides.

DISCUSSION

By expression in E.coli minicells of cloned sym plasmid fragments from R.leguminosarum PRE we could deduce the location of the nifA regulatory locus. Synthesis of a 35 kD fusion protein from pRleH24 and of truncated polypeptides from Tn5 insertions 5 and 6 determined the C-terminus of the nifA coding region. The complete nifA gene product was synthesized from pRleH12.1 (see Figs. 1 and 3); the observed molecular weight of 59 kD (recently confirmed in our laboratory by expression of this gene from a T7 RNA polymerase dependent promoter; P. Roelvink unpublished result) is in good accordance with a nucleotide sequence of 1692 basepairs as reported for the R. meliloti nifA gene (Buikema et al. 1985; Weber et al. 1985). No synthesis of a complete nifA gene protein was detected from a presumptive Rhizobium nifA promoter (in pRleH12 or pRleH12.2) or by transcriptional readthrough from weak endogenous promoter signals located more upstream (e.g. in pRleH20/21) (see Kim et al., 1985). Thus translation of the complete nifA protein depends on the presence of a fusion mRNA initiated from a strong vector promoter (in pRleH12.1); the same has been observed for R.meliloti nifA (Weber et al. 1985).
Remarkably the 35 kD fusion protein, synthesized from pRleH24 yields two protein bands on gel electrophoresis (Fig. 2, lane 4); this is probably due to a second termination signal, because Tn5 insertions 5 and 6 give only single truncated protein bands. The synthesis of two polypeptide chains from one gene upon expression of Rhizobium

sequences in E.coli minicells was reported earlier for the nifH locus in R.meliloti (Pühler et al. 1983) and in B. japonicum (Fuhrmann and Hennecke, 1984) and for the R. meliloti nifA/fixD gene (Weber et al. 1985).

A nifB/fixZ homologous gene was located, based on the synthesis of a CAT-fusion protein from pRleH24 consisting of the major N-terminal part of the nifB polypeptide chain; the position of the C-terminus was estimated with the help of nifB protein molecular weights reported in the literature (see Figs. 1 and 3). Tn5 insertions 3 and 4, supposedly localized in nifB, would be expected to confer a Fix^- phenotype but were reported earlier (Schetgens et al. 1985a) to be Fix^+.

Reexamination of total DNA digests of these mutants (and also number 11) revealed that Tn5 was not located in the expected positions, but probably had undergone transposition instead of double homologous recombination. The nifA - nifB intergenic region contains approximately 200 nucleotides in R.leguminosarum PRE, which is in accordance with the distances reported for K. pneumoniae (Beynon et al. 1983) and R. leguminosarum 248 (Rossen et al. 1984).

For nifB as well as nifA no endogenous promoter was found to be active in E.coli but the Rhizobium ribosome binding sites are recognized efficiently in this heterologous system.

Expression of pRleH23 in minicells shows the synthesis of a 30 kD CAT-chimaeric polypeptide (see Figs. 1 and 2); this implies that an open reading frame of about 22 kD is located within the left hand part of the 1.85 kb EcoRI-fragment and transcribed from the strand opposite to and overlapping nifB/fixZ. The significance of this phenomenon is unclear.

Expression of the 5.60 kb EcoRI fragment (Fig. 3) in minicells resulted in four polypeptides, apparently synthesized from a contiguous gene cluster. From the effects of Tn5 insertions on protein synthesis the positions of the 50, 42, 29 and 23 kD protein coding regions were determined. This cluster of fix-genes is expressed at a comparable level in both insert orientations (pRleH21 vs pRleH20, see Fig. 2 lanes 14 and 15; the lower intensity in lane 15 is due to the incidental loading of a smaller sample). This implies that expression is probably not dependent on vector sequences and is controlled by

transcription initiation signals present on the Rhizobium insert, i.e. one or more Rhizobium promoters may be efficiently recognized by E.coli RNA polymerase. This was also observed, though to a much weaker extent, for the nifH promoter (Pühler et al. 1983; Schetgens et al. 1984) and for some nod genes (Downie et al. 1985). Alternatively transcription may start from a vector promoter on the strand opposite to the CAT-promoter. We consider the latter to be less likely as the intensities of the polypeptide bands produced in the two orientations are similar; transcription from the CAT promoter would be expected to proceed much more efficiently than from other promoters in pACYC184. The well-detectable initiation of transcription from a Rhizobium-specific promoter in E.coli, as noticed for the 5.60 kb EcoRI-fragment of the PRE sym-plasmid, was unexpected, as fix genes are supposed to be subject to a complicated genetic control. Consequently expression of Rhizobium sym genes in E.coli in most cases depends on transcriptional or translational fusions with a strong vector promoter (Pühler et al., 1983; Weber et al., 1985; Fuhrmann and Hennecke, 1982 and 1984; Schmidt et al., 1984). Presumably thus a polycistronic mRNA (4.35 kb) directs the synthesis of four polypeptides (23, 29, 42 and 50 kD) in E.coli. We conclude this from the observation that Tn5 insertions in this region are polar on all distal genes (see Fig. 1 and 2, mutations 11, 12 and 13/14). This does not necessarily imply that the same is true in vivo. Bacteroid mRNA hybridizes more strongly to the DNA encoding the 23, 29 and part of the 42 kD protein, than to the 50 kD region. This may be due to differences in transcription rates, but also to differences in stability of regions of the polycistronic mRNA due to selective degradation of the mRNA during translation (Platt, 1978). Possibly one or more additional promoters, which are not active in E.coli, are present downstream from the one preceding the 23 kD protein coding region; the more distally encoded proteins could then still be synthesized in minicells by readthrough transcription.

