STUDIES ON THE EXPRESSION OF PLASMID-BORNE GENES IN THE ENDOSYMBIOTIC STATE OF *RHIZOBIUM LEGUMINOSARUM*

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STUDIES ON THE EXPRESSION OF PLASMID-BORNE GENES IN THE ENDOSYMBIOTIC STATE OF *RHIZOBIUM LEGUMINOSARUM*

Proefschrift

ter verkrijging van de graad van
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STELLINGEN

1. Wanneer in een *Rhizobium* stam één plasmide ongeveer twee maal zo groot is als een ander is dat niet een voldoende reden om te veronderstellen dat het grotere plasmide een dimeer is van het kleinere.

   Tichy, H.V. & Lotz, W.C. (1981) FEMS Microbiol. Letters 10, 203-207.

2. Yamada en Sakaguchi (1980) hebben onvoldoende duidelijk gemaakt dat de door hun geisoleerde stikstofbindende algen niet in symbiose leven met een stikstofbindende prokaryoot.

   Yamada, T. & Sakaguchi, K. (1980) Arch. Mircobiol. 124, 161-167.

3. De conclusie van Leonard en Zaitlin dat het 30.000 dalton polypeptide van tabaksmozaiekvirus betrokken is bij het cel-tot-cel transport van dit virus is zeer voorbarig.

   Leonard, D.A. & Zaitlin, M. (1982) Virology 117, 416-424.

4. *In vitro* weefselkweek is een goede methode voor de selectie van koude resistente tomatenplanten.

   Templeton-Somers, K.M., Sharp, W.R. & Pfister, R.M. (1981) Zeitschrift für Pflanzenphysiol. 103, 139-148.

5. De observatie van Gordon *et al.* dat de nitrogenase reaktie in *Azotobacter vinelandii* niet geremd wordt door ammonia kan verklaart worden door de geringe depolariserende werking van ammonia onder de door hun gebruikte experimentele condities.

   Gordon, J.K., Shah, V.K. & Brill, W.J. (1981) J. Bacteriol. 148, 884-888.

6. De conclusie van Albrecht *et al.* (1979) dat hydrogenase bevattende *Rhizobium* stammen de opbrengst van vlinderbloemige gewassen zouden kunnen verbeteren mist iedere grond.

   Albrecht, S.L., Maier, R.J., Hanus, F.J., Russell, S.A., Emerich, D.W. & Evans, H.J. (1979) Science 203, 1255-1257. Vergelijk: Carter, K.R., Jennings, N.T., Hanus, J. & Evans, H.J. (1978) Can. J. Microbiol. 24, 307-311.

7. Het ontwikkelen van stikstofbindende tarwe-rassen zal niet bijdragen aan het oplossen van het wereldvoedselprobleem.

Wageningen, 25 mei 1982 A.J.M. Krol

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I INTRODUCTION

1.1 NITROGEN FIXATION

Nitrogenous compounds are an important part of the nutrition of all organisms. Only a few species however have the capacity to convert nitrogen gas N_2 from the atmosphere into NH_3 and use it as the sole source of nitrogen. This process is called nitrogen fixation; for a detailed review we refer to Burns (1979). The reaction equation for nitrogen fixation is:

$N_2 + 6e^- + 12ATP \rightarrow 2NH_3 + 12ADP + 12Pi$.

In all nitrogen fixing organisms this reaction is catalyzed by the enzym nitrogenase. Nitrogenase is composed of two components: component I, a FeMo protein, composed of two α and two β subunits, and component II, a Fe protein, composed of two identical subunits.
The FeMo cofactor is a complex cluster of molybdenum, iron and sulfur atoms (Burgess *et al.*, 1981) and is located in the active centre of the enzyme. Component I is the actual nitrogenase: it is the site of the binding and reduction of N_2. Component II mediates the flow of electrons to component I. Although the reduction of N_2 is exergonic, 12 molecules of ATP (adenosine triphosphate) are hydrolysed per molecule of N_2. Probably the ATP is hydrolised to overcome the high activation energy to break the triple bond in N_2.
As a side reaction nitrogenase produces besides NH_3 also H_2:

$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4Pi$.

At maximal electron flux through nitrogenase at least 25% of the ATP utilized by the nitrogenase is consumed by hydrogen evolution, but this process can use up to 60% of the ATP at lower rates. Some nitrogen fixing strains possess a hydrogenase and in these organisms a part of the ATP which was hydrolysed during H_2 evolution can be recovered by oxidation of the H_2 (Stouthamer, 1981).

N_2 is an inert molecule and its reduction requires electrons of sufficient low potential. These electrons are possibly generated by the potential difference between the inner and outer surface of the

cytoplasmic membrane (Laane *et al.*, 1979). Maintenance of the membrane potential also requires the hydrolysis of ATP. In *Rhizobium* the nitrogenase reaction, the hydrogen evolution and the maintenance of the membrane potential use up to 40 molecules of ATP per molecule N_2 fixed (Stouthamer, 1981).

Possibly with the exeption of an eukaryotic alga (Yamada and Sakagucki, 1980) all nitrogen fixers are prokaryotic organisms (Table 1). They can be divided into two groups: the organisms which fix nitrogen in a free living state, and the organisms which must live in symbiosis or intimate assosiation with a particular plant or fungus before they develop the ability to fix nitrogen.

Nitrogenase is inactivated by oxygen. Therefore all nitrogen fixers have provisions to protect their nitrogenase against oxygen. Anaerobic, facultative anaerobic and phototrophic bacteria only fix nitrogen under anaerobic conditions. Some aerobic bacteria, e.g. *Mycobacterium flavum* can only fix nitrogen under low oxygen tension. An obligate aerobic bacterium like *Azotobacter vinelandii* maintains a low intracellular oxygen tension by a high respiration rate. Moreover it possesses a FeS-protein which binds to nitrogenase and protects it against oxygen (Haaker and Veeger, 1977). In filamentous blue green algae, e.g. *Anabaena cylindrica*, nitrogenase is only synthesized in heterocysts. These specialized cells lack an oxygen producing photosystem and respire all incoming oxygen, whereas the energy for nitrogen fixation is supplied by photosynthesis in the vegetative cells. Also symbiotic nitrogen fixers have provisions for protection of nitrogenase against inactivation. In root nodules of legumes the plant protein leghemoglobin facilitates a high flux of oxygen at a low pressure by binding free oxygen. In other symbiotic systems like actinomycetous root nodules of alder such a protein has not been demonstrated.

It will be obvious that as a consequence of the high energy demand of nitrogen fixation, nitrogen fixation will in general not occur when fixed nitrogen is available. In nature a large part of the fixed nitrogen is recycled. Only the fixed nitrogen which is removed

from the nitrogen cycle by denitrification, leaching or other processes must be replenished. In most ecosystems nitrogen fixing organisms are not abundant, indicating that the amount of nitrogen lost from the nitrogen cycle is relatively small. Only in areas where little fixed nitrogen is present, organisms possessing a nitrogen fixing apparatus can become abundant. Akkermans and van Dijk (1976) estimated that in a stand of alders (*Alnus glutinosa*) 58 kg N_2 per

Table I Nitrogen fixing organisms (Eady and Smith, 1979, Vincent, 1980, Akkermans, 1978a, Döbereiner and Boddey, 1981).

	Example:
1. Free living microorganisms	
a Obligate aerobic bacteria	*Azotobacter vinelandii*
b Facultative anaerobic bacteria (only fix anaerobically)	*Klebsiella pneumoniae*
c Obligate anaerobic bacteria	*Clostridium pasteurianum*
d Phototrophic bacteria (only fix anaerobically)	*Rhodospirillum rubrum*
e Blue green algae	*Anabaena cylindrica*
2. Symbiotic nitrogen fixing organisms	
a Blue green algae	*Nostoc* (in lichens) *Anabaena azollae* (in the waterfern Azolla)
b Bacteria	*Azotobacter paspali* (in the rhizosphere of *Paspalum notatum*)
c Actinomycetes	*Frankia* (root nodules on *Alnus, Ceanothus* and some other higher plants)
d *Rhizobium*	*Rhizobium leguminosarum* (root nodules on *Pisum sativum*) *Rhizobium* "Parasponia" (root nodules on *Parasponia parviflora*)

ha per year was fixed. When most fixed nitrogen has been removed, as is the case in agricultural fields, legumes can fix as much as 300 kg N_2/ha/yr (Quispel, 1974). Because of the limiting supply of energy for bacteria living in the soil free living nitrogen fixing bacteria probably fix not more than 2 kg N_2/ha/yr (Jordan, 1981).

1.2 THE *RHIZOBIUM* - LEGUME SYMBIOSIS

As is indicated above the *Rhizobium* - legume symbiosis can fix a large amount of N_2. Moreover legumes are an important crop and the interaction of *Rhizobium* with a plant is an interesting example of symbiosis. Therefore the *Rhizobium*-legume symbiosis is intensively studied. The genus *Rhizobium* consists of gram negative bacteria, which are able to form root nodules on leguminous plants. Also the non-legume *Parasponia parviflora* is nodulated by a *Rhizobium* species (Akkermans *et al.*, 1978b). In the root nodules the bacteria differentiate into bacteroids, which are the actual nitrogen fixers.

Nodule induction and development is a complex process which is dependent on genetic functions of both the *Rhizobium* bacterium (Maier and Brill, 1976) and the host plant (Nutman, 1956). The various steps in nodule formation have been reviewed in detail by Vincent (1980). Nodulation is started by colonization of the root surface and adhesion of the bacteria to a root hair. This adhesion and the subsequent nodule formation only occurs with specific combinations of *Rhizobium* species and host plants. A *Rhizobium* strain isolated from *Pisum sativum* normally also nodulates other *Pisum* species and *Vicia*, *Lathyrus*, *Lens* and *Cicer* species, but other legumes are not nodulated by this strain. Non nodulating or ineffective combinations with a particular host within this group often occur. All *Rhizobium* strains nodulating plants from the genera mentioned above have been classified as one species: *Rhizobium leguminosarum*. The group of plants nodulated by a single *Rhizobium* species is called a cross inoculation group. Other cross inoculation groups are e.g. *Trifolium* species, nodulated by *Rhizobium trifolii*, *Phaseolus* species, nodulated by *Rhizobium phaseoli* and *Melilotus*, *Medicago* and *Trigonella* species, nodulated by *Rhizobium meliloti*. Host specificity will be further discussed in section 3. The specific adhesion of *Rhizobium* to a root hair is supposed to happen via a

multi-valent lectin, which recognizes similar glycosylated receptors on the bacterium and the host (Dazzo *et al.*, 1981). It was suggested that extracellular polysaccharides play a role in adhesion (Napoli and Alberheim, 1980) but Sanders *et al.*, (1981) found no correlation between production of extracellular polysaccharides and nodulating ability. After binding of the rhizobium the root hair curls, and by an invagination of the cell wall the bacteria grow into the plant. The bacteria continue to divide and the invagination develops into an infection thread which penetrates into the root tissue up to the inner cortex. Some cortical cells dedifferentiate and start to divide. The final nodule tissue is polyploid; there are indications that the primary cells from which the nodules originated were already polyploid, but polyploidy might also arise during nodule development. The infection thread branches and grows into the newly formed meristemic tissue. Bacteria are released from the infection threads and fill the cells completely. They remain surrounded by a membrane, which is of plant origin. The bacteria increase in size and some strains become Y shaped. Their amount of DNA increases (Bisseling *et al.*, 1977) and they are no longer viable (van den Bos and Broughton, 1981). Because of the marked difference between free living rhizobia and their endosymbiotic counterparts, the latter are called bacteroids. At this stage nitrogen fixation starts and the ammonia produced is exported to the plant. Both in the bacterium (Krol *et al.*, 1980, Prakash, 1981 and this thesis) and in the plant (Legocki and Verma, 1980) a number of genes are only expressed in the symbiotic state. One of the nodule-specific plant proteins is leghemoglobin, which is very abundant in root nodules. The function of leghemoglobin in protecting nitrogenase against oxygen was mentioned above. Other nodule-specific proteins, the so-called nodulins, were detected by Bisseling (to be published). The function of the nodulins is still unknown. That a number of proteins from plant and from bacterium are only present in nodules indicates that the formation of an effective symbiosis is achieved both by the differentiation of plant cells into nodule tissue as well as by differentiation of bacteria into bacteroids.

1.3 INVOLVEMENT OF *RHIZOBIUM* PLASMIDS IN SYMBIOSIS AND NITROGEN FIXATION

Rhizobium contains large plasmids, as was first demonstrated by Nuti *et al.*, (1977). Because fast growing *Rhizobium* species are closely related to *Agrobacterium tumefaciens*, the plasmids in *Rhizobium* drew special attention. For *Agrobacterium tumefaciens* induces Crown Gall tumors on plants by transfer of a part of a large plasmid to a plant cell, where it becomes incorporated in the plant genome (Drummont *et al.*, 1977). In the relation between *Rhizobium* and a plant a plasmid might function in a similar way, but untill now no transfer of bacterial DNA to the plant has been demonstrated. Nuti *et al.* (1977) showed the occurrence of large plasmids in *Rhizobium leguminosarum* (2 strains), *Rhizobium trifolii* (2 strains), *Rhizobium japonicum* (1 strain) and *Rhizobium* "dolichos" (1 strain). By conjugation experiments Johnston *et al.* (1978) found indications for the invqlvement of a large plasmid in nodule formation. Nuti *et al.* (1979) demonstrated that at least a part of the *nif* (nitrogen fixation) genes are located on a large plasmid in *Rhizobium leguminosarum* by hybridization with pSA30, a recombinant plasmid containing the structural *nif* genes of *Klebsiella pneumoniae* (see section 4). Conjugation experiments using *Rhizobium leguminosarum* demonstrated that genes controlling nodulation and genes coding for a hydrogenase, which apparently improves the efficienty of nitrogen fixation, are located on a single plasmid (Brewin *et al.* (1980a). Also genes coding for bacteriocin production, which might be important during colonization of the root hair, appeared to be linked to the nodulating properties of *Rhizobium leguminosarum* and were located on a single *nod* plasmid (Brewin *et al.*, 1980b). Using deletion mutants Dénarié *et al.* (1981) demonstrated that genes controlling nodule formation are located on a large plasmid in *Rhizobium meliloti*. By hybridization with pSA30 the *nif* genes were also located on this plasmid. Hooykaas *et al.*, (1981) transferred a plasmid from *Rhizobium trifolii* to *Rhizobium leguminosarum* and to *Agrobacterium tumefaciens*. In these experiments it was shown that this plasmid contained genes for host specificity and nodule formation, as both transconjugants nodulated *Trifolium*. By transfer to a fix$^-$ (not fixing) strain of *Rhizobium leguminosarum* which lacked a part of the structural *nif*

genes this strain was rendered fix^+, but the *Agrobacterium* transconjugant was fix^-. So at least a part of the *nif* genes are located on this plasmid, but some genes involved in nitrogen fixation are either not expressed in *Agrobacterium* or are located either on the chromosome or on another plasmid. The last possibility is not likely, because hybridization experiments of bacteroid RNA with the two plasmids present in *Rhizobium leguminosarum* strain PRE showed only hybridization with the plasmid carrying the *nif* genes (Krol *et al.*, 1982 and this thesis). As there are strong indications that all bacterial genes involved in nodule formation and nitrogen fixation are located on a single plasmid, Hooykaas *et al.* (1981) proposed to call these plasmids *Sym* plasmids.

It has been shown that host specificity, which has been used to classify *Rhizobium* species (Vincent, 1974) can be transferred to other *Rhizobium* species (Johnston *et al.*, 1978) and even to *Agrobacterium* (Hooykaas *et al.*, 1981). On the other hand transfer of a plasmid from *Agrobacterium tumefaciens* to *Rhizobium trifolii* rendered the transconjugant Crown Gall inducing (Hooykaas, 1979). So symbiotic or phytopathogenic properties and host range depend on the plasmid content of the bacterium and are therefore not species determined. This led Hooykaas (1979) to propose a new classification of *Rhizobium* species based on a number of other mostly metabolic properties (Table 2). The classification and host range determination of the slow growing *Rhizobium* species are still very incomplete. As the old classification is still generally in use, the older nomenclature also has been used in this thesis.

