CHARACTERIZATION OF THE NIFA REGULATORY GENE OF RHIZOBIUM LEGUMINOSARUM PRE

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CHARACTERIZATION OF THE NIFA REGULATORY GENE OF RHIZOBIUM LEGUMINOSARUM PRE

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen
op gezag van de rector magnificus,
dr H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 30 mei 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Het omslag-ontwerp toont een 35.000 maal vergrote bacteroide van Rhizobium leguminosarum.

Het ontwerp werd gemaakt door Walter Spek.

1. Het activeringsmodel voor het twee componenten regulatiesysteem voor bacteriële genen, bestaande uit een omgevingssensor en een activator is niet van toepassing op de door David et al. (1988) geidentificeerde fixLJ genen.

   Ronson et al. (1987) Cell 49: 579-581
   David et al. (1988) Cell 54: 671-683
   Dit proefschrift: Hoofdstuk 2

2. De suggestie van Nees et al. (1988) dat het asparagine residue in positie 593 het draaipunt vormt tussen de twee DNA bindende helices in het NifA eiwit van Azorhizobium caulinodans is onjuist.

   Nees et al. (1988) Nucl. Acids Res. 16: 9839-9853
   Dit proefschrift: Hoofdstuk 3

3. Het transcriptie activeringsmodel voor het Azorhizobium caulinodans nifA gen is gebaseerd op een onjuiste interpretatie van de eigen experimentele gegevens en gegevens uit de literatuur.

   Nees et al. (1988) Nucl. Acids Res. 16: 9839-9853

4. Kaminski et al. (1988) hebben ten onrechte het door hen geidentificeerde stikstoffixatie gen aangeduid als nifO; dit moet zijn fixO.

   Kaminski et al. (1988) Mol. Gen. Genet. 214: 496-502

5. De consensus sequentie CTGGYRYRN$_4$TTGCA van RpoN afhankelijke nif en fix promoters dient te worden veranderd in NNGGYRYRN$_4$TTG$\begin{matrix}C\\A\end{matrix}$N.

   Gussin et al. (1986) Ann. Rev. Gen. 20: 567-591
   Dit proefschrift: Hoofdstuk 5

6. Het is onwaarschijnlijk dat van de twee mogelijke R. meliloti NifA eiwitten van 59.9 kD en 57.5 kD, alleen het grootste actief is.

   Beynon et al. (1988) EMBO J. 7: 7-14

7. De constatering dat schizophrenie in een aantal gevallen is gekoppeld aan een locus op chromosoom 5 van het menselijk genoom, biedt de mogelijkheid dit psychisch ziektebeeld nader te definiëren en op langere termijn, gericht therapeutisch te behandelen.

   Sherrington et al. (1988) Nature 336: 164-167
   Kennedy et al. (1988) Nature 336: 167-170

8. De conclusie van Carrington et al. (1989) dat het C-terminale gedeelte van de 56 kD bladluis transmissie helper component van het tabaks-etsvirus protease activiteit bezit, lijkt voorbarig zolang een andere mogelijkheid niet kan worden uitgesloten.

   Carrington et al. (1989) EMBO J. 8: 365-370

9. De constatering dat het 4 kD prothoracicotroop hormoon van Bombyx mori een onduidelijke fysiologische betekenis heeft, is gebaseerd op een verkeerde bioassay.

   Ishizaki et al. (1987) in J. Law (ed): Molecular Entomology: 119-128

10. Na ingebruikname van de nieuwe analyse apparatuur voor hoge energie experimenten van het Centre Europeène de Rechèrche Nucleaire te Genève, zullen de meeste botsingen op Frans grondgebied worden geregistreerd.

11. Politiek beleid is toekomstgericht. De jeugd heeft de toekomst. Daarom dienen belangrijke politieke beslissingen ter beoordeling worden voorgelegd aan het Nederlandse volk en dient aan de visie van de Nederlandse jeugd groot gewicht te worden toegekend.

12. Het principe "de vervuiler betaalt" zou ook van toepassing moeten zijn op het schriftelijk en mondeling taalgebruik van Nederlandse politici, ambtenaren, journalisten, radio en TV presentatoren en nota-producenten.