The fact that in minicells the 42 and 50 kD protein bands are less intense than those of 23 and 29 kD (see Fig. 2) may be due to diminished stability of distal parts of long readthrough mRNA molecules, or to partial readthrough transcription through termination

signals between the 29 and 42 kD genes, or to differences in the activity of the respective ribosome binding sites. The cluster of four fix-genes, upstream of the regulatory nifA-locus in R.leguminosarum PRE, resembles the fixABC operon in R.meliloti which encodes proteins with molecular weights of 30, 37 and 43 kD respectively (Ausubel et al. 1985; Weber et al. 1985). Hybridization of different R.meliloti fixABC probes against digests of the 5.60 kb EcoRI PRE fragment indeed showed significant homology with the genes encoding the 29, 42 and 50 kD proteins (J. Hontelez, unpublished results). Besides a probe containing the R.meliloti P2 promoter showed sequence homology to the 23-29 kD intergenic region of the PRE sym plasmid. If this region indeed contains a symbiotic promoter then the 23 kD gene must form a separate transcription unit in vivo.

The function of the nifA locus on the sym plasmid is defined by the effect of Tn5 mutations in this gene. R.leguminosarum mutants with such mutations did not produce the polypeptides of the components I and II of nitrogenase in bacteroids, indicating that the nifHDK operon is not expressed. The Tn5 mutations in the nifA locus on the sym plasmid are not complemented in vivo by a recombinant plasmid directing constitutive synthesis of K.pneumoniae nifA protein. There may be several reasons for this lack of complementation. It is possible that the nifA protein of K.pneumoniae is not active in Rhizobium even if there is considerable sequence homology between the genes in both species. Other possibilities are that the amount of Klebsiella nifA protein produced in Rhizobium bacteroids is not sufficient to detect activation of the nifHDK operon, or that an additional factor is required for nifA protein activity in Rhizobium, which is lacking in the mutants or inactive with the K.pneumoniae nifA protein. Our observation that K.pneumoniae nifA protein does not induce nifHDK expression in free-living R.leguminosarum agrees with the results of Better et al. (1985) but is in contrast with the results of similar experiments of Szeto et al. (1984). We have no explanation for this discrepancy but we can only indicate that Szeto et al. determined nifHDK expression by measuring the nifHDK mRNA levels, whereas Better et al. and we looked for the nitrogenase proteins encoded by nifHDK. If that would be the only reason for the

discrepancy in our results and those of Szeto et al., it might indicate that there is a posttranscriptional regulatory mechanism influencing the translation of the mRNA for nitrogenase. Further evidence for such a mechanism is however entirely lacking.

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Chapter V
Organization and phenotypical characterization of *R. leguminosarum* PRE symbiotic genes as detected by specific expression in nodules

SUMMARY

Regions on the sym-plasmid of R.leguminosarum PRE which hybridized with labeled bacteroid RNA from 17 days old nodules were isolated from a clone bank in phage λEMBL3. Two non-overlapping stretches of DNA surrounding the earlier identified nifHDK nitrogenase operon and the "fixABC"-nifA-nifB cluster respectively were thus characterized. Site-directed transposon Tn5 mutagenesis of subfragments showing different levels of expression during symbiosis revealed the presence of a number of functional fix genes. Furthermore a nodulation gene region was detected by homology with a R.meliloti nod gene probe downstream and adjacent to the fix cluster containing nifA. Three distinct Nod$^-$ phenotypes resulted from mutations in this region. An estimate of the number of symbiotic genes within these two regions of the R.leguminosarum PRE sym-plasmid was made and the organization of the sym plasmid compared with that in other Rhizobium species.

INTRODUCTION

In the previous chapters we described the molecular genetic analysis of R.leguminosarum PRE sym-plasmid sequences encoding the nitrogenase structural genes (nifHDK), a nif-regulatory gene (nifA) and a gene homologous to nifB in K.pneumoniae and therefore probably involved in FeMo-cofactor synthesis. In addition we described some genes involved in nitrogen fixation (fix) but with a yet unspecified function.
In this chapter we describe the identification of several other clusters of symbiotic genes on the R.leguminosarum PRE sym-plasmid and their organization and expression.
The detection of these other symbiotic genes was based on the observation of Krol et al. (1980) that certain sym-plasmid regions are transcribed in nitrogen-fixing nodules but not to any significant extent in free-living rhizobia. We further report the detection of a genomic region required for

nodule development (nod) by DNA hybridization to a specific R.meliloti nod probe. A clone bank of PRE sym-plasmid DNA fragments was screened with ^{32}P-labeled bacteroid RNA from 17 days old pea root nodules; different levels of expression during endosymbiosis could thus be assigned to several sym-plasmid fragments.
By site-directed transposon mutagenesis of the cloned sym-plasmid fragments it was shown that these regions contained several functional fix and nod genes. In some cases operon structures were deduced from the effects of Tn5 insertions.