1.4 ORGANISATION AND REGULATION OF *NIF* GENES IN *RHIZOBIUM* and *KLEBSIELLA*

The large plasmids found in *Rhizobium* carry at least a part of the genes involved in nitrogen fixation (Nuti *et al.*, 1979). Prakash (1981) made a physical map of such a *nif* plasmid from *Rhizobium leguminosarum*. On this map he localized regions which are expressed in bacteroids and in stationary phase bacteria, and regions which are homologous to the Ti plasmid of *Agrobacterium tumefaciens*. The precise organisation of the *nif* genes in *Rhizobium* is difficult to assess. In most strains nitrogenase is only expressed in the sym-

Table 2 Classification of *Rhizobium* species as proposed by Hooykaas (1979)

Genus	Species	Former name and plasmid mediated phenotype	
1. *Rhizobium* (fast growing, generation time 2-4h, acid producing)	A. *Agrobacterium* (several biotypes)	*A. tumefaciens*	Crown gall induction
		A. rubi	Cane gall induction
		A. rhizogenes	Hairy root induction
	B. *Rhizobium meliloti*	*R. meliloti*	Nodule formation on *Meliloti, Medicago, Trigonella*
	C. *Rhizobium leguminosarum*	R. leguminosarum	Nodule formation on *Pisum, Lathyrus, Vicia, Lens, Cicer*
		R. trifolii	Nodule formation on *Trifolium*
		R. phaseoli	Nodule formation on *Phaseolus*
2. *Phytomyxa* (slow growing, generation time 6-10h, alkali producing		*R. japonicum*	Nodule formation on *Glycine*
		R. lupinii	Nodule formation on *Lupinus*

biotic state. Some strains are able to fix nitrogen in a free living state (Kurz and La Rue, 1975, McComb and Dilworth, 1975, Pagan *et al.*, 1975), but these strains do so only in the presence of fixed nitrogen. Therefore it is not possible to select mutants which are defective in nitrogenase using selective growth conditions; all mutants must be individually tested. A genetic map of the *nif* genes of *Rhizobium* has untill now not been constructed.

The free living nitrogen fixing bacterium *Klebsiella pneumoniae* is much better known. *Klebsiella pneumoniae* is closely related to *Escherichia coli*. Therefore genetic techniques developed for *Escherichia coli* could be applied to *Klebsiella pneumoniae* to map the genes involved in nitrogen fixation (MacNeil et al., 1978). The genes are located in a single region on the chromosome, approximately 24 kb in size. The *nif* genes and the function of the products are listed in table 3 (Kennedy *et al.*, 1981). The *nif* genes are organized in 7 or 8 operons, which are all transcribed in the same direction as indicated in the table by the arrows. Most operons code for several proteins. The operons HDKY and LA code for related proteins of which the synthesis is probably coordinately regulated. The HDKY operon, coding for the subunits of nitrogenase, is of special interest. This operon has been cloned on a 6.9 kb fragment in the recombinant plasmid pSA30 (Cannon *et al.*, 1979), which has been used in chapter 3, 4 and 5 of this thesis.

The expression of *nif* genes of *Klebsiella pneumoniae* is subjected to different levels of control. As indicated in section 1 nitrogen fixation demands much energy in the form of ATP. This makes that the nitrogenase genes of *Klebsiella pneumoniae* are strongly repressed when sufficient fixed nitrogen is present. Also in the presence of oxygen the nitrogenase genes are repressed because oxygen inactivates nitrogenase. A key role in the regulation of amino acids is given to the enzyme glutamine synthetase. As is demonstrated by mutations in the glutamine synthetase genes A, G and F, this enzyme also mediates nitrogen starvation to the *nif* cluster (Leonardo and Goldberg, 1980). It induces the expression of the *nif* LA operon, which in turn regulates the expression of the other operons of the *nif* region. The *nif* A product is a positive activator of all other *nif* operons. On the contrary the *nif* L product prevents expression of the other *nif* operons if ammonia or oxygen are present. From both

Table 3: The *nif* genes of *K.pneumoniae* and their products. (Kennedy *et al*, 1981).

Kilobase DNA	Operon	Gene	M.W. of product	Function of product
0		Q	unknown	Unknown
		B	unknown	Essential for active FeMo-cofactor
		A	60.000 57.000	Activates the expression of all other *nif* genes.
4		L	50.000 45.000	Inhibits the expression of all other *nif* genes.
		F	17.000 10.000 20-22.000	Electron transfer to nitrogenase
		M	28.000	Activation of Kp2
8		V	42.000	Modifies substrate specificity of Kp1
		S	18.000 45.000	Unknown
		U	22.000 25/32.000(doubled)	Unknown
		X	18.000	Unknown
		N	50.000	Essential for active FeMo-cofactor
12		E	46.000 40.000	Essential for active FeMo-cofactor
		Y	24.000 19.000	Unknown
		K	60.000	Component 1 β-subunit
16		D	56.000 60.000	Component 1 α-subunit
		H	35.000 35/39.000(doubled) 31.753	Component 2 subunit
20		J	120.000	Electron transfer to nitrogenase

the *nif* A protein and the *nif* L protein the function is concluded from genetic experiments, but the precise biochemical action of the proteins is far from understood. A further regulation of *nif* genes is observed in the expression of the HDKY operon. Dixon *et al.* (1980) demonstrated that molybdenum, in combination with one or more products of the HDKY operon, increased the expression of this operon sevenfold. Presumably intact component I, which is composed of the products of the genes D and K and contains the FeMo cofactor, is involved in the autogenous regulation of this operon. If molybdenum is absent the synthesis of the subunits of nitrogenase is reduced to prevent the formation of an inactive nitrogenase.

In *Rhizobium* the regulation of the nitrogenase genes is not yet understood. A difference between *Rhizobium* and *Klebsiella* in the regulation of the *nif* genes is that in *Rhizobium* fixed nitrogen is apparently not repressing. The strains which are able to fix nitrogen in a free living state do so in the presence of fixed nitrogen, and bacteroids export a large part of their fixed nitrogen to the plant. Laane *et al.*, (1980) demonstrated that bacteroids excrete methylammonium as a result of a ΔpH across the cytoplasmic membrane. ^{14}C-labeled methylammonium was used as an analogue of ammonium; methylammonium and ammonium are supposed to react similarly in respons to the ΔpH. Ludwig (1980) found an increased ammonia export from free living *Rhizobium* under microaerobic conditions. The export of ammonia from the bacteroids might result in a low level of ammonia in the cell. This decreases the degree of adenylylation of glutamine synthetase and in this form this enzyme might induce the nitrogenase genes (Ludwig, 1980). Involvement of genes analogous to *nif* A or *nif* L of *Klebsiella pneumoniae* is still unknown, but possibly also *Rhizobium* possesses a *nif* A protein. *Rhizobium* "cowpea" strain CB756 and *Rhizobium japonicum* strain 32H1 develop a pink colour in the presence of 6-cyanopurine, an activity which is associated with *nif* A in *Klebsiella pneumoniae* (Kennedy *et al.*, 1981).

Experiments on the expression of the nitrogenase genes were done by Bisseling (1980). Radioimmunoassays showed that during the onset of nitrogen fixation in root nodules component I was synthesized before component II. Reducing the oxygen tension by placing the nodu-

les under water (waterlogging) resulted in the repression of the synthesis of component II, but not of component I. This resembles the autogenous regulation of nitrogenase in *Klebsiella pneumoniae*, where component I is supposed to regulate the expression of the nitrogenase operon HDKY. But in *Klebsiella pneumoniae* we were not able to detect component I before component II during the derepression of the *nif* genes. That component I from *Rhizobium* is synthesized independently from component II suggests an organization in different operons, or a selective translation of messenger RNA. By hybridization experiments of blotted bacteroid RNA with pSA30 it was shown that in *Rhizobium* the *nif* genes D and H are presumably located on one messenger (this thesis). This implicates that selective translation of this messenger may occur.

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II ISOLATION AND CHARACTERIZATION OF THE PLASMIDS OF *RHIZOBIUM LEGUMINOSARUM* STRAIN PRE

2.1 INTRODUCTION

In this chapter our experiences with different procedures for the isolation of large plasmids from *Rhizobium leguminosarum* are described. Agarose gel electrophoresis demonstrated that *Rhizobium leguminosarum* strain PRE contains two plasmids of approximate molecular weights of 230 x 10^6 and 570 x 10^6 respectively. Reannealing experiments and electron microscopic measurements confirmed the value of the molecular weight of the smaller plasmid. The difference in buoyant density between plasmid DNA and chromosomal DNA which was found by Spitzbart *et al.* (1979) for *Rhizobium meliloti* strain 20-1 and 20-2 was confirmed for the plasmid DNA of strain PRE. This difference in density could not be used to isolate plasmid DNA on a large scale.

2.2 MATERIALS AND METHODS

2.2.1 *Cultivation of bacteria*

Rhizobium leguminosarum strain PRE was cultured in medium containing 5 g bactotryptone (Difco, Detroit, Michigan, U.S.A.), 3 g yeast extract (Difco) and 10 mg uracil in 1 l demineralized water. After sterilization for 15 min at 120°C 0.13% Ca $(NO_3)_2$ was added. This medium will be referred to as TY medium. Bacteria were grown at 29°C. When the culture had reached an optical density of 0.15 - 0.20 at 660 nm, which corresponds to the mid-logarithmic growth phase of the culture, cells were collected by centrifugation at 16,000 x g for 10 min and resuspended in buffer containing 0.05 M Tris-HCl 0.02 M EDTA, pH = 8.0 (TE buffer). For labeling of DNA bacteria were cultured in 10 ml TY medium containing 100 µCi ^{3}H Thymidine. Bacteria were grown for 16 h at 29°C till the culture had reached an optical density of 0.15 - 0.20 at 660 nm. This

corresponds to approximately five generations of growth.

2.2.2 *Alkaline sucrose gradient centrifugation of total cellular DNA* (Ledeboer *et al.*, 1976)

^{3}H Thymidine labeled bacteria from a 10 ml culture were pelleted by centrifugation at 16,000 x g for 10 min and resuspended in 1 ml TE buffer. Cells were lysed by addition of SDS (sodium dodecylsulfate) to 1% (w/v). The lysate was made 0.3 M NaOH and sheared for 60 sec on a Vortex mixer at maximum speed. After centrifugation at 12,000 x g for 10 min the lysate was loaded on a 5 - 20% sucrose gradient in a solution containing 0.05 M Tris, 0.02 M EDTA, 0.3 M NaOH, 1 M NaCl and centrifuged at 40,000 rpm for 16 min in a Beckman SW41 rotor. Fractions of 10 drops were collected through a hole punctured in the bottom of the tube. Aliquots of 50 µl were spotted on Whatman 3MM paper, the DNA was precipitated in a solution containing 5% (w/v) TCA (trichloroacetic acid) and 2% (w/v) sodium pyrophosphate in water. The filters were washed in a solution of 2% (w/v) TCA and 2% (w/v) sodium pyrophosphate and radioactivity was assayed in 8 ml instafluor (Packard) in a liquid scintilation counter. The counting efficiency for tritium was 10 - 20%.

2.2.3 *Renaturation kinetics* (Ledeboer *et al.*, 1976)

Fractions containing ^{3}H-labeled plasmid and chromosomal DNA were pooled from an alkaline sucrose gradient (Fig. 1) and dialysed against distilled water. The DNA was sonified in a Sonifier celldisruptor (Branson Sonic Power Co., U.S.A.), at maximal output, 50% duty, for 30 sec. After sonification the water was evaporated *in vacuo* and the DNA was dissolved in 0.12 M sodium phosphate buffer, pH 6.8, to a concentration of approximately 1 µg/ml for plasmid DNA and 30 µg/ml for chromosomal DNA. Samples of 1-5 µl were taken into micropipettes (Brand, Germany) which were sealed by melting both ends. The DNA in the micropipettes was denatured at 100°C for 10 min, and then the micropipettes were transferred to a waterbath of 66°C for renaturation of the DNA. DNA samples were taken at t = o and further at regular intervals, every interval being approximately twice as long as the preceeding interval. The last sample was taken at 48 h. Each

time the DNA samples were diluted to 1 ml with 0.15 M sodium phosphate buffer and applied to a 0.25 ml hydroxylapatite (Biorad, U.S.A.) column in a 2 ml syringe at 60°C. The column was washed two times with 1 ml 0.15 M sodium phosphate buffer; all three ml's were collected in the same vial. Then the column was washed two times with 1 ml 0.3 M sodium phosphate buffer and the effluent was collected in another vial. The contents of both vials were filled up to 4 ml with a final concentration of 0.15 M sodium phosphate buffer, 16 ml hydroluma (Packard) were added and the radioactivity in the samples was assayed in a liquid scintillation counter.

2.2.4 *Isolation of bacterial DNA enriched in plasmid DNA*

Three different methods were used to isolate DNA enriched in plasmid DNA:

1) The method as described by Currier and Nester (1976).
Bacteria from 1 l of culture were resuspended in 1 l TE and lysed by addition of SDS to 1% (w/v) and proteinase K (Merck, Darmstadt F.R.G., preincubated for 1 h at 37°C) to 10 µg/ml. After incubation for 20 min at room temperature the lysate was made pH 12.1 - 12.3 by dropwise addition of 3M NaOH, under gently stirring. After 20 min. incubation at room temperature the pH was lowered to pH 8.5 by dropwise addition of a solution of 2 M Tris - HCl pH 7.0. Solid NaCl was dissolved to a final concentration of 3% (w/v) and the lysate was extracted once with 1 vol of phenol/cresol (phenol/cresol = phenol: m-cresol: hydroxyquinoline = 1000 : 140 : 0.4 (w/w/w)), and once with chloroform. DNA enriched in plasmid DNA, which will be referred to as crude plasmid DNA, was precipitated from the aqueous layer by addition of 1 vol of ethanol and the mixture was kept at -20°C for 2 h.

2) A modification of the method described by Hansen and Ohlson (1978).
A lysate was prepared, titrated with NaOH and neutralized as described for the method of Currier and Nester. The lysate was made 1 M NaCl from a 5 M stock solution, kept at 0°C for 5 h and then precipitated material was removed by centrifugation at 16,000 x g for 15 min.

Floating material was removed with a strip of Whatman 3 MM paper and to the remaining supernatant a 50% solution (w/v) of polyethylene glycol 6000 in water was added to a final concentration of 10% to precipitate plasmid DNA. After 16 h at 0°C crude plasmid DNA was collected by centrifugation at 16,000 x g for 10 min.

3) The method as described by Casse *et al.*, 1979.
Bacteria from 1 l of culture were resuspended in 5 ml of water and the slurry was added to 95 ml of TE, previously made 1% SDS (w/v) and adjusted to pH 12.4 a short time before the addition of the cells. The suspension was kept at room temperature for 30 min to lyse the cells, and then 15 ml 2M Tris-HCL pH 7.0 were added dropwise, followed by 25 ml 5M NaCL and 100 ml phenol/cresol, all dropwise without stirring. After addition of the phenol/cresol the mixture was stirred very gently using a glas rod and then centrifuged at 16,000 x g for 10 min. The aqueous layer was removed and mixed with 1 volume of ethanol. After 16 h at -20°C crude plasmid DNA was collected by centrifugation at 16,000 x g for 10 min.

2.2.5 *CsCl-ethidium bromide gradients*

Crude plasmid DNA from 1 l of culture was dissolved in 8.5 ml of TE buffer, 9 g CsCl and 0.5 ml ethidium bromide (10 mg/ml) were added and centrifugation was performed in a 75 Ti rotor (Spinco) at 30,000 rpm for 64 h at 20°C. Crude plasmid DNA prepared by the method of Hansen and Ohlson was dissolved in 8.5 ml TE buffer containing 0.5% diethylpyrocarbonate. After centrifugation the DNA bands were visualised with long wavelength U.V. light (300 nm) from a C63 transilluminator (U.V. products Inc., San Gabriel, California, U.S.A.) and the lower band containing plasmid DNA was sucked into a syringe through a hole punctured in the side of tube. The DNA collected from the gradients was diluted with 1 vol of water and precipitated with 2 volumes of ethanol.

2.2.6 *Agarose gel electrophoresis*

Crude plasmid DNA from 0.5 l bacterial culture was dissolved in 0.5 ml TE buffer. This was mixed with 50 µl 50% Ficoll (w/v) (containing xylene cyanol FF as a tracking dye) and samples of 20 µl per slot were loaded onto a horizontal 0.6% agarose gel in 89 mM Tris 89 mM EDTA pH 8.0 (Meyers *et al.*, 1976). The gel was submerged in the same buffer and the DNA was separated by electrophoresis at 6 V/cm for 16 h. After staining with ethidium bromide (0.5 µg/ml) the separated DNA bands were visualised with long wavelength U.V. light and photographed using an orange filter.