13. De gewoonte van het Nederlands concertpubliek om na het eerste en het laatste deel van een klassiek concert op te staan voor een staande ovatie, wordt eerder ingegeven door de kwaliteit van het in de foyers gebodene, of de bereikbaarheid van de parkeerplaats, dan de kwaliteit van de muziekuitvoering.

14. De Nederlandse Spoorwegen dient terstond over te gaan tot inrichting van niet Walkman coupés.

Stellingen behorende bij het proefschrift:

"Characterization of the nifA regulatory gene of Rhizobium leguminosarum PRE"

Wageningen, 30 mei 1989 Peter W. Roelvink

τῆς τε γὰρ ὑπαρχούσης φύσεως μὴ χείροσι γενέσται ὑμῖν
μεγάλη ἡ δόξα καὶ ἧς ἂν ἐπ' ἐλάχιστον ἀρετῆς πέρι ἢ
ψόγον ἐν τοῖσ ἄρσεσι κλέος ᾖ.

(Thucidides, ii. 45§2)

Aan mijn moeder
Voor Gerda, Tineke, Ruud en Paul

CONTENTS

Met dit sop zijn ook zij overgoten die door het publiceren van boeken naar onsterfelijke roem streven. Zij hebben allen zeer veel aan mij te danken, vooral echter degenen die hun boeken met zuivere onzin vullen. Want degenen die in geleerde trant schrijven naar de smaak van een handjevol deskundigen..... die komen mij eerder beklagenswaardig dan gelukzalig voor. Zij martelen zich immers voortdurend af: ze voegen toe, veranderen, strepen door, schrijven het toch weer, beginnen opnieuw, werken om, laten het lezen, leggen het voor jaren in de la, en nooit is het hun naar de zin. Zo betalen zij voor hun onbeduidende beloning, d.w.z. een lofprijzing (maar dan van heel weinigen) zó'n hoge tol: zoveel nachtwerk, het aanzienlijk verlies van dromen- het heerlijkste dat er bestaat- zoveel zweet, zoveel martelingen! Tel daar nog bij op het verlies aan gezondheid, de aantasting van het uiterlijk voorkomen oogziekte of zelfs blindheid, armoede, nijd, het gemis aan plezier, voortijdige ouderdom, een vroege dood en wat dies meer zij. Zoveel ellende meent die wijze te moeten betalen om door de een of andere leepoog te worden gewaardeerd.

Desiderius Erasmus: Laus Stultitiae

CHAPTER 1

SCOPE OF THE THESIS

Few of us are vegetarians or even seriously consider the alternative during our lives. Is it because we feel at ease with the idea of killing cows and pigs and so on? Hardly. Few of us want to be reminded that there is a hunk of dead animal on our plate when we are served a steak. Mostly, we protect ourselves by a coy use of language and an elaborate set of conventions that allow us to maintain a double standard. The true nature of meat eating, like the true nature of sex and excretion, is only easy to refer to implicitly, hidden in euphemistic synonyms and allusions: "Veal cutlets", "making love", "going to the bathroom". Somehow we sense that there soul killing going on in slaughterhouses, but our palates don't want to be reminded of it.