MATERIALS AND METHODS

Microbiological techniques

The strains of R. leguminosarum and Escherichia coli, λ phage and plasmids used are listed in Table I. Culture media, concentrations of antibiotics and growth conditions were as described by Schetgens et al. (1984 and chapter II of this thesis).

DNA methodology

The isolation of sym-plasmid DNA from R.leguminosarum PRE, the isolation of total DNA from R.leguminosarum PRE, the isolation of E.coli plasmids, restriction endonuclease digestion, agarose gel electrophoresis, fragment elution, nick translation, Southern blot hybridization, ligation and E.coli transformation have been described by Schetgens et al. (1984; chapter II of this thesis). Purification of λEMBL3 plaques and the preparation of recombinant phage DNA were performed according to Maniatis et al. (1982).

Construction of λEMBL3 gene libraries

Partial digestion of R. leguminosarum genomic or sym-plasmid DNA, size fractionation by sucrose gradient centrifugation and the dephosphorylation of DNA fragments (15-20 kb), as well as the preparation of packaging extracts and the in vitro packaging procedure for λEMBL3 recombinant DNAs were performed as described by Grosveld et al. (1981).

Table 1. Bacterial strains, plasmids and bacteriophage.

Designation	Relevant character/genotype	Reference/source
-Bacterial strains.		
R.leguminosarum PRE	wild type	Lie et al., 1979
PRE Str^r	streptomycin and acriflavin resistance	Lie et al., 1979
E.coli MM294	endoI hsdR hsdM pro	Ruvkun and Ausubel, 1981
Q358	$R_k^-M_k^+Su_{II}^+$80R	Karn et al., 1980
Q359	$R_k^-M_k^+Su_{II}^+$80R P2	Karn et al., 1980
N5387	a lysogenic strain of λb211 rex::Tn5 CI857 with a chromosomal Tn5	From A. Krol, Amsterdam
-Bacteriophage.		
λEMBL3	λ1059-derived cloning vector (Q358 and Q359 hosts)	Karn et al., 1980
-Plasmids.		
pACYC184	Cm^{r}Tc^r	Chang and Cohen, 1978
pRK290	Tc^r; conjugative broad host range plasmid	Ditta et al., 1980
pRK2013	Nm^r; complements tra genes of pRK290 in conjugations	Figurski and Helinski, 1979
pPH1JI	Gm^r; incompatible with pRK290	Beringer et al., 1978
pRmSL26	R.meliloti nod genes cloned in pLAFR1	Long et al., 1982

Isolation and labeling of bacteroid RNA

Total bacteroid RNA purified from 17 days old nodules was labeled with ^{32}P at the 5'end by T4 polynucleotide kinase (Krol et al. 1980) and subsequently used for screening of the R.leguminosarum sym-plasmid clone bank in phage λEMBL3 by plaque hybridization (Karn et al. 1980; Maniatis et al. 1982) or for probing Southern blots.

Transposon mutagenesis

The construction and testing of R.leguminosarum PRE with site-specific Tn5 mutations in the sym-plasmid have been described (chapters II and III of this thesis; see also Schetgens et al., 1984 and 1985).

RESULTS AND DISCUSSION

Screening of a sym-plasmid gene library from R. leguminosarum PRE with bacteroid RNA as a probe.

The 350 kb sym-plasmid of R.leguminosarum PRE carries the genes involved in nodule development and symbiotic nitrogen fixation. For the cloning of large sym-plasmid fragments sym-plasmid DNA was isolated from vegetatively grown bacteria and partially digested by EcoRI; the resulting fragments were size-fractionated, dephosphorylated and ligated into the EcoRI-site of phage λEMBL3. Alternatively a partial BamHI digest of total DNA from R.leguminosarum was cloned in the same vector. After in vitro packaging and transfection, the plaques obtained from both gene libraries were blotted onto nitrocellulose filters and the blots were hybridized with ^{32}P-kinase-labeled RNA from actively nitrogen-fixing bacteroids, isolated from pea root nodules at 17 days after inoculation.
Six positive plaques with an average insert length of 15 kb were selected from the EcoRI sym-plasmid clone bank and further characterized. Restriction enzyme analysis of the inserts and hybridization of the resulting fragment patterns on Southern blots with ^{32}P-labeled bacteroid RNA (not shown) revealed that five clones overlap, while one showed no

overlap with the others. These two non-overlapping regions of the R.leguminosarum PRE sym-plasmid detected by hybridization with bacteroid RNA plausibly carry genes with a function in the symbiosis. Physical maps of both sym-plasmid regions are given in Fig. 1.