2.2.7 *Electron microscopy*

Plasmid DNA isolated by CsCl-ethidium bromide centrifugation was dialysed against a suspension of 10 g Dowex X50 in 50 ml 0.8 M NaCl, 0.01 M EDTA, 0.05 M Tris-HCl pH 8.0, to remove the ethidium bromide (Thompson *et al.*, 1974). After dialysis against 100 mM Tris-HCl pH 8.5 10 mM EDTA the plasmid DNA solution was made 50% formamide and 0.2 mg/ml cytochrome C (Davis *et al.*, 1971). 0.5 µg/ml ØX 174 DNA was added as an internal standard. The DNA was spread on water, picked up on a grid, stained with 0.125 mM uranyl acetate, shadowed with platinum-iridium and examined in an Elmiskoop electron microscope from Siemens.

2.2.8 *Buoyant density*

Plasmid DNA purified by CsCl-ethidium bromide centrifugation was ethanol precipitated, dissolved in TE buffer and then 1.30 g CsCl per ml were added. The solution was centrifuged in a Beckman 75 Ti rotor at 30,000 rpm at 20°C for 64 h. The tube was punctured at the bottom and fractions of 10 drops were collected. The density profile of the gradient was determined by measuring the refraction index of every fifth fraction, using density (25°C) = (10.8601 x refraction index) - 13.4974 (Ifft *et al.*, 1961). The DNA in each fraction was stained by the addition of ethidium bromide to a final concentration of approximately 0.5 µl/ml and visualised with long wavelength U.V. light.

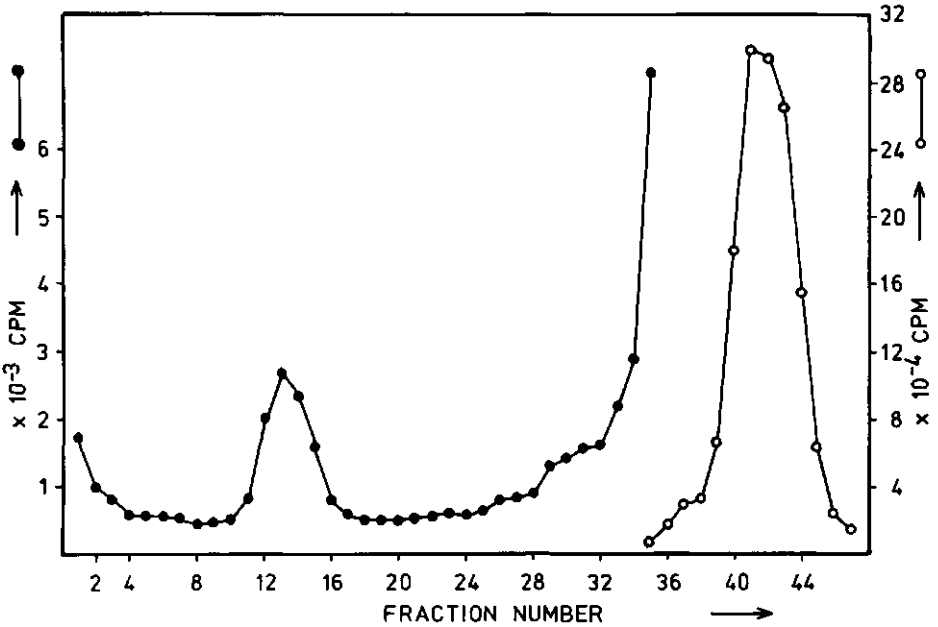

Fig 1. Alkaline sucrose gradient.
^{3}H-Thymidine labeled DNA in a lysate of Rhizobium leguminosarum strain PRE was sheared by mixing vigorously for 60 sec and loaded on a 5-20% alkaline sucrose gradient. After centrifugation at 200,00 x g for 16 min fractions containing 10 drops were collected after puncturing the bottom of the tube and the radioactivity in each fraction was determined. Fractions 11-16 were pooled as plasmid DNA, fractions 40-43 as chromosomal DNA.

2.3 RESULTS

2.3.1 *Alkaline sucrose gradients (Fig. 1)*

If a ^{3}H-Thymidine labeled lysate of *Rhizobium leguminosarum* PRE bacteria is applied to a 5-20% alkaline sucrose gradient and centrifuged at 16,000 x g for 16 min a radioactivity profile as shown in Fig. 1 is obtained. The fractions 11-16 represent plasmid DNA, as will be demonstrated below. Because it is necessary to shear the DNA to achieve a good separation of plasmid DNA and chromosomal DNA, the yield of plasmid DNA was low and amounted to only 0.5% of the total amount of DNA, which corresponds with 60 ng plasmid DNA per gradient.

2.3.2 *Renaturation kinetics*

^{3}H-Thymidine labeled plasmid DNA and chromosomal DNA isolated by alkaline sucrose gradient centrifugation were broken by sonification to fragments of approximately 900 bases in lenght. The solution of plasmid DNA had a concentration of 1 µg/ml and that of chromosomal DNA a concentration of 30 µg/ml. These concentrations will be referred to as Co for each of the DNA preparations respectively. The DNA was heated at 100°C to separate the two bands and allowed to reanneal at 66°C. After different periods of reannealing a sample was taken and analysed on a hydroxylapatite column. The DNA eluting at 0.15 M sodium phosphate was regarded to be single stranded. The amount of single stranded DNA eluting from the column was expressed as a percentage of the total amount of DNA in the sample applied to the column and plotted against Cot. This is the initial DNA concentration Co multiplied by the incubation time of the sample. The experimental values for the renaturation of the chromosomal DNA and the plasmid DNA fitted to a curve representing second order kinitics

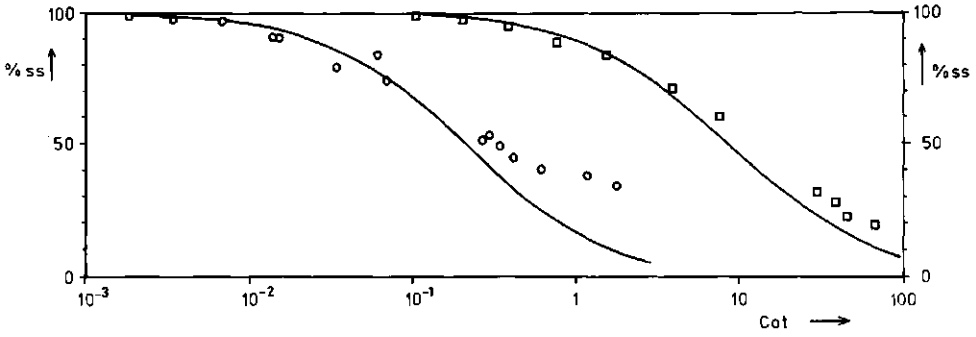

Fig. 2. Renaturation kinetics.
Chromosomal DNA (¤) or plasmid DNA (0) was melted and allowed to reanneal at 66°C in 0.12 M sodium phosphate buffer. At regular times t a sample was taken and the percentage single stranded (%SS) DNA was determined. This value was plotted as a function of the initial DNA concentration multiplied by the time (Cot), expressed as mol nucleotides per liter x time in sec. The drawn curves represent ideal second order kinetics.

as is shown by the drawn lines in Fig. 2. In drawing these lines it was assumed that in the samples taken at longer incubation times

the percentage of single stranded DNA is overestimated due to degradation of DNA. In the course of the experiment some DNA is degraded as a result of the high temperature, and this DNA will be eluted in the single stranded DNA fraction. The value of Cot at which 50% of the chromosomal DNA has reannealed ($Cot_{\frac{1}{2}}$) is approximately 29 times higher than the value of $Cot_{\frac{1}{2}}$ for plasmid DNA. Based on a value of 6×10^9 for the molecular weight of chromosomal DNA (Reynders *et al.*, 1975, Bisseling *et al.*, 1977) a value of 207×10^6 can be calculated for the molecular weight of plasmid DNA.

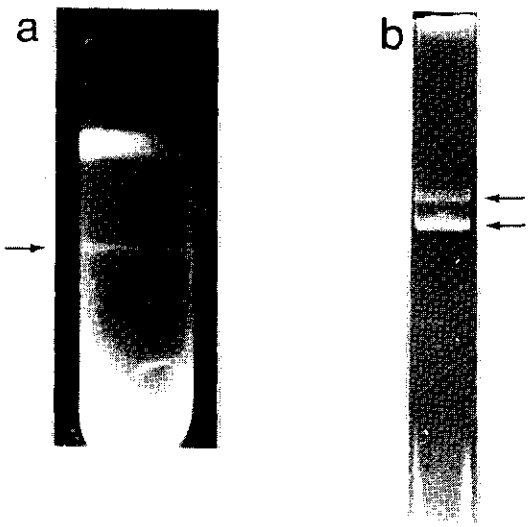

Fig. 3. Purification of plasmid DNA.
Bacterial DNA enriched in plasmid DNA (see text) was dissolved in buffer and centrifuged in a CsCl-ethidium bromide gradient (a) or electrophoresed in an agarose gel (b). The DNA was visualised with long wave-lenght U.V. light. The arrows indicate the position of circular supertwisted plasmid DNA.

2.3.3 *Isolation of plasmid DNA on a large scale*

Plasmid DNA was isolated on a large scale by a procedure producing DNA enriched in plasmid DNA, followed by further purification by CsCl-ethidium bromide gradient centrifugation. The methods used were essentially those described by Currier and Nester (1976), Hansen and Ohlsen (1978) and Casse *et al.* (1979), which are based on denaturation of the chromosomal DNA at pH 12.1 - 12.4, followed by

neutralisation which results in precipitation of the chromosomal DNA. The DNA remaining in the supernatant is enriched in plasmid DNA and can be concentrated by precipitation with polyethylene glycol or ethanol. The plasmid DNA was further purified by centrifugation in a CsCl-ethidium bromide gradient (Fig. 3a) or agarose gel electrophorises was performed to separate the plasmids (Fig. 3b). With all three methods to isolate plasmid DNA the yields of purified plasmid DNA ranged between 0 and 25 µg after CsCl-ethidium bromide centrifugation.

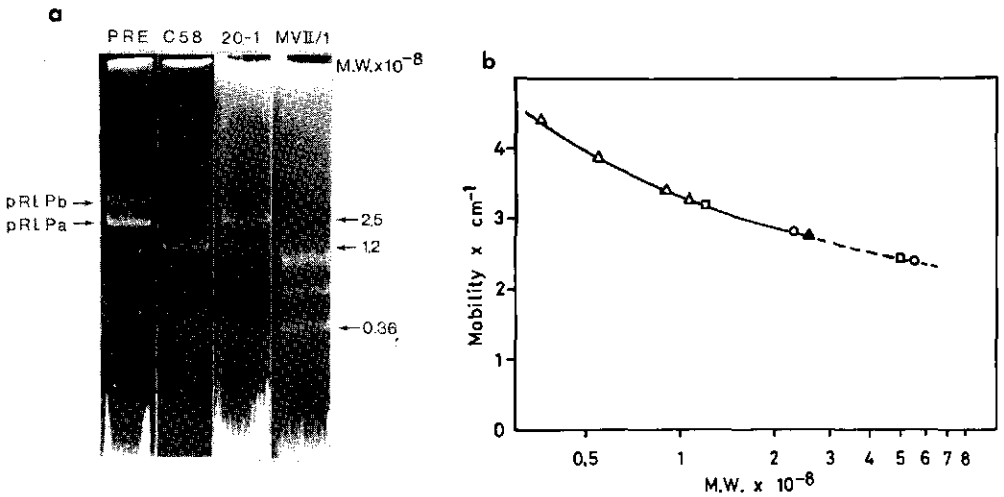

Fig. 4. Molecular weight estimation of plasmid DNA by agarose gel electrophoresis. Plasmid DNA from Rhizobium leguminosarum strain PRE, Agrobacterium tumefaciens strain C58 and Rhizobium meliloti strain 20-1 and strain MVII/3 were separated in a 0.6% agarose gel and stained with ethidium bromide, as shown in Fig. 4a. The two plasmids of PRE are referred to as pR1Pa and pR1Pb. The molecular weights of the marker molecules are: 2.5×10^8 (20-1), 1.2×10^8 (Ti plasmid of C58) and 1.04×10^8, $0.90 \times 10.^8$, 0.54×10^8 and 0.36×10^8 (MVII/1). The mobility of the marker plasmids was plotted against the logarithm of their molecular weight (Fig. 4b). From the mobility of the plasmids of PRE their molecular weight was estimated; for pR1Pb it was necessary to extrapolate the figure (broken line). O: PRE, □: C58, ▲: C20-1, Δ: MVII/1.

in a CsCl gradient without ethidium bromide. The gradient was fractionated and the density profile was determined by measuring the refraction index of every fifth fraction (Ifft *et al.*, 1961). The position of the DNA was determined by U.V. fluorescence after addition of ethidium bromide to every fraction. The buoyant density of the plasmid DNA was found to be 1.717 g/cm^3, compared with 1.722 g/cm^3 for chromosomal DNA centrifuged simultaneously in a separate gradient. The density of plasmid DNA corresponds to a G-C content of 56% compared with 63% for chromosomal DNA. Similar values were found by Spitzbart *et al.* (1979). To investigate if the difference in buoyant density between plasmid DNA and chromosomal DNA could be used for the isolation of plasmid DNA on a large scale, crude plasmid DNA from 1 l of culture was centrifuged in a CsCl gradient containing 0.5 µg/ml ethidium bromide to visualize the DNA. This is less than 1/1000 of the amount used in CsCl ethidium bromide gradients and not sufficient to saturate the DNA. Therefore the difference in buoyant density between plasmid and chromosomal DNA will not change very much. As shown in Fig. 6 plasmid DNA bands closely above the chromosomal DNA and it was not possible to remove it from the gradient without contamination with chromosomal DNA.

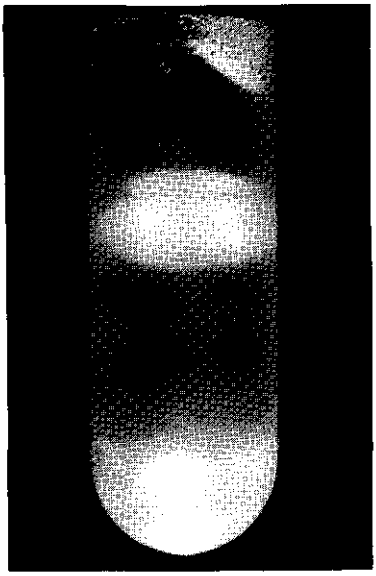

Fig. 6. Density centrifugation of plasmid DNA.
Bacterial DNA, enriched in plasmid DNA was centrifuged in a CsCl gradient containing 0.5 µg/ml ethidium bromide to stain the DNA. The upper band is plasmid DNA, as indicated in the text.