Douglas Hofstadter / Daniel Dennett: The Mind's Eye

Scope of the thesis

Biological nitrogen fixation is an essential link in the nitrogen cycle. The ability to fix atmospheric nitrogen into ammonia is limited to a number of bacterial genera and blue green algae. Some bacterial species, like Klebsiella pneumoniae and Azotobacter vinelandii can fix nitrogen in the free living state. Other bacteria fix nitrogen when in a symbiotic association with a specific host plant. Examples are Bradyrhizobium japonicum with Glycine max L. (soybean), Azorhizobium caulinodans with Sesbania rostrata (a tropical legume) and Rhizobium meliloti with Medicago sativa L. (alfalfa). The soil bacterium R. leguminosarum PRE can infect the roots of its host plant, Pisum sativum L., the pea. This infection results in the formation of new anatomical structures on the roots, socalled root nodules. In these nodules the bacteroids, a modified form of the bacteria, fix atmospheric nitrogen into ammonia. The ammonia produced is made available to the host plant, which in its turn supplies the bacteroids with sugars, other metabolic compounds, salts etc. This process of interdependent living apart together is known as symbiotic nitrogen fixation. The genomes of both bacteria and plant have to interact to establish a functional root nodule. This interaction is hallmarked by the specific expression of both bacterial and plant genes. Among the genes specifically expressed in the bacteria are those needed for nodulation (nod genes) and for nitrogen fixation (the nif and fix genes). The nitrogen fixation genes are involved in the synthesis and assembly of the nitrogenase enzyme complex that fixes atmospheric nitrogen. Their expression is regulated by the product of the nifA regulatory gene. The nifA regulatory gene of R. leguminosarum PRE is the subject of this thesis. In recent years the nifA genes of several nitrogen fixing bacteria have been the subject of detailed investigations. The studies on Klebsiella NifA protein resulted in a model for the regulation of nitrogen fixation genes by this activator.

Chapter 1 reviews the regulation of transcription of the nifA gene itself and the regulation of nitrogen fixation genes by the NifA protein. The review focuses on two bacterial species: The free-living K. pneumoniae and the endosymbiotic R. meliloti. The model that emerges for nif gene regulation by NifA in Klebsiella is discussed.

Only few data is available concerning the nifA gene and the NifA protein in Rhizobiaceae. Given the fact that nifA genes from various diazotrophs are quite homologous, and appear to have the same target sites in the promoter region of the nitrogen fixation genes, it seems likely that the model will apply, albeit with modifications.

Chapter 2 deals with the nifA regulatory gene of R. leguminosarum PRE. The nucleotide sequence of this gene and the amino acid sequence, derived by computer, are given. Furthermore, the transcription start and termination sites were determined. The sequence of the nifA open reading frame reveals interesting features. It contains two translational initiation sites, which, in an E. coli background, are both functional. The relevance of this finding with respect to experimental results reported for R. meliloti nifA is discussed. The NifA polypeptide has a DNA binding domain at its C-terminal end, which consists of two helices separated by a linker of four amino acids.

Chapter 3 addresses the question why R. leguminosarum nifA::Tn5 mutants cannot be complemented by plasmids, which have the DNA fragment encoding the nifA gene. NifA::Tn5 mutants can be complemented though by plasmids carrying a DNA fragment encoding nifA and downstream genes. This shows that the transposon has a polar effect on the downstream nifB gene resulting in lack of complementation by nifA. It is shown that the nifA gene has a ρ-independent terminator, which is only partly efficient and allows approximately 50% readthrough from the nifA promoter.

Chapter 4 deals with the analysis of one of the target sites of the nifA gene product: the nifH promoter region. The sequence of this region is given and the significance of the promoter elements is evaluated in heterologous and homologous backgrounds.

CHAPTER 2

REGULATION OF NITROGEN FIXATION IN DIAZOTROPHS: THE REGULATORY *NIFA* GENE AND ITS CHARACTERISTICS

Iets is nooit het een of het ander, maar altijd zowel het een als het ander, het een noch het ander, en het een èn het ander.