Screening of the BamHI library with ^{32}P-bacteroid RNA resulted in the isolation of clone λRleH1, which contained part of one of the regions isolated from the EcoRI bank. A physical map of the region from which the five EcoRI clones and the BamH1 clone λRleH1 originate is given in the upper part of Fig. 1. The nifHDK genes, coding for component II (nifH) and the two subunits of component I, Iα (nifD) and Iβ (nifK) of the nitrogenase enzyme complex, are also located in this region. The mapping of these genes and their characterization has been described in chapter II. Tn5 mutations located in the nifHDK region and the coding regions analysed in that chapter are also indicated in Fig. 1.

The physical map of the other sym-plasmid region isolated from the EcoR1 clone bank in λRleH6 is given in the lower part of Fig. 1. This region appeared to contain the 7.45 kb part with the 23 kD gene, the cluster fixABC, nifA and nifB already described in chapters III and IV. From our clone bank in λEMBL3 (this chapter) and a cosmid clone bank prepared by R. Klein Lankhorst (unpublished results) parts of the sym plasmid adjacent to the region in λRleH6 were isolated and in the lower part of Fig. 1 the physical map of a stretch of 48 kb is given. This also contains a region of nod genes, which will be discussed in more detail further in this chapter.

By hybridizing Southern blots of DNA fragments obtained with various restriction enzymes using bacteroid RNA as a probe the relative rates of transcription of different parts of the sym plasmid were determined. This is shown by the shading of the bar above the physical map in Fig. 1. The high level of expression observed for the nifHDK operon is in accordance with the finding that about 20% of the total protein in mature bacteroids is nitrogenase (Bisseling et al. 1978). Immediately upstream from the nifH promoter in a 6.0 kb EcoRI-fragment as well as further downstream in 4.3 and 1.35 kb EcoRI-fragments sym-plasmid regions were found to hybridize also relatively strongly with bacteroid RNA. Other fragments hybridized moderately or only weakly and in between there were parts that did not show detectable hybridization with bacteroid RNA from 17 days old nodules.

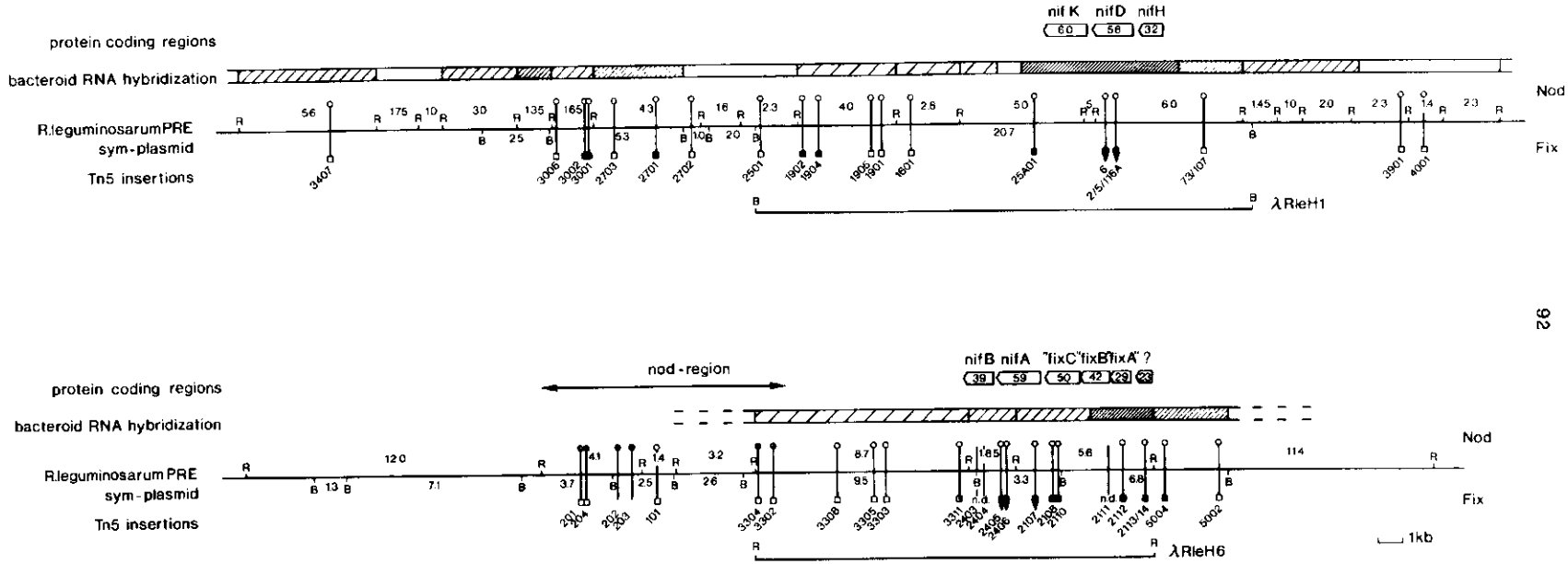
nifK nifD nifH
60 56 32
protein coding regions
bacteroid RNA hybridization
Nod
R.leguminosarum PRE
sym-plasmid
Fix
Tn5 insertions
λRleH1
nod-region
nifB nifA "fixC" fixB fixA" ?
39 59 50 42 29 23
λRleH6
1kb

Fig. 1

Genetic organization of two non-overlapping regions of the R.leguminosarum PRE sym-plasmid.