2.4 DISCUSSION

In the beginning of our study on *Rhizobium* plasmids we used alkaline sucrose gradients according to Nuti *et al.* (1977) to demonstrate the occurrence of plasmid DNA. From these gradients small amounts of radioactively labeled plasmid DNA could be isolated which were used for molecular weight estimations by renaturation kinetics. Later agarose gel electrophoresis was used to separate plasmid DNA (Casse *et al.*, 1979), giving a better molecular weight estimation by comparison with marker molecules. The amount of plasmid DNA which can be isolated by alkaline sucrose gradient centrifugation is too small for restriction enzyme analysis or hybridization experiments. Therefore procedures were developed to isolate plasmid DNA on a larger scale. As a first step in purification a DNA fraction enriched in plasmid DNA was isolated, because covalently closed circular plasmid DNA represents only a small part of the total amount of DNA in the cell. To prepare DNA enriched in plasmid DNA the methods described by Currier and Nester (1976), Hansen and Ohlsen (1978) and Casse *et al.* (1979), respectively, were used. All three methods are based on denaturation of DNA at pH 12.1 - 12.4. The strands of intact circular plasmid DNA cannot separate completely and will again form a double stranded molecule upon rapid neutralization. Currier and Nester and Hansen and Ohlsen titrate the lysate with NaOH to pH 12.1 - 12.3. To avoid locally high pH's when the NaOH is added dropwise stirring is essential. If the bacterial cells are suspended and lysed in a small volume of buffer a very viscous lysate is obtained, making stirring difficult. By adding the bacteria in a small volume of water directly to a much larger volume of buffer of pH 12.4 stirring can be avoided (Casse *et al.*, 1979). After neutralization and addition of NaCl, which is the same in all three procedures, the viscosity of the lysate is much reduced because the denatured chromosomal DNA precipitates. In the method of Hansen and Ohlsen the lysate is placed at 0°C, which causes SDS and proteins to precipitate. At the same time chromosomal DNA, SDS and proteins are removed by centrifugation, after which plasmid DNA is precipitated by complexation with polyethylene glycol. This precipitate contains beside DNA also a large amount of proteins and polyethylene glycol. To avoid this contamination is it preferable to precipitate

plasmid DNA with ethanol after extraction of the lysate with phenol/cresol (Currier and Nester, 1976, Casse *et al.*, 1979). After precipitation plasmid DNA was further purified by CsCl-ethidium bromide centrifugation (Fig. 3a), or electrophoresed in an agarose gel (Fig. 3b). It has remained difficult to isolate the large plasmids from *Rhizobium leguminosarum* strain PRE using each of the three methods. The most important problem in isolating large plasmid DNA is the stirring of the lysate. It is important to achieve a good mixture with as little stirring as possible, not only to avoid shearing of plasmid DNA, but also to keep the chromosomal DNA as long as possible. If the chromosomal DNA is too short, it will not precipitate upon neutralization and may cause overloading of the CsCl-ethidium bromide gradient if the amount of DNA applied to the gradient is too large. Therefore the method as described by Casse *et al.* (1979) appears the most suitable because it caused less shearing of the DNA, it is less labourious and it uses less material. With this method it was possible to isolate plasmids with a molecular weight up to 600 x 10^6 from different *Rhizobium leguminosarum* strains as is described in Chapter 4, but many isolations did not produce plasmid DNA at all.

If plasmid DNA from *Rhizobium leguminosarum* is separated by agarose gel electrophoresis two plasmid bands are shown (Fig. 4). From this gel a value of 230 x 10^6 is found for the molecular weight of pRlPa. This value was also found by electron microscopic measurements, whereas by renaturation kinetics a value of 207 x 10^6 was obtained. The values for the molecular weight of plasmid DNA obtained by renaturation kinetics showed a large variation with 90 x 10^6 and 480 x 10^6 as extremes. This indicates that these values are not very precise. That pRlPb is not detected in CsCl-ethidium bromide gradients using electron microscopy or in alkaline sucrose gradients using renaturation kinetics might imply that the band indicated with pRlPb in an agarose gel is a dimer or an open circular or linear form of pRlPa. This is not the case, as will be demonstrated in Chapter 4. It appears probable that pRlPb is lost in alkaline sucrose gradients or CsCl gradients due to shear forces during the

isolation. That pRlPb is not isolated using these gradients indicates the increasing difficulty to isolate such large DNA molecules by these methods. The value of 570 x 10^6 for pRlPb is a rough estimation, because no accurate molecular weight markers are available in this range. A difference of 20% with the real molecular weight should be considered. We have found no indications for the occurence of still larger plasmids in PRE, like the plasmids with a molecular weight of more than 800 x 10^6 which were found in *Rhizobium meliloti* by Dénarié *et al.* (1981).

As the plasmid band in a CsCl-ethidium bromide gradient consists for a large part of pRlPa, we determined only the buoyant density of pRlPa. The difference in buoyant density between plasmid DNA and chromosomal DNA means a difference in G-C content of 7%. The reason for this difference is unknown.

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III EXPRESSION OF LARGE PLASMIDS IN THE ENDOSYMBIOTIC FORM OF *RHIZOBIUM LEGUMINOSARUM*

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ABSTRACT

Isolated plasmid DNA from *Rhizobium leguminosarum* was hybridised with cellular RNA from broth-cultured bacteria and endosymbiotic bacteroids. From these hybridisation experiments it is concluded that plasmid genes are strongly expressed in bacteroids and only weakly or not at all in bacteria. From the hybridisation of plasmid DNA with the cloned structural *nif* genes of *Klebsiella pneumoniae* it is shown that at least part of the *nif* genes are located on a plasmid.

INTRODUCTION

Upon the transition of free living *Rhizobium leguminosarum* into nitrogen fixing bacteroids in root nodules, marked changes occur in the morphology, DNA content and the patterns of protein synthesis in the cells (1, 2). Since the presence of large plasmids (M.W. 100 - 250 x 10^6) in different rhizobium species has been demonstrated by several authors (3 - 6), these plasmids have been attributed a key role in the infectivity of the Rhizobium bacteria, the induction of root nodules and the achievement of nitrogen fixation. Evidence for such a role of plasmids has been found in genetic experiments (7, 8), demonstrating that genes involved in host specific nodulation are located on a large plasmid, and in biochemical experiments (9, 10) which indicate that the structural genes for nitrogenase are at least partly plasmid borne.

This paper describes experiments to examine the extent to which plasmid genes are transcribed in free-living rhizobia or in bacteroids. For that purpose RNA from bacteria or bacteroids was hybridized to restriction enzyme-digested plasmid DNA using the Southern blotting technique (11). The results show that the large plasmids are actively transcribed in the bacteroid form, whereas transcription of plasmid genes remains undetectable in broth-cultured bacteria. The location of *nif* genes on the rhizobium plasmids was demonstrated by using cloned *Klebsiella pneumoniae nif*-sequences (12) as a probe.

MATERIALS AND METHODS

Cultivation of Rhizobium bacteria and bacteroids. Rhizobium leguminosarum (strain PRE) was grown in TY medium (5 g bactotryptone, Difco, Detroit Michigan U.S.A., 3 g yeast extract, Difco, 10 mg uracil in 1 l, plus separately sterilized 10 ml 13% $Ca(NO_3)_2$) at 30°C to A_{660}= 0.15 - 0.20. The bacteria were pelleted by centrifugation for 10 min at 16,000 x*g*.

Pea plants were grown in gravel as described by Bisseling *et al.*(13). Nodules from 20 day old plants were picked and immediately frozen in liquid nitrogen; 25 g of nodules were crushed in a cold mortar in 50 ml of cold 0.15 M sucrose, 25 mM Tris-HCl pH 7.4, 100 mg/l chloramphenicol (Sigma, St.Louis, MO, U.S.A.), and filtered through Miracloth. Bacteroids were pelleted by centrifugation for 7 min at 27,000 x*g* prior to RNA isolation, or purified by sucrose gradient centrifugation (2) for DNA isolation.

DNA isolation. Cell pellets from 1 l cultures were suspended in 30 ml of TE buffer (0.05 M Tris-HCl, 0.02 M EDTA pH 8.0). After addition of SDS (SDS: sodium dodecylsulfate) to 1% (w/v) (from 20% stock) and proteinase K (Merck , Darmstadt F.R.G., preincubated for 1 h at 37°C) to 10 µg/ml (from 200 µg/ml stock), the suspension was incubated for 20 min at room temperature. The resulting lysate was extracted 3 - 4 times with phenol/cresol (phenol: m-cresol: hydroxyquinoline = 1000 : 140 : 4 (w/w/w)), until the aqueous layer was clear. After extraction of the aquous layer with 1 vol of chloroform the solution was made up to 0.2 M sodium acetate. Nucleic acids were precipitated overnight at -20°C after adding 2 vols of ethanol. After centrifugation the pellet was dissolved in 5 ml 0.1 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate pH 7.0). RNA was digested with 100 µg/ml RNAse A (Sigma) (preincubated for 10 min at 80°C) and 250 units/ml RNAse T1 (Sigma) for 30 min at 37°C. After incubation with 10 µg/ml proteinase K (30 min at 37°C in 1% SDS) and phenol/cresol and chloroform extraction the DNA was ethanol-precipitated.

Isolation of large plasmid DNA. The procedure used was a modification of the procedure of Hansen and Ohlsen (14). Bacteria were grown and lysed as above. Cells from 1 l cultures were suspended in 900 ml TE to give 1 l lysate. The pH of the lysate was increased to 12.3 by addition of 3 M NaOH with gently stirring. After 20 min the pH was lowered to 8.5 with 2 M Tris-HCl pH 7.0 and 5 M NaCl was added to a final concentration of 1 M. After 5 h at 0°C precipitated material was removed by centrifugation (15 min at 16,000 x*g*). Floating material was removed with a strip of Whatmann 3 MM paper, and 50% (w/v) polyethylene glycol (PEG) was added to the remaining supernatant to a final concentration of 10% (w/v). After leaving overnight at 0°C crude plasmid DNA was pelleted

(10 min at 16,000 x*g*) and dissolved in 8.5 ml TE + 0.5% diethylpyrocarbonate (DEP). 9 g CsCl and 0.5 ml ethidium bromide (10 mg/ml) were added and equilibrium centrifugation was performed in a 75 Ti rotor (30,000 rpm, 64 h, 20°C) in a Spinco centrifuge. DNA bands were visualized with long wavelength UV light. The lower band was sucked into a syringe after puncturing the tube. The DNA sample was diluted with 1 vol of water and precipitated with 2 vols of ethanol.

Isolation of plasmid pSA 30 (15). All manipulations with bacteria containing recombinant DNA were performed under the guidelines of the Dutch Committee in charge of the control on genetic manipulation. *E.coli* K 12 strain GTM-4, containing plasmid pSA 30, was grown in a medium containing 1% bacto-tryptone, 1% NaCl, 0.5% yeast extract and 0.1% glucose (w/v) at 37°C in a shaking incubator. Chloramphenicol (170 mg/l) was added and incubation was continued for 16 h at 37°C. The cells were pelleted, suspended in 50 ml TE buffer and lysed by addition of SDS to a final concentration of 1% (w/v). The lysate was made up to 1 M NaCl (from 5 M stock solution) and incubated at 4°C for 5 h. Precipitated material was removed by centrifugation (15 min at 16,000 x*g*), the supernatant was extracted once with phenol/cresol and plasmid was precipitated at -20°C overnight by the addition of 1 vol of ethanol. The plasmid was further purified by CsCl equilibrium centrifugation as described above.

Restriction enzyme digestion and gel electrophoresis. 1 - 5 µg of DNA was incubated in 20 µl of 90 mM Tris-HCl, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol pH 7.9 and 6 units of EcoRI or HindIII (both from Boehringer, Mannheim FRG). After a 2 h incubation at 37°C ficoll (5% w/v final concentration) was added and electrophoresis was performed in a horizontal 0.7% agarose (Miles Laboratories, England) gel (autoclaved before pouring) buffered with 40 mM Tris-acetate, 10 mM EDTA pH 7.7 (16). After electrophoresis (2 V/cm) for 16 h the gel was stained in electrophoresis buffer containing 0.5 µg/ml ethidium bromide. The DNA fragments were visualised with long wavelength UV light and photographed. If restriction fragments were to be isolated from a gel, electrophoresis buffer without EDTA was used. The DNA was freeze-squeezed from frozen gel slices (17) and used directly for nick translation.

Southern blotting. DNA bands were transferred from the agarose gel to a nitrocellulose filter essentially according to Southern (11). After blotting overnight at 4°C the slots were marked on the nitrocellulose filter; the filter was washed in 3 x SSC and baked for 2 h at 80°C.

RNA isolation. For RNA isolation, bacteria were cultured to A_{660} =

0.15. Chloramphenicol (75 mg/l) and NaN_3 (final conc. 10 mM) were then added and the culture was poured onto ice. The cells were collected by centrifugation at 16,000 *xg* for 10 min. To isolate the bacteroid RNA the sucrose gradient centrifugation step was omitted. Lysis was performed as described for DNA isolation, exept for the proteinase K treatment. The lysate was immediately extracted with phenol/cresol. After ethanol precipitation the nucleic acids were dissolved in 10 mM Tris-HCl, 10 mM $MgCl_2$ pH 7.6. DNA was digested with 50 µg/ml DNase I (Sigma) for 30 min at 37°C. After phenol/cresol extraction, RNA was precipitated with ethanol.

In vitro labelling of RNA. The method used was essentially that of Goldbach *et al.* (18). Specific activities obtained were 2 - 10 x 10^6 dpm/µg RNA.

DNA-RNA hybridisations. Southern blots were preincubated for at least 6 h in 3 x SSC, 0.1 % SDS, 100 µg/ml yeast RNA at 63°C. The filters were sealed in a plastic bag with 10^7 dpm (0,5 - 2 µg) *in vitro* labelled RNA in a volume of 1 ml 3 x SSC, 0.1% SDS, 100 µg/ml yeast RNA, 50 µg/ml rhizobium ribosomal RNA and hybridised for 38 - 40 h at 63°C. The filters were washed three times for 2 h in 50 ml of 3 x SSC at 63°C, dried and autoradiographed. In some cases a Fast Tungstate intensifying screen (Ilford, England) was used.

In vitro labelling of DNA. Nick translation of DNA was essentially according to Rigby *et al.* (19). Specific activities obtained ranged from 2 - 5 x 10^7 dpm/µg DNA.

DNA-DNA hybridisation. Southern blots were preincubated at 63°C for 30 min in 3 x SSC, then for 3 h in 3 x SSC, 0.2% (w/v) ficoll, 0.2% (w/v) bovine serum albumin, 0.2% (w/v) polyvinylpyrrolidone (Denhardts solution, ref. 20) and finally for 1 h in Denhardts solution supplemented with 0.1% (w/v) SDS, 50 µg/ml low molecular weight denatured calf thymus DNA and 10 µg/ml poly (A). Nick translated DNA was denatured in 0.1 x SSC at 100°C for 10 min. Hybridisation was performed in 1 ml of final incubation buffer supplemented with 0.2 - 1 x 10^6 dpm denatured DNA, in a sealed plastic bag at 63°C for 40 h. After hybridisation the filters were washed three times for 2 h in 3 x SSC at 63°C, dried and autoradiographed.

RESULTS

To study the expression of large plasmids in *Rhizobium*, plasmid DNA was purified from broth-cultured *R. leguminosarum*, digested with EcoRI restriction endonuclease and subjected to agarose gel electrophoresis. The DNA fragment pattern was blotted onto nitrocellulose filters and hybridised with *in vitro*

labelled ^{32}P-RNA from both broth-cultured rhizobium bacteria and endosymbiotic bacteroids. The autoradiograms of these blots are shown in fig. 1, lanes 1 and 4. It can be seen that bacteroids (lane 4) contain RNA molecules which hybridise with plasmid DNA, whereas in free-living bacteria such RNA is not detectable (lane 1). The results indicate that plasmid DNA is actively transcribed in bacteroids but not in free-living bacteria. It has been verified that the bacteroid RNAs hybridisable to the DNA fragments are indeed plasmid transcripts and not transcripts from chromosonal DNA, which might contaminate the plasmid preparation used. For what purpose EcoR1 digests of total DNA from rhizobium bacteroids were separated in an agarose gel, transferred to nitrocellulose filters and hybridised with ^{32}P-RNA from both bacteria and bacteroids (fig. 1, lanes 2 and 3). The hybridisation pattern of bacteroid RNA to plasmid DNA (lane 4) is markedly different from that of bacteroid RNA to total DNA (lane 3). This indicates that the plasmid DNA is (almost) free of chromosal DNA. Thus the RNA molecules hybridised in lane 4 must be trans-

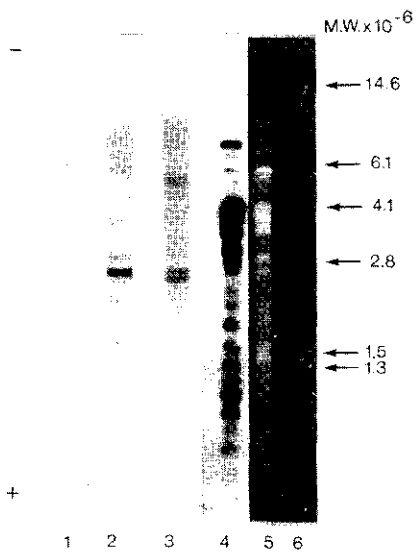

Fig. 1: Hybridisation of *Rhizobium leguminosarum* plasmid DNA and total DNA with RNA from free-living and endosymbiotic cells. Plasmid DNA (lanes 1, 4 and 5;5 µg) and total DNA (lanes 2 and 3; 1 µg) were digested with EcoR1, separated in an agarose gel and blotted on a nitrocellulose filter. Hybridisations were carried out with 10^7 dpm *in vitro* labelled RNA from broth-cultured bacteria (lanes 1 and 2; 0.5 µg RNA) or endosymbiotic bacteroids (lanes 3 and 4; 2 µg RNA). Lane 5 shows the pattern of the ethidium bromide-stained restriction fragments of the plasmid DNA, prior to the blotting. Lane 6 contains DNA, digested with HindIII, as a marker.

cripts of plasmid DNA. In contrast to this the hybridisation of bacterial RNA to plasmid DNA develops a hybridisation pattern identical to bacterial RNA hybridised to total DNA, if exposed for a much longer period (results not shown). This must be a result of a minor contamination of plasmid DNA with chromosal DNA. From the intensity of the hybridisation we estimate that the plasmid preparation used contains 2 - 4% chromosal DNA. The differences in the hybridisation patterns of bacterial or bacteroid RNA to total DNA (cf. lanes 2 and 3) are probably due to dissimilarities in gene expression in bacteria and bacteroids; *e.g.* the fragment of M.W. 2.5 x 10^6 which is strongly expressed in bacteroids but not in bacteria possibly contains a gene which is functional during the symbiosis. The amount of plasmid DNA in the total DNA of *Rhizobium* has been estimated to be 3 - 4% of the total DNA (3). This amount is apparently too small to be able to detect plasmid specific hybridisation with bacteroid RNA (Fig. 1, lane 3).