A.G. Kloppers

REGULATION OF NITROGEN FIXATION IN DIAZOTROPHS: THE REGULATORY NIFA GENE AND ITS CHARACTERISTICS

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1. INTRODUCTION

Biological nitrogen fixation is an important link in the nitrogen cycle. The ability to converse atmospheric nitrogen to metabolically usable compounds is confined to a few classes of bacteria and blue green algae. Nitrogen-fixing organisms can be divided in two groups: the free living species that fix nitrogen for their own purpose, like Klebsiella pneumoniae and Azotobacter vinelandii, and the species that depend on an intimate association with plants, like the cyanobacterium Anabaena azollae with the waterfern Azolla, or on intracellular symbiosis, like the bacterial genera Rhizobium, Bradyrhizobium and Azorhizobium with various, specific leguminous host plants, and the actinomycete Frankia with alders. Nitrogen fixation by Rhizobium species in symbiosis with members of the plant family Leguminosae, among others pea, (Pisum sativum L.), soybean (Glycine max L.) and alfalfa (Medicago sativa L.) is of great importance to agriculture. The interaction of bacteria and plants is a very complicated multistep process that involves gene products from both symbiotic partners. Rhizobium bacteria interact with their specific host plants, which results in the formation of newly developed anatomical structures on the main and lateral roots of the plants, the root nodules. In some cases, e.g. Azorhizobium caulinodans with its host plant Sesbania rostrata nodules are also formed on the stem. In the nodules some cells contain bacteroids, specialized forms of the bacterium, capable of fixing nitrogen by means of the oxygen sensitive enzyme nitrogenase. This enzyme complex, which consists of three different polypeptides, fixes atmospheric nitrogen into ammonia. The symbiotic bacteria-plant interaction is marked by the symbiosis specific expression of both bacterial and plant genes.

In this chapter we will summarize and discuss data specifically concerning the regulation of the genes that are expressed in nitrogen fixing bacteria. We will focus upon two bacterial species that are exemplary: the facultative anaerobic K. pneumoniae and the obligate aerobe R. meliloti, the symbiont of alfalfa (M. sativa L.).

We will first discuss the basic nitrogen regulation in Klebsiella: the chain of reactions that leads to the activation of genes involved in nitrogen fixation. Then, we will focus upon the operon that has a regulatory role in nitrogen fixation, nifLA. Activation of nitrogen fixation genes in R. meliloti follows a path that differs in several aspects from that in Klebsiella. An emerging model for this activation will be discussed. The regulatory nifA gene plays a key role in all nitrogen fixing species. We will summarize the data on the structure and function of the nifA gene product. A general model for the activation of nitrogen fixation genes will be discussed that, for the lack of data for Rhizobium species, will be mainly based on data published for K. pneumoniae.

The nitrogen fixation genes fall into two groups: nif and fix genes. Nif genes were originally described in Klebsiella and have homologues in other nitrogen fixing species. One example is nifA, the gene that encodes the regulatory NifA protein. Fix genes were originally identified in nitrogen fixing species other than Klebsiella and have no homologue in this species. Examples are the fixABC genes thought to be involved in electron transport to the nitrogenase complex. As described below other genes involved in the nitrogen metabolism are ntr and gln (see table 1). In designating genes and their products we will follow the primeval genetic rule: non capitalized underlined designations refer to genes (e.g. nifA), capitalized ones to products (e.g. NifA).

Many features of both the structure and the regulation mechanism of nitrogen fixation genes have been conserved in evolution. It is the aim of this review to give a general overview of both similarities and differences in the nitrogen fixing apparatus of both the free living K. pneumoniae and a symbiotic nitrogen fixer like R. meliloti. Reviews on nodule formation and involvement of plant genes in this process have been presented elsewhere (Long 1984; Downie & Johnson 1986; Nap 1988).