Restriction enzyme sites are abbreviated as R (EcoRI) and B (BamHI); fragment lengths are given in kb.
Intensities of hybridization with bacteroid RNA are indicated by differentially shaded bars (dark to lighter shadings correspond with very strong to moderate or weak expression levels, empty bars with undetectable hybridization and broken lines with not determined regions).
Protein coding sequences and transcriptional directions are indicated by open arrows with gene designations and protein molecular weights (in kD). The approximate location of the nod region is indicated.
Locations of two recombinant phage clone inserts, in either of the two sym-plasmid regions, are marked (λR1eH1 and λR1eH6), other λEMBL3 inserts and different subclones have not been indicated.
The position of Tn5 insertions are given by numbered vertical bars. Nod and Fix phenotypes are indicated by the following symbols: ○ Nod^{+} (normal); ◐ $Nod^{+/-}$ (delayed); ◑ $Nod^{-/+}$ (reduced); ● Nod^{-} (deficient); □ Fix^{+}; ◨ $Fix^{-/+}$; ■ Fix^{-}; ▼ $Nif(HDK)^{-}$; ▽ $NifH^{(+)}D^{-}K^{-}$; n.d. is not determined.

In the region of the sym plasmid represented in the lower part of Fig. 1 we have so far only examined the relative rate of transcription of the part present in clone λRleH6. A 5.6 kb EcoRI fragment showed an almost equally strong hybridization with bacteroid RNA as the nifHDK genes did. This is in agreement with our observations described in chapters III and IV, that several fix genes are located in this region. In comparison the degree of hybridization of the nifA and the nifB genes with bacteroid RNA was moderate. Other parts such as the lefthand part of the 11.4 kb EcoRI fragment or the 8.7 kb EcoRI fragment showed more intense or weaker hybridization with bacteroid RNA. It is clear however that the whole region present in clone λRleH6 and adjacent parts is actively expressed during symbiosis.
Possible functions of the sym-plasmid regions which are actively expressed during symbiosis were analysed by constructing Tn5 mutations using the procedure for site-directed Tn5 mutagenesis described in chapters II and III.

Phenotypes of Tn5 mutants

Nif and Fix phenotypes

The positions of the Tn5 mutations in the two regions of the sym-plasmid presented in Fig. 1 are also indicated in this figure. In chapter II a number of these mutants have already been described such as the mutants 2, 5, 116A and 6, all in nifD, which have Fix^- $NifH^{(+)}D^-K^-$ phenotype, i.e. they are defective in nitrogen fixation, do not synthesize the two CI polypeptides and show a reduced production of nitrogenase CII. Likewise in chapter III we have reported on mutants 2405, 2406 and 2107 in the nifA gene having a Fix^- $Nif(HDK)^-$ phenotype and mutants 2108, 2110, 2112 and 2113/14 in the 23kD-fixABC cluster having a $Fix^-Nif(HDK)^+$ phenotype.
A $Fix^-Nif(HDK)^+$ phenotype was also observed for many of the mutants obtained by inserting Tn5 in parts of the sym-plasmid which were found to be actively transcribed during symbiosis. These include in the region represented in the upper part of Fig. 1 the mutants 25A01 (adjacent to the nifK coding region and within the same strongly expressed fragment), 1902

and 1904 (in a weakly expressed 4.0 kb EcoRI fragment), 2701 (in a strongly expressed 4.3 kb EcoRI fragment) and 3001 and 3002 (both in a moderately expressed 1.65 kb EcoRI fragment). Tn5-insertions 4001, 3901, 73/107, 1601, 1901, 1905, 2501, 2702, 2703, 3006 and 3407 in fragments of the sym-plasmid region shown in the upper part of Fig. 1, which are transcribed at different levels in bacteroids, had no effect on the acetylene-reduction capacity.

In the region represented in the lower part of Fig. 1 insertion 5004 (in a strongly expressed sequence of an 11.4 kb EcoRI fragment) conferred a Fix^- $Nif(HDK)^+$ phenotype, whereas mutants 5002, 3303, 3305 and 3308 had no effect on symbiotic properties. Mutant 3311 remarkably showed a reduced nitrogen fixing ability ($Fix^{-/+}$; approximately 8% of the wild type level), while normal quantities of CI and CII were observed. This region might be homologous to nifQ in K.pneumoniae, which plays a role in the sequestration of molybdate for FeMo-cofactor synthesis, because of its position relative to nifA and nifB and the leaky Fix^- phenotype (Dixon, 1984).