The degree of expression of plasmid DNA in endosymbiotic bacteroids is further illustrated by the stained EcoR1 fragment pattern of the plasmid shown in lane 5. To understand the pattern it is important to know that the plasmid preparation is a mixture of two plasmids. If the crude plasmid preparation (after the PEG precipitation) is separated in an agarose gel (ref. 5) two plasmid bands are visible of molecular weights of approximately 210 and 240 x 10^6
(Fig. 2). Because of the large size of both plasmids an EcoR1 digestion of purified plasmid will result in a relatively large number of fragments of similar size, which in an agarose gel will migrate to similar positions. As a result most bands in fig. 1, lane 5 will be composed of more than one fragment. Comparison of the hybridisation pattern (lane 4) and the stained fragment pattern (lane 5) demonstrates that the RNA used in lane 4 hybridises with many DNA fragments. The reason for the difference in the amount of hybridisation of the various DNA fragments may be caused by a dissimilarity in the expression of the plasmid genes, or alternatively that the respective mRNAs are stable to different degrees.

Hybridisation of Rhizobium RNA and DNA with pSA 30. Plasmid pSA 30 (12) was used as a probe to detect the presence of *nif* specific mRNA in RNA prepared from broth-cultured rhizobia and bacteroids and subsequently to identify the rhizobium plasmid restriction fragment from which this RNA has been transcribed; pSA 30 contains the structural genes for nitrogenase of *Klebsiella pneumoniae* (12). The experiment shown in Fig. 3 indicates that the EcoR1 fragment of pSA 30 which contains the *nif* genes hybridises with ^{32}P-RNA from

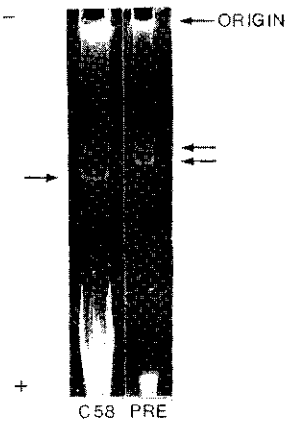

Fig. 2: Evidence for two plasmids in *Rhizobium leguminosarum* strain PRE. A plasmid preparation (after the PEG precipitation) was separated in an agarose gel and stained with ethidium bromide; the UV pattern is shown in lane 2. The arrows indicate the two Rhizobium plasmids (estimated molecular weights approximately 210 and 240 x 10^6, based on renaturation kinetics, data not shown). Lane 1 contains a plasmid preparation from *Agrobacterium tumefaciens*. The arrow indicates the Ti plasmid of M.W. 120 x 10^6, the upper band is a criptic plasmid of M.W. >214 x 10^6 (ref. 5).

rhizobium bacteroids, but there is no hybridisation detectable with RNA from broth-cultured bacteria (cf. lanes 1 ans 2). This result demonstrates that there is *nif* specific mRNA in the bacteroid RNA preparation. As expected such RNA is absent in RNA from broth-cultured bacteria because the free-living bacteria do not synthesize nitrogenase (21).

Plasmid pSA 30 was furthermore used to identify the rhizobium DNA fragment from which the nitrogenase mRNA is transcribed. For that purpose purified plasmid DNA and total DNA of *Rhizobium leguminosarum* were digested with EcoR1, electrophoresed and blotted onto nitrocellulose, similary to the method described above. However DNA-DNA hybridisation was now performed using ^{32}P-labelled pSA 30. The results of the hybridisation are shown in the autoradiograms presented in Fig. 4. It is clear that only one EcoR1 fragment of the rhizobium plasmids shows hybridisation with pSA 30 (Fig. 4, lane 3). The fragment pattern of total DNA shows a faint hybridisation band in a similar position, caused bij the 3 - 4% of plasmid DNA in the preparation (lane 1). To determine which part of pSA 30 hybridises with the rhizobium plasmid fragment, pSA 30 was digested with EcoR1 and HindIII. This results in two vector fragments, a fragment with gene K and a fragment with the genes D and H (12).

Nucleic Acids Research

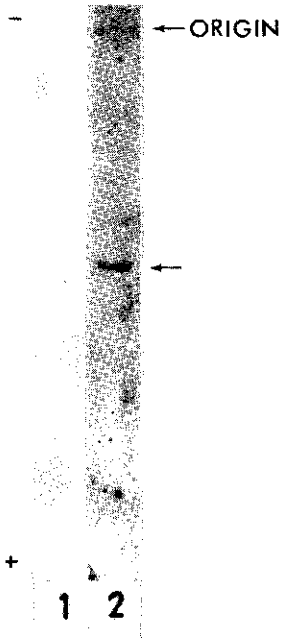

Fig. 3: Hybridisation of rhizobium RNA to *Klebsiella pneumoniae nif* genes. Cloned *nif* genes of *K. pneumoniae* were digested with EcoR1, separated in an agarose gel, blotted on a nitrocellulose filter and hybridised to *in vitro* labelled RNA from rhizobium bacteria (lane 1) and bacteroids (lane 2). The arrow indicates the *nif* fragment (M.W. 4.6×10^6) which hybridises with bacteroid RNA.

These fragments were separated in an agarose gel, isolated from the gel (17) and labelled *in vitro* (19). Only fragment D + H appeared to hybridise with the plasmid fragment (Fig. 4, lane 2); gene K or the vector failed to hybridise (results not shown). This confirms the results of Ruvkun and Ausubel (22), who also found that only fragment D + H is homologous to rhizobium DNA. Thus, at least a part of gene D, coding for one of the subunits of component I, or gene H, coding for the subunit or component II, or both, are located on a rhizobium plasmid. Fig. 4, lane 4 (identical to fig 1, lane 4) shows that this fragment is relatively strongly expressed in nitrogen-fixing bacteroids.

DISCUSSION

In this paper evidence has been presented that in endosymbiotic *Rhizobium leguminosarum*, large plasmids are extensively transcribed. As a large number of EcoR1 fragments of the plasmids hybridise to total bacteroid RNA a substan-

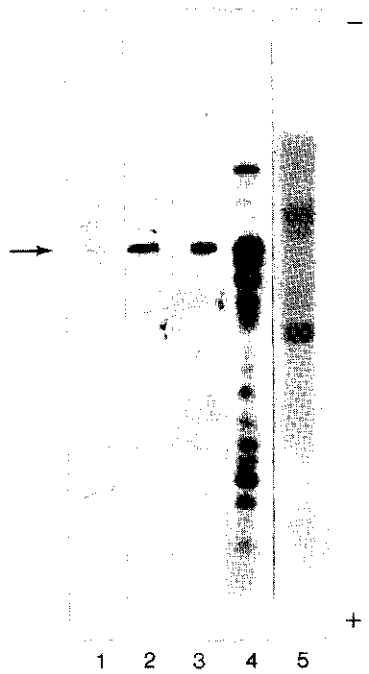

Fig. 4: Detection of *nif* genes in *Rhizobium*. Plasmid DNA (lanes 2 and 3; 1 µg and 4; 5 µg) and total DNA (lanes 1 and 5; 1 µg) from rhizobium were digested with EcoR1, separated in an agarose gel and blotted on a nitrocellulose filter. Hybridisations were performed with *in vitro* labelled preparations of pSA 30 (lanes 1 and 3; 0.2 x 10^6 dpm, 4 - 10 ng DNA), fragment D + H (lane 2; 0.2 x 10^6 dpm, 4 - 10 ng DNA), and rhizobium bacteroid RNA (lanes 4 and 5; 10^7 dpm, 2 µg RNA). The arrow indicates the position of the *nif* genes (approx. M.W. 4.3 x 10^6).

tial part of these plasmids seems to code for proteins playing a role during the symbiosis. The results presented substantiate the conclusions of other authors (7 - 10) that plasmids in *Rhizobium* must play an important role in the process of infection and nitrogen fixation. We were not able to detect transcription of plasmid DNA in broth-cultured rhizobia, suggesting that under these conditions plasmid genes are not expressed or only so to a limited extent. A plasmid function which is reported to be expressed in bacteria is the production of bacteriocin (23). The amount of mRNA transcribed from these genes is possibly too low to be detected above the background. We conclude that the large rhizobium plasmids contain at least part of the genes which are functional during the differentiation of bacteria into bacteroids and which are necessary for the infection process in legumes. Genetic and

biochemical analysis of plasmid borne genes will therefore be of great importance for the understanding of these phenomena.

Preparations of total DNA, consisting of about 96% chromosal DNA, were found to be expressed in free-living as well as in endosymbiotic rhizobia, although the restriction fragments showed different degrees of hybridisation. We suppose that in both broth-cultured and endosymbiotic rhizobia similar genes involved in metabolic processes are expressed, although probabely at different rates as a result of the different conditions to which the bacteria or bacteroids are subjected. From the difference in the hybridisation patterns of lanes 2 and 3 it is not possible to conclude whether or not chromosomal genes are exclusively expressed during the symbiosis, especially in this case (bacteroid RNA hybridised to bacteroid DNA) because some hybridisation of contaminating plant material cannot be excluded. Experiments are in progress to decide whether the hybridisation at M.W. 2.5×10^6 (Fig. 1, lane 3) originates from *Rhizobium* genes.

The presence of *nif* specific mRNA in preparations from endosymbiotic rhizobia was shown by hybridisation to the cloned structural *nif* genes from *Klebsiella pneumoniae* (Fig. 3). This mRNA is transcribed from a plasmid, as is shown by the hybridisation of *K. pneumoniae nif* DNA to rhizobium DNA (Fig. 4). Not all the *nif* genes are homologous; only the EcoR1 + HindIII fragment D + H, containing the *K. pneumoniae* genes coding for one of the component I subunits plus component II, hybridises (lane 2), as also described by Ruvkun and Ausubel (22). It cannot be ruled out however that only a part of the genes D or H is sufficient homologous to the corresponding gene in *Rhizobium* to hybridise.

Our results indicate that in *Rhizobium* not only part of the genes for nitrogenase are localised on a plasmid, but many other genes involved in symbiosis and nitrogen fixation.

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IV ONLY ONE OF THE LARGE PLASMIDS IN *RHIZOBIUM LEGUMINOSARUM* STRAIN PRE IS STRONGLY EXPRESSED IN THE ENDOSYMBIOTIC STATE

ABSTRACT

Plasmid DNA from six strains of *Rhizobium leguminosarum* was separated by agarose gel electrophoresis and showed a large variation in the number and size of the plasmids. Blotting onto nitrocellulose and hybridization with a probe containing *nif* (nitrogen fixation) genes from *Rhizobium meliloti* demonstrated that all strains contain only one plasmid carrying *nif* genes. The size of the *nif* plasmids ranged between approximately 130 x 10^6 and 550 x 10^6. Using DNA-DNA hybridization no homologous sequences were detected between the *nif* plasmid of strain PRE and the other plasmid present in this strain. Hybridization with RNA from bacteroids showed that only the *nif* plasmid is strongly expressed in the endosymbiotic state. No selective amplification of plasmid DNA occurs during the transition of free living bacteria into bacteroids.

INTRODUCTION

Bacteria of the genus *Rhizobium* are able to fix nitrogen in the root nodules of leguminous plants. Conjugation experiments have demonstrated that bacterial genes involved in the formation of root nodules are located on a large plasmid in *Rhizobium leguminosarum* (Johnston *et al.*, 1978), and also at least a part of the structural *nif* (nitrogen fixation) genes of *R. leguminosarum* appear to be located on a large plasmid (Nuti *et al.*, 1979). *Nif* genes and genes involved in nodule formation are located on one of the plasmids occurring in *R. meliloti* (Dénarié *et al.*, 1981). Brewin *et al.* (1980) found that nodulating ability and hydrogenase, which enhances the efficiency of nitrogen fixation, were coded by one of the large plasmids in *R. leguminosarum*. Hooykaas *et al.* (1981) demonstrated

that at least a part of the structural *nif* genes and host specificity concerned with nodulating ability in *R. trifolii* are located on one plasmid. Since *Rhizobium* bacteria contain more than one plasmid, it has been postulated that larger plasmids may be multimers of the smaller ones (Tichy & Lotz, 1981) or that the plasmids have otherwise homologous sequences. This would render the bacteria polyploid for certain symbiotic genes. The plasmids might be different, carrying complementary information necessary for the establishment of a nitrogen fixing nodule, or alternatively, only the plasmid carrying the nodulation genes and *nif* genes is important for symbiosis, while the other plasmids are not expressed in bacteroids. In this paper experiments are described which support the last hypothesis.

METHODS

Bacterial strains. The six *Rhizobium leguminosarum* strains used are listed in Table 1. Strains PRE, PF2, TOM, S310a and P8 are from the Laboratory of Microbiology, Wageningen, The Netherlands. Strain 1001 originates from the Rothamsted collection and was obtained from Dr. R.K. Prakash, Department of Biochemistry, Leiden, The Netherlands. All strains except P8 are Nod^+ and Fix^+ on *Pisum sativum* cv. Rondo.

Cultivation of *Rhizobium* bacteria and pea and isolation of bacteroids was as described by Krol *et al.* (1980).

Plasmid isolation and agarose gel electrophoresis. Plasmid isolation was as described by Casse *et al.* (1978).

Isolation of plasmid DNA from a gel. Large plasmid DNA was isolated from a gel of low melting agarose (Marine Colloids, Rockland U.S.A.). Plasmid bands were cut out of the 0.6% (w/v) gel, made up to 0.5 M NaCl (from a 5 M stock solution), melted at 65°C, extracted with phenol/cresol (phenol: m-cresol: hydroxyquinoline = 1000: 140 : 0.4 (w/w/w/)) (Wieslander, 1979) and the DNA was precipitated with 2 vols of ethanol at -20°C. To remove traces of agarose from

the DNA the pellet was dissolved in a small volume of 0.25 M ammonium acetate, centrifuged 5 min at 18,000 x g and the DNA was again precipitated from the supernatant with 2 vols of ethanol at -20°C.

Southern blotting of large plasmid DNA. Prior to blotting intact plasmids were fragmented by incubating the gel in 0.25 M HCl for 20 min. at 20°C (Wahl *et al.*, 1979). The gel was transferred to 0.5 M NaOH and blotting onto nitrocellulose filters, (Sartorius, 0,1 µ) was performed essentially according to Southern (1975).

Preparation of filters for hybridizations. Total cellular DNA from free living bacteria and from bacteroids was isolated as described before (Krol *et al.*, 1980). After 10 min at 100°C in 0.1 x SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.0) portions containing DNA (10 µg) were made up to 6 x SSC and filtered three times through a nitrocellulose filter (Sartorius, 0.1 µ). The filters were baked at 80°C for 2 h.

Plasmids pSA30 and pRmR1 were obtained from Dr. F.M. Ausubel, Harvard University, Cambridge MA, U.S.A. Plasmid pSA30 contains the HDKY operon of *Klebsiella pneumoniae*, of which gene H codes for the subunits of component II and gene D and K for the α and β subunits of component I of nitrogenase (Cannon *et al.*, 1979). Plasmid pRmR1 contains DNA from R. meliloti homologous to pSA30. Plasmids were isolated as described before (Krol *et al.*, 1980) and *in vitro* labelled with ^{32}P by nick translation according to Rigby *et al.* (1977).