2. REGULATION OF NITROGEN FIXATION IN THE FREE-LIVING DIAZOTROPH K. PNEUMONIAE

2.1. Basic nitrogen regulation in K. pneumoniae

K. pneumoniae is a bacterium that fixes atmospheric nitrogen into ammonia in response to low fixed nitrogen levels under microaerobic conditions. The 21 nif-genes, organized in 8 distinct operons (Merrick 1988), are located on a 24 kb chromosomal fragment, the sequence of which has been determined (Arnold et al. 1988). The nif genes are subject to two levels of regulation: one global and one nif-specific. Global regulation is brought about by the nitrogen regulation system (ntr-system), which also controls the expression of several other nitrogen assimilatory genes in enteric bacteria (Magasanik 1982; Merrick 1987; Dixon 1987). Figure 1 illustrates the ntr-control of nitrogen assimilation in K. pneumoniae. Central to the ntr-circuitry is a set of (modified) gln-gene products that act in a cascade fashion to biochemical changes in the cell (see Table 1). The glnD product, an uridylyltransferase (UTase) responds directly to the intracellular ratio of glutamine to 2-ketoglutarate; a high ratio (Gln>>2KG) indicates a surplus of fixed ammonia, a low ratio (2KG>>Gln) a shortage (Magasanik 1982). The UTase is activated by 2-ketoglutarate and inhibited by glutamine. Thus, during nitrogen limitation, the UTase is activated and uridylylates P_{II}, the product of the glnB gene (Bueno et al. 1985). P_{II} exists in the cell as a tetrameric polypeptide. During the activation reaction, a uridylyl-group from UTP is coupled to all four subunits of the UTase (Holtel & Merrick 1988). If all four subunits are uridylylated, P_{II} is maximally active. The activated P_{II}, P_{II}-UMP, interacts with the ntrB product, which leads to phosphorylation of NtrC (Ninfa & Magasanik 1986). The resulting NtrC-P is a DNA-binding activator that interacts with RNAP (RNA-polymerase) complexed with RpoN, a sigma factor encoded by the rpoN gene (also known as ntrA, glnF and rpoE) that recognises nif and fix promoters. From the sensing of the N-content of the cell by the UTase and the resulting activating modifications of other proteins in the cascade, the regulatory system, that first reacted to a biochemical signal, now influences other operons at the transcriptional level.

Basic nitrogen regulation in *Klebsiella pneumoniae*

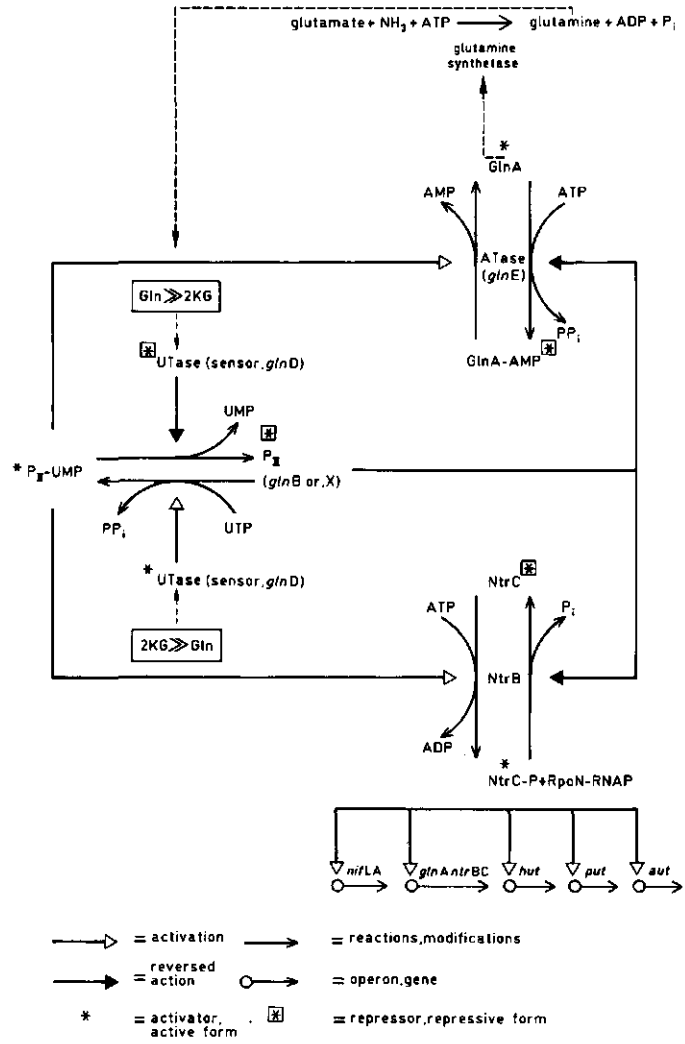

Fig. 1: **Schematic representation of global nitrogen regulation in K. pneumoniae. Different symbols are explained in the figure. The genes and gene products involved as well their functions are explained in Table 1. This figure is intended to be self-explanatory; the reader is advised to follow the different pathways starting from either of the two initial situations (Gln>>2KG or 2KG>>Gln).**