Nod phenotypes

It has recently become clear that only a limited number of Rhizobium genes is involved in recognition of the host plant and nodule development, and that there are common nodulation genes required for the root hair curling (hac) response which appear to be highly conserved among Rhizobium species (Long et al. 1982; Downie et al. 1983a; Kondorosi et al. 1984; Schofield et al. 1984; Djordjevic et al. 1985; Lamb et al. 1985). We took advantage of this interspecies homology for detection of the nod region in PRE, by screening the sym plasmid bank with pRmSL26 carrying hac and common nodulation genes from R.meliloti (Long et al. 1982) as a probe. In this way we selected BamH1 clone λRleM1, the insert of which consists of the successive BamH1 fragments of 7.1 kb, 3.7 kb, 2.5 kb and 2.6 kb indicated in the lower part of Fig. 1. The homology with R.meliloti nod genes mainly resides in the 4.1 kb and 1.4 kb EcoRI fragments of the insert. Among the Tn5 mutants in this region three different types of variation in nodulation behaviour were observed upon inoculation of pea seeds. At three times between 14 and 30 days after inoculation two plants at a time were examined for nodulation with each Tn5 mutant. The mutants 202 and 203 (see Fig. 1, lower

part) were completely defective in nodulation (Nod^-). Two other mutants, 201 and 204, showed delayed nodule development: in these two cases the nodules did not appear before 21 days after inoculation, which is at least 5 days later than with the parent wild type bacteria under our conditions. The number of nodules was about the same as upon inoculation with wild type PRE bacteria, but the nodules appeared mainly on young lateral roots. This type of delayed nodulation response is indicated with $Nod^{+/-}$. The two Nod^- and two $Nod^{+/-}$ mutations occur in the 4.1 kb EcoR1 fragment. Mutant 101 with a Tn5 insertion in the adjacent 1.4 kb EcoR1 fragment did not show aberrant nodulation behaviour, but further to the right, in the left hand portion of the 8.7 kb EcoR1 fragment two other mutations 3302 and 3304 with an effect on nodulation were obtained. These mutants showed a strong reduction in the number of nodules produced on the roots. The nodules showed effective nitrogen fixation (Fix^+) but in comparison with the wild type the number of nodules was only 5 to 10% at day 15 after inoculation. This phenotype is indicated with $Nod^{-/+}$.
The $Nod^{+/-}$ mutants 201 and 204 have a wild type Hac (hair curling) phenotype, whereas the Nod^- mutants 202 and 203 were Hac^{++} i.e. they showed extremely strong root hair curling. Mutants 3302 and 3304 ($Nod^{-/+}$) were not examined for their root hair curling behaviour.
The three different types of nod mutations in PRE roughly correspond with the five classes of nodulation mutants described by Downie et al. (1985) for R.leguminosarum 248. Our Nod^- mutants seem to belong to either his class I (nodC or hac), class II (nodD) or class III (nodAB); the $Nod^{-/+}$ mutants resemble class IV mutants, whereas the $Nod^{+/-}$ mutants are similar to class V mutants (Rossen et al. 1984a; Schmidt et al. 1984; Török et al. 1984; Downie et al. 1985).

Organization of symbiotic genes on the R.leguminosarum PRE sym plasmid

We have identified two regions of 52 kb and 48 kb respectively on the 350 kb sym-plasmid of R.leguminosarum PRE. The region of 52 kb harbors the nifHDK operon and at least seven other functional fix regions. This latter conclusion is based on the various rates of transcription found for various parts of the 52 kb region. The region of 48 kb contains a nodulation region and a cluster of fix genes including the nifA gene which regulates the expression of all other nif genes. For nifA, its direct neighbours nifB and the fixABC cluster and the 23 kD gene the coding regions have been identified as described in chapter IV. In this

chapter we have shown that the 48 kb segment contains at least two additonal fix regions, based on the determination of the rates of transcription of different parts. The nod region has a total length of 10 kb and is located approximately 7 kb downstream from nifB. We have not yet established physical linkage between the 52 kb and 48 kb sym-plasmid regions. It is apparent that the distance between the nifHDK operon and the nod region and the second fix cluster is considerable and amounts to at least 35 kb. This implies that the functional sym regions are more spread over the sym-plasmid than in R.leguminosarum 248 or in R.meliloti, where the sym genes have a more compact organization. In the 200 kb sym-plasmid of R.leguminosarum 248 the nifHDK operon and a second fix cluster, which contains nifA and nifB (indicated with fixY and fixZ in R.leguminosarum 248) are only 27 kb apart and in this 27 kb stretch the 10 kb nod region (Downie et al., 1983b; Rossen et al., 1984b)is comprised. In R.meliloti the cluster with nifA, nifB and fixABC is located about 1 kb upstream from the nifH promoter, whereas the nod region is found within 30 kb downstream from nifK (Long et al., 1982; Zimmerman et al. 1983; Kondorosi et al., 1984).
In order to establish the physical map of the entire sym-plasmid and to characterize other R.leguminosarum PRE genes involved in symbiotic nitrogen fixation in future experiments gene libraries will have to be screened with RNA probes, purified from bacteroids in various stages of nodule development. This will hopefully lead to a better insight in the sequential order of expression of symbiotic genes and in the regulatory mechanisms acting during the interaction of Rhizobium with legume hosts.

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Chapter VI
Summary and conclusions

Bacteria of the genera Rhizobium and Bradyrhizobium are unique in their quality to form nitrogen-fixing root nodules in symbiosis with leguminous plants. In fast-growing Rhizobium bacteria the genes involved in host recognition and nodule development (nod) and in nitrogen fixation (nif or fix) are located on large sym-plasmids (for recent review see e.g. Ausubel, 1984).