Total cellular RNA from bacteroids was isolated as described by Krol *et al.* (1980) and *in vitro* labelled with ^{32}P according to Goldbach *et al.* (1978).

Hybridizations. DNA blots were prehybridized at 63°C for at least 6 h in 3 x SSC, 0.1% (w/v) SDS, 2 µg/ml bovine serum albumin, 2 µg/ml polyvinylpyrrolidone, 2 µg/ml ficoll (Denhardt, 1966), supplemented with 50 µg/ml denatured low molecular weight calf thymus DNA and 10 µg/ml poly (A) (for DNA-DNA hybridizations) or 100 µg/ml yeast RNA (for DNA-RNA hybridizations). Hybridization was performed

in 1 ml of prehybridization buffer with *in vitro* labelled pRmR1, pSA30, *Rhizobium* large plasmid DNA or bacteroid RNA at 63°C for up to 24 h. After hybridization the filters were washed three times for 2 h in 3 x SSC at 63°C and then dried and autoradiographed using Sakura X-ray film (Konishiroku Photo Ind. Co. Ltd, Japan). In the DNA-DNA filter hybridization experiments radioactivity was assayed in 8 ml Instafluor (Packard Instrument Inc.) in a liquid scintilation counter.

RESULTS

Plasmid content of strains

Six different isolates of *R. leguminosarum* were compared with regard to their plasmid content (Table 1). *R. leguminosarum* PRE is the strain commonly used in our experiments. The other strains can be distinguished from PRE by differences in their symbiotic properties such as the shape of the bacteroids (PF2 and P8), ability to nodulate *P. sativum* cv. Afganistan (TOM), possession of a hydrogenase (S310a) or absence of nitrogen fixation (P8). Electrophoresis in 0.6% (w/v) agarose gels showed that the six isolates also differ in plasmid content (Fig. 1a). PRE, PF2 and 1001 each carry two plasmids, S310a three, P8 four and TOM probably also four, two of which are probably of similar size. The latter conclusion is based on the much stronger fluorescence of the middle band. The molecular weights of the plasmids of each strain were estimated by comparing their electrophoretic mobility in agarose gels with those of the two plasmids of PRE, for which molecular weights of approximately 230 x 10^6 and 500 x 10^6 have been determined (H.V. Tichy and W. Lotz, personal communication). The molecular weights of the different plasmids (Table 1) vary widely from approximately 130 x 10^6 (1001) to 600 x 10^6 (S310a). In five strains a plasmid with a molecular weight of 500-550 x 10^6 was found, and in four strains a plasmid with a molecular weight of 200 - 240 x 10^6. No plasmids of exactly the same size were found in any of the strains.

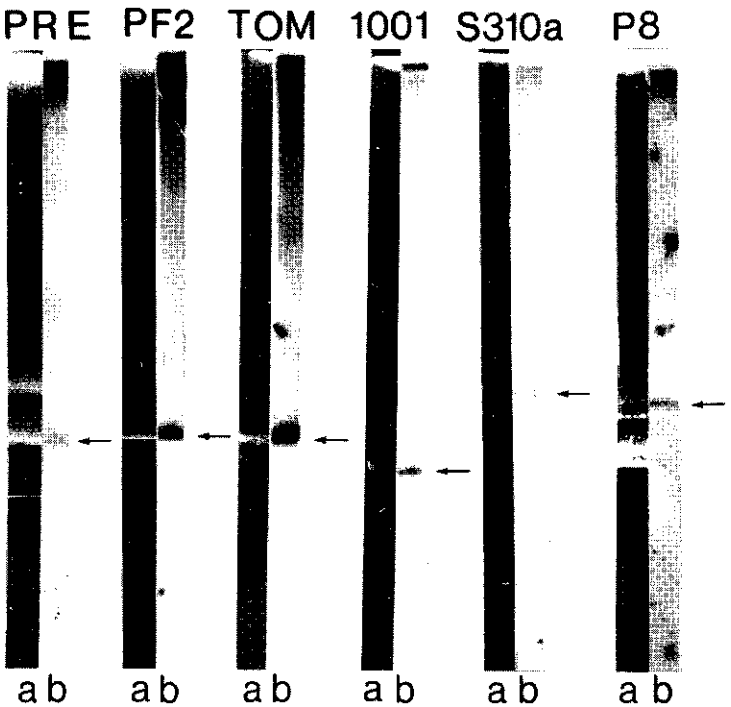

Fig. 1. Hybridization of Rhizobium leguminosarum plasmid DNA with pRmR1. Plasmid DNA from strains PRE, PF2, TOM, 1001, S310a and P8 was separated by agarose gel electrophoresis and stained with ethidium bromide. The U.V. pattern is shown in lane a. After blotting onto nitrocellulose the plasmids were hybridized with in vitro ^{32}P-labelled pRmR1 carrying nif genes from R. meliloti. The autoradiograph is shown in lanes b. The arrows indicate the position of the nif plasmids.

Hybridization studies

To determine which of the plasmids in each strain carries *nif* genes the plasmids were blotted onto nitrocellulose filters and hybridized with *in vitro* labeled pRmR1. pRmR1 is a recombinant plasmid containing a 3.9 Kb insert with at least a part of the structural *nif* genes of *R. meliloti* (Ruvkun & Ausubel), 1980). The autoradiograph (Fig. 1b) shows that in each isolate only one plasmid hybridized with pRmR1, suggesting that only one of the plasmids carries *nif* genes. There is a large variation in size between the plasmids carrying *nif* genes: in 1001 the *nif* plasmid is nearly half the size of the *nif* plasmid of PRE while in S310a it is more than twice as large. Also strain P8 appeared to have a *nif* plasmid, although it is Fix$^-$ on all hosts tested.

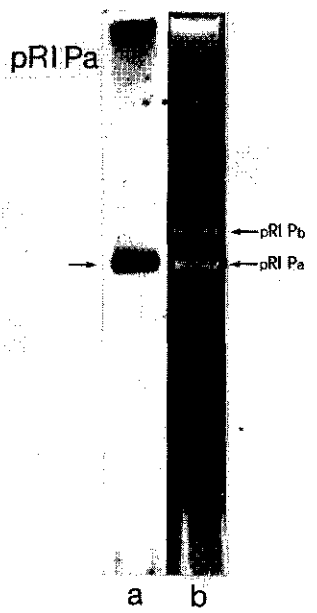

Fig. 2. Comparison of the two plasmids of Rhizobium leguminosarum strain PRE. The two plasmids of PRE were separated by agarose gel electrophoresis and stained with ethidium bromide; the U.V. pattern is shown in lane b. After blotting onto nitrocellulose the plasmids were hybridized with in vitro ^{32}P-labelled pRlPa, isolated from an agarose gel (lane a).

Comparison of pRlPa and pRlPb

The two plasmids detected in strain PRE were compared in more detail. The *nif* plasmid of PRE, referred to as pRlPa, was isolated from an agarose gel, labelled *in vitro* with ^{32}P and used for hybridization with the two separated PRE plasmids, blotted onto a nitrocellulose filter. The autoradiograph, shown in Fig. 2, shows that pRlPa only annealed to itself, but not to the larger plasmid, referred to as pRlPb. This indicates the absence of appreciable homology between the two plasmids. The hybridization of pRlPb with a blot of the separated plasmids was not done, because pRlPb is contaminated with pRlPa. This is due to breaking of supercoiled plasmids, causing tailing toward the origin. Therefore a preparation of pRlPb will always show some hybridization with pRlPa.

Transcription of plasmids

To examine if the extensive transcription of plasmid-born genes which we found earlier (Krol *et al.*, 1980) is from a single plasmid or from both, the two plasmids of PRE were isolated from an agarose gel. The amount of pR1Pb obtained from a gel is less than the amount of pR1Pa: in this experiment approximately 0.5 µg pR1Pb and 1 µg pR1Pa were isolated. EcoR1 digests of the plasmids were separated in a 0.7% (w/v) agarose gel, blotted onto nitrocellulose filters and hybridized with *in vitro* ^{32}P-labelled RNA from bacteroids.

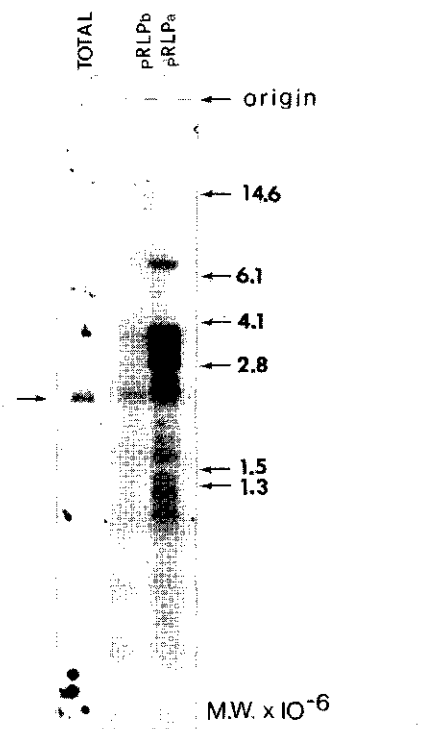

Fig. 3. Autoradiographs showing hybridization of isolated plasmids of Rhizobium leguminosarum strain PRE with RNA from bacteroids.
Plasmid pR1Pa and pR1Pb (see Fig. 2) were isolated from an agarose gel and digested with EcoR1 (lanes b and c). The fragments were separated by agarose gel electrophoresis, blotted onto nitrocellulose and hybridized with in vitro ^{32}P-labelled RNA from bacteroids. The autoradiographs are shown in lanes b and c. For comparison total DNA from free living bacteria was digested with EcoR1 and after separation by agarose gel electrophoresis, hybridized with in vitro ^{32}P-labelled ribosomal RNA from free living bacteria (lane a). The arrow at the left indicates the position of the fragment of chromosomal DNA strongly hybridizing with ribosomal RNA. The arrows at the right indicate the position of phage lambda DNA Hind III fragments used as molecular weight markers.

The autoradiograph is shown in Fig. 3, lane c. Many fragments of pR1Pa (the *nif* plasmid) are hybridizing; the pattern is in agreement with the pattern obtained earlier by hybridization of bacteroid RNA with plasmid DNA isolated by CsCl-ethidium bromide centrifugation (Krol *et al.*, 1980). In lane b showing the EcoR1 fragments from pR1Pb a weaker hybridization is probably due to contamination of pR1Pb with pR1Pa as is indicated above, since the pattern of bands in lane b corresponds exactly to that of the most strongly hybridizing bands in lane c. The weaker hybridizing bands in lane c are not, or hardly, visible in b. An exception is the band at a position a little below 2.8×10^6 which is more intense in lane b relative to the other bands than in lane c. In lane a of Fig. 3 the hybridization of EcoR1-digested total bacterial DNA with purified bacterial ribosomal RNA is shown. It can be seen that ribosomal RNA hybridized strongly to a fragment with a molecular weight of approximately 2.8×10^6, presumably the location of the ribosomal RNA gene on the chromosome. In the EcoR1 pattern of pR1Pa and pR1Pb a fragment of similar size also hybridizes strongly with the ribosomal RNA, indicating that the isolated plasmids are contaminated with chromosomal DNA. Therefore the hybridization observed in lane b of Fig. 3 must be attributed to contamination of pR1Pb with pR1Pa and chromosomal DNA. No specific hybridization of bacteroid RNA with pR1Pb is detectable, suggesting that the large plasmid is not expressed in root nodules. This conclusion was confirmed by an experiment in which plasmids were separated in an agarose gel, blotted onto nitrocellulose and hybridized with bacteroid RNA. The autoradiograph, shown in Fig. 4, shows only hybridization with pR1Pa.

No selective amplification of *nif* plasmid

The use of cloned *nif* genes makes it possible to examine whether a small amplification of *nif* plasmid might occur during the differentiation of bacteria into bacteroids. Therefore total DNA from bacteria and bacteroids was isolated, blotted onto nitrocellulose filters and hybridized with *in vitro* ^{32}P-labelled pSA30. The results (Table 2) show that the rates of hybridization of pSA30 with both DNAs are equal. This indicates that there is no selective amplification of *nif* plasmid.

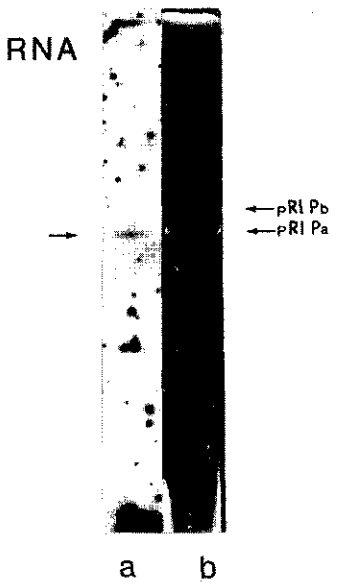

Fig. 4. Hybridization of plasmid DNA of Rhizobium leguminosarum strain PRE with RNA from bacteroids. The two plasmids of PRE were separated by agarose gel electrophoresis and stained with ethidium bromide. The U.V. pattern is shown in lane b. After blotting onto nitrocellulose the plasmids were hybridized with in vitro ^{32}P-labelled RNA from endosymbiotic bacteroids. The autoradiograph is shown in lane a.

DISCUSSION

Rhizobium strains have been shown to contain several plasmids varying in size (Tichy & Lotz, 1981). If a CsCl-ethidium bromide gradient is used for the isolation of large plasmids from *Rhizobium* (Casse *et al.*, 1978; Spitzbarth *et al.*, 1979; Nuti *et al.*, 1979; Krol *et al.*, 1980; Dénarié *et al.*, 1981) a mixture of the plasmids present is obtained, in which the larger plasmids will be underrepresented, if they occur at all, because they are more sentitive to shearing forces (Dénarié *et al.*, 1981). To study the different plasmids separately we therefore used agarose gel electrophoresis, which makes it possible to detect the presence and the relative amount of the different plasmids by ethidium bromide staining

(Table1). The separated plasmids can be blotted onto a nitrocellulose filter, but it is necessary to reduce the size of the DNA before blotting by incubation of the gel in 0.25 M HCl (Wahl *et al.*, 1979). Such blots make it possible to localize properties on each of the plasmids by annealing with DNA probes or with RNA. By hybridization with pRmR1 as a probe we have shown that different strains of *R. leguminosarum*, varying in plasmid content, contain only a single *nif* plasmid. The *nif* plasmids differ remarkably in size: from 130 x 10^6 in strain 1001 to 550 x 10^6 in strain S310a. There is no correlation between the size of the *nif* plasmid and the phenotype of the symbiosis: PRE and 1001 have similar symbiotic properties, but differ approximately 100 x 10^6 in the size of their *nif* plasmids. On the contrary PRE, PF2 and TOM differ in symbiotic properties, but have a *nif* plasmid of approximately the same size. Strain S310a has a very large *nif* plasmid (550 x 10^6) and also possesses genes for hydrogen uptake (*hup* genes). However, Brewin *et al.* (1980a) located genes involved in Hup^+ phenotype on a 190 x 10^6 plasmid, so that Hup^+ is not necessarily correlated with a larger size of *nif* plasmid. Strain P8 is Fix^-, and possesses a *nif* plasmid with a molecular weight of 400 x 10^6 making it unlikely that a large deletion is involved. It reduces acetylene at a very low rate, (the maximal rate for strain P8 is less that 1/1000th of the maximal rate of PRE) and only for a very short period after nodule formation; it fails to maintain leghemoglobin synthesis (T. Bisseling, unpublished results). Apparently P8 is detective not in a *nif* gene but in some other gene involved in establishing an effective symbiosis. The significance of the variation in size of the *nif* plasmids from different strains of *R. leguminosarum* remains a mystery as also does the function of the other plasmids besides the *nif* plasmid. For strain PRE we have demonstrated that there is no detectable homology between the two plasmids of this strain. The latter would render PRE diploid for certain genes. Brewin *et al.* (1980b) found for strain TOM a similar plasmid pattern as presented here: only their size estimates were smaller than ours. By plasmid transfer experiments they demonstrated that the middle one of the TOM plasmids carries genes for nodulation and fixation. By blotting of the plasmids and hybridization with pSA30 it was shown that this plasmid also contains the nitrogenase genes. (Hombrecher *et al.*, 1981). Four other plasmids

Table 1: Properties of the *Rhizobium leguminosarum* strains.
Nod^+, Fix^+ and Hup^+ indicate the possession of nodulating ability, nitrogen fixation and hydrogen uptake respectively; plasmids carrying *nif* genes are indicated by*.