TABLE 1

Gene	Product	Function	Mode of action
glnD	uridyl transferase (UTase)	"sensor" of glutamine to 2-ketoglurate ratio	regulates P_{II} mode of action by (de)uridylylation
glnB	P_{II}	regulates activities of both NtrB and ATase	activates NtrB and ATase when present as P_{II}-UMP, reverses their action when present as P_{II}
glnE	adenyl transferase (ATase)	(de)adenylylation of glutamine synthetase	acts in concert with P_{II}-UMP to deadenylylate. Acts in concert with P_{II} to adenylylate
glnA	glutamine synthetase (present in adenylylated form)	catalyzes conversion of glutamate and ammonia to glutamine	deadenylylation leads to activation. Adenylylation leads to deactivation
ntrB	NtrB	dephosphorylase/kinase	together with P_{II}-UMP, NtrB activates NtrC by phosphorylation. Together with P_{II}, NtrB deactivates NtrC by dephosphorylation
ntrC	NtrC	transcriptional activator	active when phosphorylated. Inactive when dephosphorylated.

Table 1: Genes and gene products, which play a major role in nitrogen regulation of K. pneumoniae.

Examples of such transcriptionally regulated operons are those necessary for the utilization of poor nitrogen sources like histidine (hut), proline (put) and arginine (aut) as well as glnAntrBC and nifLA. Ammonia formed as a result of the nitrogen fixation process is made available to the biochemical processes in the cell through the basic reaction catalysed by glutamine synthetase (GlnA)

$$\text{Glutamate} + NH_3 + \text{ATP} \xrightarrow{\text{GlnA}} \text{Glutamine} + \text{ADP} + P_i$$

The production of ammonia influences the glutamine to 2-ketoglutarate ratio. Sensing of the increased glutamine content (Gln>>2KG) leads to a reversal of the activation sequence. Key enzyme in this sequence is the glnA encoded glutamine synthetase which exists in the cell as an adenylylated, inactive dodecamer (Magasanik 1982). Deadenylylation, which leads to activation of GlnA, is brought about by the concerted action of an adenylyltransferase (ATase), encoded by the glnE gene, and P_{II}-UMP. The produced glutamine increases the glutamine to 2-ketoglutarate ratio resulting in partial reversal of UTase activity (glnD). The UTase thus deuridylylates P_{II}. The presence of unmodified P_{II} reverses the activity of NtrB, which dephosphorylates NtrC-P, rendering it inactive, and thereby halting the activation of the nif regulatory cascade. The presence of unmodified P_{II} also reverses the action of the ATase (glnE) resulting in adenylylation and inactivation of glutamine synthetase. Recent experiments with glnB mutants indicate that the absence of the P_{II} protein does not alter the ability of NtrB and NtrC to react to the nitrogen status of the cell. This suggests that yet another sensory (backup) system able to react to shifts in the glutamine to 2-ketoglutarate ratio is present in Klebsiella (Holtel & Merrick 1988). It is notable that several proteins in the ntr-cascade can be modified, and regulated in their activity at the protein level. Thus P_{II} is not inactive, but reverses the action of P_{II}-UMP, the uridylylated form. Both P_{II} forms can exist at the same time; the same goes for glutamine synthetase. Part of it is adenylylated and inactive, while another part is deadenylylated and active. Thus, the cell maintains a balance and is able to react swiftly to shifts in N-levels.

In summary: the global ntr-system consists of a biochemical sensor and gene products capable of reacting in a cascade fashion to sensor signals, eventually leading to transcription activation in the case of an N-shortage. The interaction of NtrC-P with RpoN-RNAP leads to activation of several important genes and operons, the glnAntrBC operon being an example. NtrC-P regulates the transcription of its own operon; this regulation is discussed elsewhere in great detail (Gussin et al. 1986; Dixon 1987). In the next paragraph we will discuss the regulatory nifLA operon. The two genes in this operon, encoding the repressor NifL and the activator NifA, form the link between the global nitrogen regulation and nif specific regulation and therefore are of major importance.