The aim of the present investigations was to identify symbiotic genes in R.leguminosarum PRE and to study their expression in bacteroids. Sym-plasmid clone banks were constructed in order to analyse its physical organization and to isolate relevant fragments (see chapters II and V). The selection of sym-plasmid regions essential for symbiotic nitrogen fixation was primarily based on DNA sequence homology with specific cloned genes (probes) from other Rhizobium species or Klebsiella.

In chapter II the detection of the nitrogenase structural genes by Southern hybridization with a R.meliloti nifHDK probe, pRmR2 (Ruvkun and Ausubel, 1980) is described. The nifHDK operon was functionally characterized by site-directed transposon Tn5 mutagenesis and by mapping of protein coding regions after expression of cloned Rhizobium DNA sequences in E.coli mini-cells.

The nif-regulatory nifA gene, which in K.pneumoniae is involved in the activation of all other nif operons, also appears to be evolutionary conserved among nitrogen-fixing organisms (Dixon, 1984; Ausubel et al., 1985). In chapters III and IV the identification and characterization of the R.leguminosarum PRE nifA locus is reported. Significant homology was found with K.pneumoniae nifA (Riedel et al., 1979), a R.meliloti symbiotic regulatory gene and E.coli ntrC, a gene involved in the regulation of several nitrogen assimilation pathways (Szeto et al., 1984). Tn5 insertions in the nifA gene resulted in mutants which produced ineffective root nodules with bacteroids in which the synthesis of components I and II of nitrogenase encoded by the nifHDK operon was inhibited. This observation confirmed that the nifA type locus in the sym plasmid of R.leguminosarum has indeed a function in the regulation of the expression of the

nitrogenase proteins. The regulatory nifA gene in PRE encodes a 59 kD protein. There was no functional complementation of Rhizobium nifA mutations by the K.pneumoniae nifA polypeptide in bacteroids or in free-living cultures. In addition a coding region immediately downstream from nifA hybridized specifically with R.leguminosarum 248 fixZ, a homologue of the K.pneumoniae nifB locus involved in FeMo-cofactor production (Rossen et al., 1984).

Furthermore, as described in chapter V, a 10 kb nod region has been mapped on the PRE sym-plasmid approximately 7 kb downstream from the nifB homologous gene, based on the homology with an R.meliloti probe carrying nod-genes pRmSL26 (Long et al., 1982). Three different classes of Nod phenotypes, i.e. deficient, reduced and delayed in nodulation, resulted from Tn5 mutations in this region, and confirmed that this region of the sym plasmid contains nodulation genes.

Besides the determination of interspecies DNA homologies, we also used an alternative approach for the detection of sym genes. This was based on the observation of Krol (1982) that certain sym-plasmid regions are exclusively transcribed during endosymbiosis. In chapters III and V we show that screening of a sym-plasmid clone bank of PRE with labeled bacteroid RNA from 17 days old pea root nodules resulted in the isolation of two non-overlapping stretches of DNA each about 50 kb long and including the nifHDK operon and the nifA - nifB - nod cluster respectively.

Subfragments of the two sym-plasmid segments showed different levels of hybridization with bacteroid RNA suggesting the presence of a number of different genes. Tn5 mutations in these subfragments further demonstrated that indeed they carry different functional fix genes.

A contiguous cluster of such fix loci has been analysed in more detail and was shown to encode 23, 29, 42 and 50 kD polypeptides (chapter IV). This region of the PRE sym-plasmid resembles the fixABC operon in R.meliloti, as judged from hybridization data, molecular weights of proteins and the location upstream from nifA - nifB (Ausubel et al., 1985; Weber et al., 1985); the 23 kD fix gene presumably represents a distinct transcriptional unit.

Fig. 1 in chapter V gives an overall picture of the present knowledge of the organization of the symbiotic genes on the sym-plasmid of R.leguminosarum PRE. The genetic organization of sym-plasmids has been

compared for different rhizobial species; remarkable similarities exist e.g. conservation of the fixABC - nifA - nifB cluster, but in general the R.leguminosarum PRE sym-plàsmid shows larger distances between and shifts in the location of symbiotic genes.

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Chapter VII
Samenvatting

Rhizobium bacteriën bezitten het vermogen om stikstof uit de lucht te binden en om te zetten in ammonia en wel uitsluitend in symbiose met vlinderbloemige planten. De waardplant wordt op die manier voor een belangrijk gedeelte in zijn stikstofbehoefte voorzien. Deze symbiontische stikstoffixatie is dan ook van grote betekenis in de landbouw, zowel voor de produktie van eiwitrijke peulvruchten als voor de toepassing als groenbemesting.

De interactie van rhizobia met een specifieke gastheer leidt tot de vorming van gespecialiseerde structuren op de plant, de wortelknollen. Binnen deze organen bevinden zich in bepaalde cellen gedifferentieerde vormen van de bacterie, de bacteroiden welke de stikstoffixatie verzorgen met behulp van het enzym nitrogenase. Dit proces en vooral de genetische achtergronden hiervan worden uitgebreid behandeld in hoofdstuk I. De genen in Rhizobium die betrokken zijn bij de waardplant-herkenning, infectie en knolontwikkeling (nod) en bij de stikstoffixatie (fix of nif) liggen op een groot zogenaamd sym-plasmide.