Strain	Approximate size of the plasmids x 10^{-6}	Shape of the bacteroids	Symbiotic properties on host plant *Pisum sativum* cv. Rondo	*Pisum sativum* cv. Afghanistan	Remarks
PRE	230*; 500	Y	$Nod^+Fix^+Hup^-$	Nod^-	
PF2	240*; 500	spheroplasts	$Nod^+Fix^+Hup^-$	Nod^-	Broughton *et al.* 1980
TOM	180; 220; 220*; 500	Y	$Nod^+Fix^+Hup^-$	$Nod^+Fix^+Hup^-$	Broughton *et al.* 1980
1001	130*; 550	Y	Nod^+Fix^+	not tested	Nuti *et al.* 1977
S310a	400; 550*; 600	Y	$Nod^+Fix^+Hup^+$	Nod^-	
P8	150; 200; 350; 400*	small rods	$Nod^+Fix^-Hup^-$	Nod^-	also Nod^+Fix^- on *Vicia faba* and *Lathyrus sylvestris*

of different strains of *R. leguminosarum* or *R. phaseoli* showed the same linkage of *nif* genes and nodulating ability. Also in *R. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981) and in *R. trifolii* (Hooykaas *et al.*, 1981) nodulating genes and nitrogenase genes appear to be located on a single plasmid. Of the two plasmids of *R. leguminosarum* strain PRE, only the *nif* plasmid is strongly expressed in bacteroids, suggesting that only the *nif* plasmid carries information for the formation of effective nodules. It should be recognized that genes which are only expressed during the early stages of root infection and nodule formation and genes showing reduced expression have probably not been detected in the present experiments. Our experiments with strain PRE do not exclude that with one of the other strains of *R. leguminosarum* or with one of the other species of *Rhizobium*, other plasmids, besides the *nif* plasmid, are involved in symbiotic nitrogen fixation. Our results suggest that the information for nodule formation is located on a single plasmid and that, besides this plasmid, a variable number of cryptic plasmids may occur in *Rhizobium*.

A reason for the location of symbiotic genes on a single plasmid might be the possibility of amplification of this plasmid, making the bacteroids polyploid for symbiotic genes. During the development of *Rhizobium* into mature bacteroids the total DNA content per cell increases five-to sevenfold (Bisseling *et al.*, 1977). Reynders *et al.* (1975) demonstrated that amplification of a part of the DNA during the development of bacteroids does not occur, but because he used reannealing kinetics a two- or three-fold amplification of a small portion of the DNA, e.g. a plasmid would have remained undetected. Using cloned *nif* genes it was possible to demonstrate that the amount of *nif* genes in bacteria and bacteroids is the same, so that there is no amplification of *nif* plasmids. Thus the strong expression of genes on the *nif* plasmid is apparently not caused by amplification of *nif* plasmid DNA.

The authors thank Dr. F.M. Ausubel for supplying plasmids pRmR1 and pSA30, Dr. H.V. Tichy and Dr. W. Lotz for molecular weight determinations of the plasmids of PRE, Dr. T. Bisseling for encouraging discussions, Dr. R.C. van den Bos for critical reading of the manuscript, A. Hoogeveen for printing the photographs and M.J. van Neerven for preparing the manuscript.

Table 2: Hybridization of pSA30 with total DNA from *Rhizobium leguminosarum* PRE bacteria and bacteroids. Total DNA from bacteria and bacteroids was blotted onto nitrocellulose filters (10 µg DNA per filter), prehybridized with calf thymus DNA and hybridized with *in vitro* labelled pSA30 (specific activity 7 x 10^6 dpm/µg) in 3 x SSC at 63°C for the time indicated. After washing in 3 x SSC the filters were counted in a liquid scintillation counter. The numbers are in dpm/µg DNA and corrected for background.

DNA on filter	Amount of pSA30 hybridized to the DNA on the filters (dpm/µg DNA)		
	Time of hybridization		
	7 h	14 h	24 h
Total bacterial DNA	43.2	99.0	140.9
Total bacteroid DNA	42.7	84.8	132.8

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V ON THE OPERON STRUCTURE OF THE NITROGENASE GENES OF *RHIZOBIUM LEGUMINOSARUM* AND *AZOTOBACTER VINELANDII*

ABSTRACT

The transcription of the nitrogenase genes in *Rhizobium leguminosarum* was studied by analyzing total cellular RNA from bacteroids for the presence of nitrogenase messenger RNA. The RNA was separated by agarose gel electrophoresis and blotted onto nitrocellulose filters. Messenger RNA for nitrogenase was detected by hybridization with probes derived from plasmid pSA30, a recombinant plasmid carrying the nitrogenase genes of *Klebsiella pneumoniae*. In the same way nitrogenase mRNA was detected in RNA isolated from *Azotobacter vinelandii* and from *Klebsiella pneumoniae*, but only if both were cultured under conditions of derepression of the nitrogenase genes. The size of the RNA hybridizing with the probes indicates that in all three organisms the genes for the subunits of the two components of nitrogenase constitute a single operon.

INTRODUCTION

The organization and the regulation of the expression of the nitrogen fixation (*nif*) genes in the root nodule bacterium *Rhizobium* are hard to study since normally these genes are only expressed in the endosymbiotic state. In the facultative anaerobic bacterium *Klebsiella pneumoniae*, which is able to fix nitrogen in the free living state, the *nif* genes have been thoroughly investigated. By means of genetic techniques it has been possible to map the *nif* genes on the chromosome and to determine the number of transcription units (1-3). In *K. pneumoniae* there are seventeen *nif* genes organized in seven or eight transcription units. The structural genes for nitrogenase *nif* H, *nif* D and *nif* K of *K. pneumoniae* constitute one operon, together with *nif* Y (4, 5). *Nif* H codes for the identical subunits of component II of nitrogenase, *nif* D and *nif* K code for the α and β subunits of component I of nitrogenase; the function of *nif* Y is

Electrophoresis and blotting of RNA. Nucleic acids were collected by centrifugation and the pellet was dried *in vacuo*. The pellet was dissolved in 0.2 ml of 50% (v/v) DMSO, 1 M glyoxal, 10 mM sodium phosphate buffer pH 7.0 and the mixture was incubated at 37°C for 15 min (15). The preparations were made up to 5% ficoll, 0.01% (w/v) bromophenolblue (from a 10 x stock solution) and loaded onto a 1.1% (w/v) horizontal agarose gel in 10 mM sodium phosphate pH 7.2. The gel was submerged in the same buffer and electrophoresis was performed at 10 V/cm for 4 h. After electrophoresis the separated nucleic acids were blotted from the gel onto a nitrocellulose filter (0.1 μ, Sartorius) as described by Thomas (16). After blotting the RNA was baked onto the filters at 80°C for 2 h.

Isolation of restriction fragments of plasmid pSA30. Plasmid pSA30 was isolated as described before (17). The DNA was digested in 50 mM Tris-HCl, 10 mM $MgCl_2$, 6 mM β-mercaptoethanol pH 7.8 with a combination of the restriction enzymes EcoR1, Hind III, Bam H1 and Bgl II at 37°C for 2 h. The generated fragments were separated in a 1.2% (w/v) agarose gel in 40 mM Tris-acetate, 10 mM EDTA pH 7.7 (18) and stained with 0.5 μg/ml ethidium bromide for 30 min. The DNA bands were visualized with long wavelength U.V. light and the required fragments, further specified in Fig. 1, were cut out of the gel. The DNA was isolated from the pieces of gel by freeze-squeezing (19) and labelled with ^{32}P by nick-translation (20). The specific activity of the labelled DNA was approximately 1.6×10^7 dpm/μg.

Hybridizations. The nitrocellulose filters with RNA were prehybridized in 3 x SSC (SSC = 0.15 M NaCl 0.015 M sodium citrate pH 7.0) 0.1% (w/v) SDS, 2 mg/ml ficoll, 2 mg/ml polyvinylpyrrolidone, 2 mg/ml bovine serum albumin (21), 50 μg/ml low molecular weight single stranded calf thymus DNA, 10 μg/ml poly (A), at 63°C for 16 h. Hybridizations were performed in 1 ml of the same buffer supplemented with approximately 8×10^6 dpm of the labelled DNA probe. After hybridization at 63°C for 24 h the filters were washed 3 times in 3 x SSC at 63°C for 1 h, dried and autoradiographed using an Ilford intensifying screen.

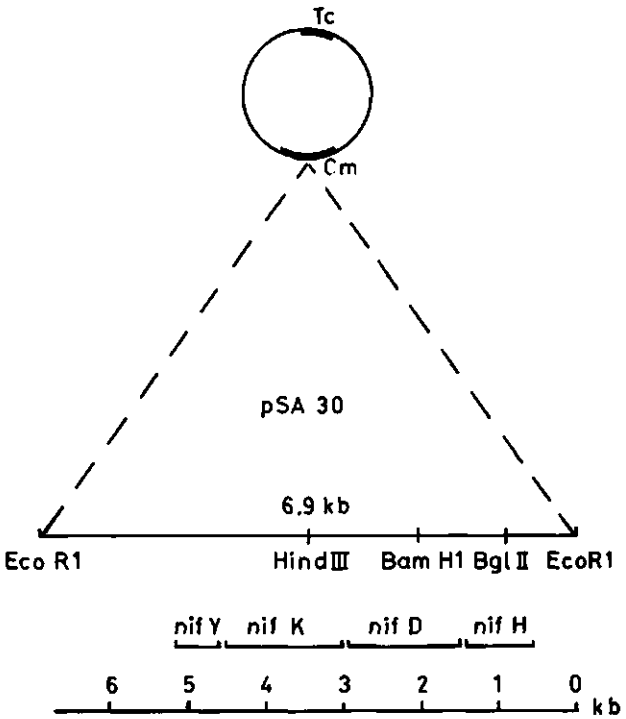

Fig. 1. Physical and genetic map of the nif DNA of Klebsiella pneumoniae cloned in plasmid pSA30 (4, 22). The structural nif genes of Klebsiella pneumoniae have been clone in the recombinant plasmid pSA30 (6). Nif H codes for the subunits of component II of nitrogenase, nif K and D code for the α and β subunits, respectively, of component I of nitrogenase. The function of nif Y, which is located in the same operon as nif H, nif D and nif K (5), is still unknown. pSA30 also contains a small part of nif E, which is involved in the construction of the FeMo cofactor. Using the restriction enzymes EcoR1 and Hind III the fragment DH was isolated, coding for nif H and nif D. Also the EcoR1-Bgl II fragment, containing a part of nif H, and the Bam H1 Hind III fragment containing a part of nif D and nif K, were used.

RESULTS

Fig. 1 represents a physical and a genetic map of plasmid pSA30, a recombinant plasmid which has a 6.9 kb insert carrying the structural *nif* genes of *K. pneumoniae* (6). Digestion of the plasmid with EcoR1 and Hind III renders a fragment DH, on which *nif* H codes for the subunits of component II and *nif* D codes for the α subunit of component I of nitrogenase. This fragment was isolated from an agarose gel, nick translated and used as a probe to detect *nif* specific RNA molecules in total RNA from *R. leguminosarum* bacteroids.

RNA was isolated from bacteroids of *R. leguminosarum* strain PRE from nodules at 13, 15 and 18 days after sowing and inoculating the pea plants. The RNA was separated in an 1.1% agarose gel, blotted onto nitrocellulose filters and hybridized with the ^{32}P-labelled fragment DH. As shown in Fig. 2a a discrete band of RNA with a molecular weight of appoximately 1.2 x 10^6 hybridized with RNA heterogenous in size in the molecular weight range of 0.4 x 10^6 and lower. Hybridization of RNA with fragment DH was hardly detectable in RNA from bacteroids of 13 d old plants, but the amount of hybridizing RNA was increased in bacteroids from 15 and 18 days old plants. Previously it has been shown (11) that under the conditions we use for growing pea oculation, and then increases rapidly during the following days. Therefore the appearance and the increase of RNA hybridizing with fragment DH parallels the development of nitrogen fixation in the root nodules. Identical blots of RNA from bacteroids of 18 d old plants were hybridized with fragment DH and with a DNA fragment carrying only a part of *nif* H and with a DNA fragment carrying parts of *nif* D and *nif* K as indicated in Fig. 1. Fig. 3 shows that both fragments D and H hybridize also with the RNA with a molecular weight of 1.2 x 10^6. At a position higher in the blot some strongly hybridizing material can be seen. This is presumably DNA. When an agarose gel with separated bacteroid RNA was incubated in 0.5 M NaOH at 50°C for 30 min, followed by neutralization and blotting only the material indicated with "DNA" in Fig. 3 showed hybridization (results not shown).

The same method was used to examine the expression of the nitrogenase genes in *A. vinelandii*. The *nif* genes of *A. vinelandii* were derepressed by transferring a culture of bacteria to a medium lacking fixed nitrogen. Samples were taken for RNA isolation at two minutes intervals after the transfer. The RNA was separated by agarose gel electrophoresis, blotted onto nitrocellulose filters and hybridized with the ^{32}P-labelled DNA fragment DH. The autoradiograph presented in Fig. 2b shows that from 34 min after the transfer to the nitrogen free medium two RNA bands with a molecular weight of approximately 1.4 x 10^6 and 0.2 x 10^6 were detected. The strong hybridization above the RNA band of 1.4 x 10^6 is due to bacterial DNA, as the hybridizing nucleic acid was alkali resistant. Weak hybridization was found with RNA with a molecular weight of 0.9 x 10^6. This

band occurs at a position close to the 23S ribosomal RNA present in large amounts and is also visible in blots of RNA from cells before the onset of nitrogen fixation. Acetylene reduction was not measurable until 40 min after the transfer of the bacteria to the low nitrogen medium and then increased rapidly. Nitrogenase activity was thus correlated with the appearance of RNA hybridizing specifically to the *nif* probe.

For comparison RNA was also isolated from *K. pneumoniae*. Cells growing anaerobically in a medium containing 1 g/l NH_4Cl, were transferred to a medium containing 1 mg/ml serine to derepress the *nif* genes and at the intervals indicated in Fig. 2c samples were taken from the culture for isolation of RNA. The RNA was again separated in a 1.1% agarose gel, blotted onto a nitrocellulose filter and hybridized with the ^{32}P-labelled fragment DH from pSA30. The autoradiograph presented in Fig. 2c shows hybridizing RNA bands with molecular weights of approximately 1.4×10^6, 1.1×10^6, 0.8×10^6 and 0.5×10^6. Also in this autoradiograph a band occurs at a molecular weight position of approximately 0.9×10^6 practically at the front of the 23S ribosomal RNA present in large amounts (not shown). Acetylene reduction was not detected until 90 min after transfer and increased rapidly. So also in *K. pneumoniae* the occurrence of hybridizing RNA was correlated with nitrogenase activity but the RNA appeared well before nitrogenase activity.