2.2. Regulation of nif gene expression: role of the nifLA operon

The nifLA operon is the master switch for nif gene expression. NifA and RpoN-RNAP together activate the nif genes that are characterized by the presence of RpoN-specific promoters (Gussin et al. 1986), and in the majority of cases reported so far upstream activator sequences (UAS), to which NifA specifically can bind (Morett & Buck 1988). The structural aspects of nif-specific promoters will be discussed in the section on nif regulation in Rhizobiaceae. NifL, in reaction to oxygen and/or ammonia, counteracts NifA activity. NifA and NtrC are rather similar in a number of respects like protein sizes and iso-electric points (Merrick 1983) and arise from operons containing genes encoding proteins involved in repression and activation. Furthermore, comparisons of the amino acid sequences derived from the DNA sequences of the activators NifA and NtrC reveal stretches of up to 50% homology located in the central and C-terminal portions of these proteins. NifA can replace NtrC-P in activation of the promoter of nifLA (Ow & Ausubel 1983; Merrick 1983; Drummond et al. 1983). It seems likely that this activation is a result of a direct NifA interaction with RpoN-RNAP at the promoter since the nifLA promoter region does not contain an UAS or NtrC binding site (see Khan et al. 1986). NtrC cannot replace NifA in activating the other nif genes (Ow & Ausubel, 1983). Taken together these results confirm the suggestion by Ow & Ausubel (1983) that the nifLA operon may have descended from an ancestral ntrBC unit.

This makes physiological sense, since the nifLA operon serves as a nitrogen fixation specific stringent control on the synthesis of the oxygensensitive nitrogenase and on the energy-intensive nitrogen fixation process independent of other systems of nitrogen assimilation that are under control of the global ntr-system (Drummond et al. 1983).
The exact nature of NifL mediated repression has not been elucidated yet. NifL represses transcription of nif genes by modulating the action of NifA directly. Several more or less attractive proposals exist for the NifL repression mechanism e.g. modification of NifA by NifL, a ligand or covalent modification, or protein-protein binding (discussed in Drummond & Wootton 1987). Collins et al. (1986) suggested that NifL is also involved in specifically destabilizing, that is, enhancing the breakdown of, the nif mRNAs as a reaction to oxygen, and, to a lesser extent, ammonia. In this respect the presence of a region in NifL with homology to the exonuclease product of the gene exo of bacteriophage lambda may be relevant (Drummond & Wootton 1987). Whether this finding has any functional significance remains to be established.

3. REGULATION OF NITROGEN FIXATION IN RHIZOBIACEAE

3.1. The regulatory role of nifA

Regulation of nitrogen fixation in Rhizobiaceae is still less well understood than in K. pneumoniae. No unified model for nif regulation in rhizobial species exists. Taking the well studied R. meliloti as a starting point a regulation model will be discussed which in part is hypothetical. It is based partly on results of regulation studies in R. meliloti, and several of the model's elements are based on the assumption that regulatory elements common to Klebsiella and Rhizobium species, at the DNA or the protein level, serve the same purpose or have the same function. Rhizobiaceae like B. japonicum and A. caulinodans differ in several aspects from R. meliloti and where necessary we will point out those differences. The NifA protein is of cucial importance for the expression of nif and most of the fix genes in all nitrogen fixing species studied so far.

Because of the lack of sufficient data on Rhizobium species the emerging model of nif gene activation in Rhizobiaceae is primarily based on results obtained with K. pneumoniae. Transcription of nif and most of the fix genes is NifA dependent; mutations in nifA lead to a Fix$^-$ phenotype (reviewed in Gussin et al. 1986).
The earlier mentioned rpoN gene encoding the sigma factor σ-54 (Hirschmann et al. 1985, Ronson et al. 1987) is common between K. pneumoniae and Rhizobiaceae and also plays an essential role in nif regulation. This gene, cloned from R. meliloti, has been sequenced and shows 38% amino acid homology with the K. pneumoniae sigma factor encoded by the ntrA gene (Ronson et al. 1987, discussed in Helman & Chamberlin 1988). In the following paragraphs the regulatory nifA gene and its product NifA will be discussed.