Doel van het onderzoek beschreven in dit proefschrift was de identificatie van symbiontische genen in R.leguminosarum PRE, met o.a. de erwt (Pisum sativum) als waardplant, en de karakterisering van hun funktie(s) in bacteroiden. Hiertoe werd een kloonbank van het betreffende sym-plasmide geconstrueerd, waaruit DNA fragmenten werden geselecteerd die essentieel zijn voor symbiontische N_2-fixatie. De selectie berustte in eerste instantie op het zoeken naar homologieën met specifieke genen uit andere Rhizobium soorten of uit de vrijlevende stikstofbinder Klebsiella pneumoniae.

In hoofdstuk II wordt de detectie beschreven van de drie genen nifH, nifD en nifK, die coderen voor de structurele eiwitten van het enzym nitrogenase. Die drie genen maken deel uit van één operon, het nifHDK operon. Dit operon werd functioneel geanalyseerd door middel van plaatsgerichte transposon Tn5 mutagenese en door localisering van eiwitcoderende sequenties middels expressie van gecloneerde DNA fragmenten in E.coli minicellen.

In de hoofdstukken III en IV wordt de identificatie (op grond van DNA-hybridisaties met K.pneumoniae en R.meliloti probes) beschreven van het nifA locus, dat verantwoordelijk is voor positieve regulatie van de expressie der overige nif operons. Tn5 mutaties in dit nifA gen, op grond van minicelresultaten coderend voor een 59 kD polypeptide, verhinderden inderdaad de synthese van de nitrogenase eiwitten. Er werd geen functionele complementatie in bacteroiden gevonden van R.leguminosarum PRE nifA gemuteerd met Tn5 door het K.pneumoniae nifA polypeptide.
Verder werd aangetoond dat stroomafwaarts aangrenzend aan nifA een gen is gelocaliseerd dat homologie bezit met R.leguminosarum 248 fixZ en K.pneumoniae nifB; dit gen is betrokken bij de synthese van een essentiële FeMo-cofactor van het nitrogenase enzym.
Ook de nodulatiegenen op het PRE sym-plasmide werden in kaart gebracht (hoofdstuk V) en wel op ongeveer 7 kb vanaf de nifB homologie. De opsporing gebeurde door hybridisatie met een R.meliloti nod probe. Deze groep nod-genen kan worden onderscheiden in drie verschillende klassen op grond van het effect van mutaties. Sommige mutanten gaven in het geheel geen knolvorming meer, andere leidden tot gereduceerde knolvorming en weer andere tot sterk vertraagde knolontwikkeling.
Een tweede methode om essentiële sym genen te ontdekken is gebaseerd op de conclusie uit het proefschrift van Krol (1982) dat bepaalde delen van het PRE sym-plasmide uitsluitend tot expressie komen tijdens endosymbiose met de erwt. De screening van de sym-plasmide kloonbank met radioactief gemerkt bacteroide RNA uit 17 dagen oude wortelknollen, leidde tot de isolatie van twee niet-overlappende DNA gebieden, die respectievelijk het nifHDK operon en de nifA - nifB cluster omvatten (hoofdstuk III en V). Subfragmenten van deze DNA kloons met verschillende expressie-niveau's in bacteroiden werden gemuteerd met Tn5. Uit de resulterende fenotypes bleek de aanwezigheid van verschillende functionele fix-genen op het sym-plasmide. Een cluster van dergelijke fix loci werd nader geanalyseerd en bleek te coderen voor 23, 29, 42 en 50 kD eiwitten (hoofdstuk IV). Dit deel van het PRE sym-plasmide dat stroomopwaarts aansluit aan nifA - nifB - nod vertoont grote gelijkenis met het fixABC operon in R.meliloti.
In Fig. 1 in hoofdstuk V wordt een overzichtsbeeld van onze huidige kennis omtrent de organisatie van symbiontische genen op het sym-plasmide van R.leguminosarum PRE gegeven. De genetische organisatie van sym-plasmiden

werd onderling vergeleken voor verschillende Rhizobium soorten; er bestaan opmerkelijke overeenkomsten zoals de conservering van het fixABC - nifA - nifB cluster, maar in het algemeen vertoont het R.leguminosarum PRE sym-plasmide grotere afstanden tussen en verschuivingen in de ligging van de verschillende symbiontische genen.

Curriculum vitae

Resie Schetgens werd op 25 april 1957 te Kerkrade geboren. In 1975 behaalde zij het diploma Gymnasium β, waarna de studie biologie werd begonnen aan de Katholieke Universiteit Nijmegen. In september 1981 deed zij doctoraal examen met als hoofdvak Moleculaire Biologie en als bijvakken Genetica en Dierfysiologie.

Van 1 oktober 1981 tot 1 oktober 1984 was zij als promovendus verbonden aan de vakgroep Moleculaire Biologie van de Landbouwhogeschool te Wageningen, in dienst van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.), onder auspiciën van de Stichting Biologisch Onderzoek in Nederland (B.I.O.N.). Vanaf 1 november 1985 is zij in dienst van de Landbouwhogeschool verbonden aan het laboratorium voor Virologie te Wageningen.