DISCUSSION

DNA fragments of plasmid pSA30 which carry parts of the structural genes for nitrogenase of *K. pneumoniae* were used to identify RNA transcripts from nitrogenase genes in *R. leguminosarum*, *A. vinelandii* and *K. pneumoniae*. Ruvkun and Ausubel (7) demonstrated that the EcoR1 - Hind III fragment carrying the genes H and D is homologous to the corresponding genes from *R. leguminosarum* and from *A. vinelandii*, but the fragment carrying a part of gene K and gene Y in not sufficiently homologous to give cross hybridization with the corresponding genes in other organisms. Using the DNA fragment DH as a probe we observed in *K. pneumoniae* the appearance of several specifically hybridizing RNA's after induction of the *nif* genes (Fig. 2c). The detection of these RNA's precedes the onset

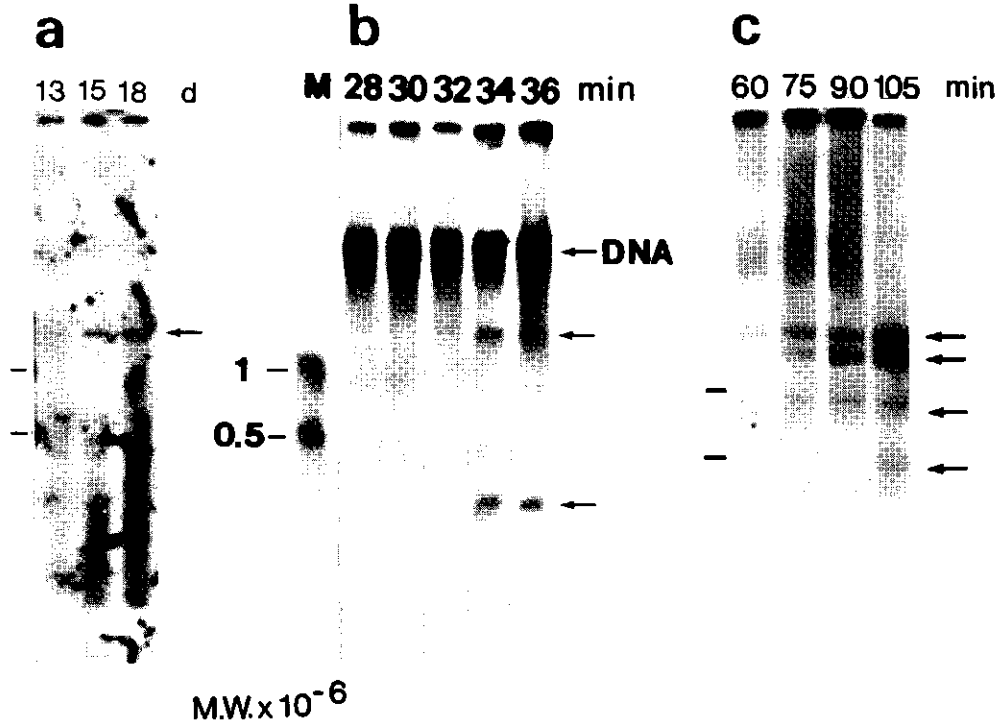

Fig. 2. Detection of nitrogenase messenger RNA in RNA from Rhizobium leguminosarum bacteroids, from Azobacter vinelandii and from Klebsiella pneumoniae. Approximately 10 µg of total cellular RNA from bacteroids of R. leguminosarum (a), from A. vinelandii (b) and from K. pneumoniae (c) was isolated at 13, 15 and 18 days (=d) after sowing or at the time indicated after the transfer of the cells to low nitrogen medium respectively. The RNA was separated by electrophoresis in an 1.1% agarose gel and blotted onto nitrocellulose filters (Sartorius, 0.1 µ) (16). As a molecular weight marker ribosomal RNA from Escherichia coli, in vivo labelled with ^{32}P-phosphate, was electrophoresed in the same gel. The blotted RNA was hybridized in 1 ml 3 x SSC at 63°C with 8 x 10^6 dpm of the ^{32}P-labelled EcoR1-Hind III fragment DH of pSA30 for 24 h. The arrows indicate the position of the hybridizing RNAs, detected by autoradiography.

of nitrogen fixation and the amount of hybridizing RNA increases with the subsequent increase in nitrogen fixing activity. This correlation suggests that the RNA hybridization with the fragment DH is messenger RNA coding for the nitrogenase components. The largest hybridizing RNA has a molecular weight of approximately 1.4 x 10^6, which is sufficient for messenger RNA carrying the information for the synthesis of the subunits of component I and II of nitrogenase with molecular weight of 60.000, 56.000 and 32.000

respectively. Given the size of approximately 4.8 kb for the HDKY operon represented in Fig. 1 a molecular weight of approximately 1.6 x 10^6 may be expected for a full size transcript of the HDKY operon. Perhaps transcription of the HDKY operon terminates before gene Y resulting in a messenger carrying the information for the nitrogenase subunits only. In addition to the presumed full size mRNA smaller RNA molecules are found to hybridize with the DH probe. Most remarkable is an RNA band with a molecular weight of approximately 1.1 x 10^6, which hybridizes as strongly as the 1.4 x 10^6 RNA and appears almost simultaneously. The smaller RNA's may represent degradation products of the full size mRNA, but the 1.1 x 10^6 RNA might also be a separate transcript comprising the genes D and K. The nature of the different RNA's has to be analyzed before any conclusion can be drawn.

In *A. vinelandii* an RNA was detected of a similar size as the largest RNA in *K. pneumoniae* (Fig. 2b). This might indicate that also in *A. vinelandii* at least the genes H, D and K are located in one operon and are transcribed in a single messenger RNA. The small RNA of approximately 0.2 x 10^6 appears to be too small for a separate messenger function and probably is a degradation product.

Since it appeared possible to isolate large messenger RNA molecules from *K. pneumoniae* and from *A. vinelandii*, we tried to isolate nitrogenase messenger RNA from bacteroids of *R. leguminosarum*. It was necessary to purify the bacteroids from contaminating plant material by using sucrose gradient centrifugation, as only diffuse RNA bands were detected with a molecular weight of 0.4 x 10^6 and less if the gradient centrifugation was omitted. In RNA from purified bacteroids a band was detected with a molecular weight of approximately 1.2 x 10^6. This was the only *nif*-RNA which could be detected and presumably represents messenger RNA transcribed from the nitrogenase operon. The synthesis of this RNA was concurrent with the development of nitrogen fixation in the pea root nodules. The size of this RNA is smaller than the largest RNA detected in *K. pneumoniae* or *A. vinelandii*. The reason for this difference may be that the molecular weights of the subunits of nitrogenase are smaller in *Rhizobium* than in *Klebsiella*. The subunits of component II and at least one of the subunits of component I are encoded on this m-RNA

as fragments of pSA30 containing a part of *nif* D and a part of *nif* H both are hybridizing with the same mRNA (Fig. 3). Since it appears plausible that both subunits of component I will be encoded on the same mRNA, it appears probable that also in *Rhizobium nif* H, *nif* D and *nif* K constitute one operon.

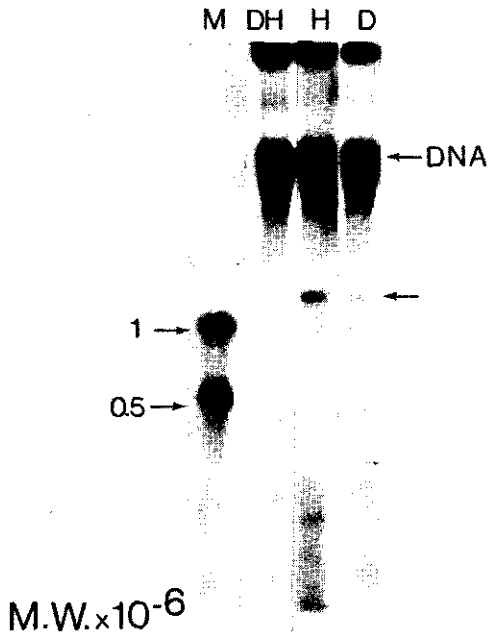

Fig. 3. Hybridizations of RNA from Rhizobium leguminosarum with fragment H and fragment D. RNA from bacteroids of Rhizobium leguminosarum was isolated 18 d after sowing. The RNA was blotted onto nitrocellulose filters (Sartorius, 0.1 μ) and hybridizations were performed under the same conditions as described in Fig. 2. The Bgl II - EcoR1 fragment containing a part of nif H and the Hind III-Bam H1 fragment containing a part of nif D and a part of nif K, respectively, were used as a probe. For comparison also the hybridization of bacteroid RNA with fragment DH is shown. The arrow indicates the position of the hybridizing RNA, detected by autoradiography.

The regulation of the synthesis of the nitrogenase components I and II of *R. leguminosarum* in pea root nodules has been studied by Bisseling *et al.* (11, 12). Waterlogging (12) caused the synthesis of component II to be repressed while the synthesis of CI was not affected. In other experiments the sequence of appearance of the

nitrogenase components in pea root nodules was followed by radioimmunoassays (11). It was shown that component I could be detected 1 or 2 days before component II. These results indicate that the synthesis of components I and II of nitrogenase of *R. leguminosarum* is regulated rather independently. Also in *A. vinelandii* component I was detected before component II during the onset of nitrogen fixation (J.G.J. Hontelez, unpublished results). In *K. pneumoniae* we have not been able to detect a separate regulation of components I and II. If indeed the genes for the subunits of the component I and II of nitrogenase are transcribed in a single messenger RNA, as we are inclined to conclude from the results presented in this paper, a regulation on the level of translation of the messenger RNA has to be postulated to explain the different rates of synthesis for component I and II in *R. leguminosarum* and *A. vinelandii*.

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GENERAL DISCUSSION

The subject matter of the research reported in this thesis is the role of plasmid-borne genes of *Rhizobium* in symbiosis and nitrogen fixation. Plasmid DNA was isolated from *Rhizobium leguminosarum strain* PRE and the expression of plasmid DNA in nitrogen fixing nodules was investigated by hybridization with RNA from bacteroids. These experiments are described in Chapters 3 and 4. From these experiments it is concluded that only one of the two plasmids present in PRE is strongly expressed in the symbiotic state. On this plasmid also the nitrogenase genes are located. Hooykaas *et al.* (1981) showed with genetic techniques that a large number, if not all, of the rhizobial genes involved in nodule formation and nitrogen fixation are carried on only one of the plasmids occurring in *R. leguminosarum*. Therefore they proposed to call such plasmids *Sym* plasmids, since obviously these plasmids are essential for the development of the symbiotic state. Our results which indicate that only one of the plasmids of *R. leguminosarum* is strongly expressed in the symbiotic state are in good agreement with the results of Hooykaas *et al.* (1981). *Sym* plasmids from different isolates of *R. leguminosarum* vary remarkably in size, as shows in Chapter 4. The reason why some *Sym* plasmids contain much more DNA then other *Sym* plasmids is still unknown. Besides the *Sym* plasmid a large number of *R. leguminosarum* strains contain at least one other plasmid, and many strains even more. No function has till now been allocated to these plasmids.

For comparison with the hybridizations of plasmid DNA with RNA from bacteroids, plasmid DNA was hybridized with RNA from free living bacteria. No plasmid specific hybridization with RNA from free living bacteria was detectable, as is shown in Chapter 3. A similar result was obtained by Prakash (1981), who could not detect hybridization of plasmid DNA with RNA from exponentially growing bacteria. However, with RNA from stationary bacteria he found hybridization with some restriction fragments of the plasmid. On the physical map of a *Sym* plasmid which he constructed the part which is expressed in stationary bacteria differs from the parts which are expressed in bacteroids (Prakash, 1981). Both his results and ours indicate that a number of plasmid-borne genes are only expressed in the symbiotic state, and that plasmid-borne genes in exponentially growing

bacteria are not expressed, or at the most at a level below detection. It is possible that in exponentially growing bacteria messenger RNA is more rapidly degraded than in bacteroids or in stationary phase bacteria. This could make the detection of specific expression of genes more difficult in exponentially growing bacteria. Rhizobia without *Sym* plasmids are still viable (Hooykaas *et al.*, 1981) so expression of this plasmid DNA is not necessary for growth of free living bacteria.

Among the plasmid-borne genes which are strongly expressed in the symbiotic state are the nitrogenase genes. This was demonstrated by hybridization of plasmid DNA with plasmid pSA30. Plasmid pSA30 is a recombinant plasmid containing the nitrogenase operon of *Klebsiella pneumoniae* (Cannon *et al.*, 1979). pSA30 is only homologous to the part of *Rhizobium nif* H, coding for the subunits of component II of nitrogenase, and to a part of *nif* D, coding for the α subunits of component I (Ruvkun and Ausubel, 1980). It is therefore not possible to determine with pSA30 as a probe in hybridizations with *Rhizobium* DNA whether *nif* K, coding for the β subunits of component I of nitrogenase, constitutes in a single operon together with *nif* D and *nif* H, as is the case in *Klebsiella pneumoniae* (MacNeal *et al.*, 1978). A decisive answer might be obtained if it would be possible to detect intact messenger RNA from the nitrogenase operon of *Rhizobium*. As described in Chapter 5 it is possible to isolate large messenger RNAs from bacteroids. Based on the molecular weight of the RNA and on hybridizations with fragments of pSA30 coding for *nif* H and *nif* D respectively it is concluded that in *Rhizobium nif* H, coding for the subunits of component II, is located in a single operon together with *nif* D and *nif* K, coding respectively for the α and β subunits of component I. Bisseling (1980) studied the regulation of the synthesis of the nitrogenase components I and II of *R. leguminosarum* in pea root nodules. It was shown by radioimmunoassays that component I could be detected 1 or 2 days before component II during the development of the root nodules. During waterlogging component I was still synthesized, whereas the synthesis of component II was repressed. These results indicate that the synthesis of component I and component II of nitrogenase of *R. leguminosarum* can be regulated rather independently. If the genes for the subunits of the components I and II are transcribed in a single messenger RNA, a regulation on the level of translation of

this messenger has to be postulated to explain the different rates of synthesis of component I and component II during nodule formation or in waterlogging root nodules. Whether this difference in regulation of the synthesis of component I and component II is on the level of transcription or translation cannot be concluded from our experiments. For that the synthesis of nitrogenase messenger RNA has to be examined under conditions which result in different rates of synthesis of component I and component II. Now the analytical devices are available isolated nitrogen fixing bacteroids may have good potentialities in further study of the mechanism of the regulation of the nitrogenase genes in *Rhizobium*.

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SAMENVATTING

Bacteriën van het geslacht *Rhizobium* zijn in staat om stikstof te binden in de wortelknollen van vlinderbloemige planten. Het is bekend dat *Rhizobium* grote plasmiden bevat, waarop genen liggen die betrokken zijn bij de herkenning van de plant, de vorming van de wortelknol en de stikstofbinding. Deze plasmiden vormen het onderwerp van dit proefschrift. In het eerste hoofdstuk is beschreven hoe plasmide-DNA werd geïsoleerd. Om kleine hoeveelheden radioactief plasmide te isoleren, werden alkalische sucrose gradiënten gebruikt. Grotere hoeveelheden plasmide werden geïsoleerd met behulp van CsCl-ethidium bromide gradiënten. De twee verschillende plasmiden die aanwezig zijn in *Rhizobium leguminosarum* PRE werden gescheiden door agarose gel electroforese. Met behulp van renaturatie kinetiek, electronen microscopische lengtemeting en agarose gel electroforese werd bepaald dat het kleinste plasmide in PRE een molecuul gewicht heeft van ongeveer 230 x 10^6. Op grond van agarose gel electroforese werd voor het grootste plasmide bij benadering een molecuul gewicht van 570 x 10^6 gevonden. Hybridisatie experimenten met RNA respectievelijk geïsoleerd uit gekweekte bacteriën en uit bacteroïden, lieten zien dat het plasmide DNA op grote schaal tot expressie komt in bacteroïde, maar niet of niet detecteerbaar in de vrij levende bacteriën. Na scheiding van de plasmiden in een agarose gel en hybridisatie met bacteroïde RNA bleek dat slechts één van de plasmiden nitrogenase genen bevatte, en dat alleen dit plasmide uitgebreid wordt overgeschreven in bacteroïden. De differentiatie van een *Rhizobium* bacterie in een stikstof bindende bacteroïde gaat gepaard met een vijf- tot zevenvoudige toename van de hoeveelheid DNA, maar de relatieve hoeveelheid nitrogenase genen blijft gelijk. Om te onderzoeken of de genen die coderen voor de subunits van de nitrogenase componenten I en II van *Rhizobium* één operon vormen, werd nitrogenase messenger RNA gezocht in het RNA van bacteroïden. Het RNA werd op grootte gescheiden met behulp van agarose gel electroforese en overgebracht op een nitrocellulose filter. Het nitrogenase messenger RNA werd gedetecteerd door hybridisatie met fragmenten van het plasmide pSA30, een recombinant plasmide dat de nitrogenase genen van *Klebsiella pneumoniae* bevat. De grootte van het RNA en de hybridisatie met gen H en gen D van *K. pneumoniae* maken aannemelijk dat in *R. leguminosa-*

rum de subunits van de twee componenten van het nitrogenase één operon vormen.

CURRICULUM VITAE

Adri Krol werd op 1 januari 1953 geboren te Leiden. In 1971 deed hij eindexamen HBS-B, waarna de studie scheikunde werd begonnen aan de Rijksuniversiteit Leiden. In maart 1977 deed hij doctoraal examen met als hoofdvak biochemie en als bijvakken tumorvirologie en reactiemechanismen in de organische chemie. Van 13 juni 1977 tot 13 juni 1981 was hij verbonden aan het laboratorium voor Moleculaire Biologie te Wageningen, eerst in een "pilot project", daarna als promovendus. Vanaf 1 november 1981 is hij in dienst van Z.W.O. verbonden aan het laboratorium voor Moleculaire Genetica van de Vrije Universiteit te Amsterdam.

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