3.2. Regulation of nifA expression in R. meliloti

Szeto et al. (1984) first identified a symbiotic regulatory gene (nifA) in R. meliloti with strong homology to E. coli ntrC and somewhat less homology to K. pneumoniae nifA. Previous experiments had already shown that a Tn5 mutation in this gene led to a Fix$^-$ phenotype and that no accumulation of *NifHDK* proteins was detectable (Zimmerman et al. 1983). Other nifA-like genes were isolated from different Rhizobiaceae like B. japonicum (Thöny et al. 1987), R. leguminosarum (Schetgens et al. 1985; Grönger et al. 1987) and A. caulinodans (Pawlowski et al. 1987). The availability of nifA genes allowed the construction of nifA::lacZ transcription and translation fusions. In combination with nif::lacZ fusions that are activated by NifA, the nifA constructs played a major role in the analysis of the first steps of the regulation of nitrogen fixation in Rhizobiaceae. Experiments to study the activation of plasmid borne nifA::lacZ and nifH::lacZ fusions together with direct mRNA measurements, revealed that nifA expression in R. meliloti is induced when the oxygen concentration is reduced to microaerobic levels (Ditta et al. 1987). This indicated that sensing of oxygen levels is a fundamental aspect of nif gene expression in R. meliloti. Using an approach in which a 310 Kb fragment of the symbiotic plasmid (pSym) from R. meliloti was analysed for transcription during symbiosis, a DNA region was identified containing fix-genes that were transcribed independently of

NifA (David et al. 1987). This region was shown to encompass at least five NifA independent fix genes (Kahn et al. 1988). Experimental results obtained by Virts et al. (1988) suggested that some of the genes in this DNA region have a regulatory function. Among these genes, fixLJ, were identified by transposon mutagenesis and genetic analyses of the DNA region showed that they are transcribed independently of NifA (David et al. 1988). The DNA sequence and amino acid homology analysis by computer (David et al. 1988) revealed that the fixLJ genes share homology with a family of bacterial regulatory proteins for which a sensor/activator model was proposed (Ronson et al. 1987). According to this model (see Fig. 2) the N-terminal part of a receptor protein receives a signal that leads to a conformational change in the conserved C-terminal part of the protein.

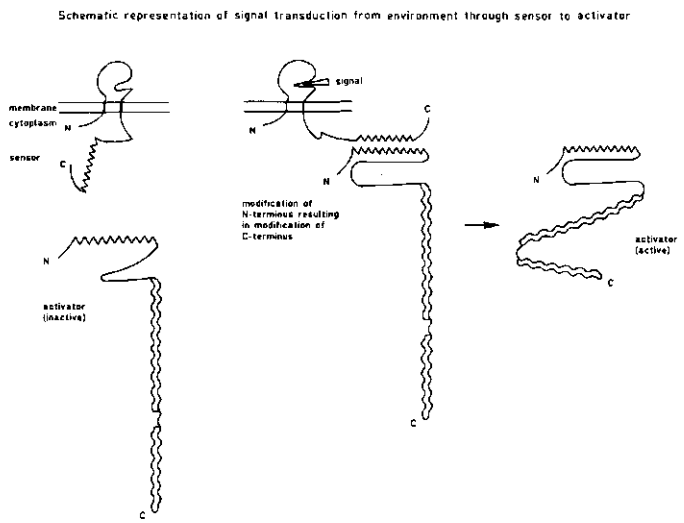

Fig. 2: Schematic representation of the sensor/activator model for bacterial regulatory protein pairs. The system consist of a sensor protein anchored in the membrane and an activator in the cytoplasm. Binding of a signal molecule to the external N-terminal part of the sensor induces a conformational change in the C-terminal part, which then interacts with the N-terminus of the activator protein. This interaction results in a conformational change in the C-terminal part of this protein causing a shift from an inactive to an active state of the activator protein.

This altered C-terminus then interacts with, and modifies, the conserved N-terminal portion of the regulator protein. It is this interaction that modulates the conformation of the non-conserved C-terminal domain of the activator causing the switch between non-active and active or repressor and activator forms (Ronson et al. 1987). Based on this general model, a regulatory model for the function of FixL and FixJ was proposed by David et al. (1988) in which FixL functions as the sensor of the oxygen level. Fig. 3 shows a hypothetical model based on their data and our hypothesis discussed below.

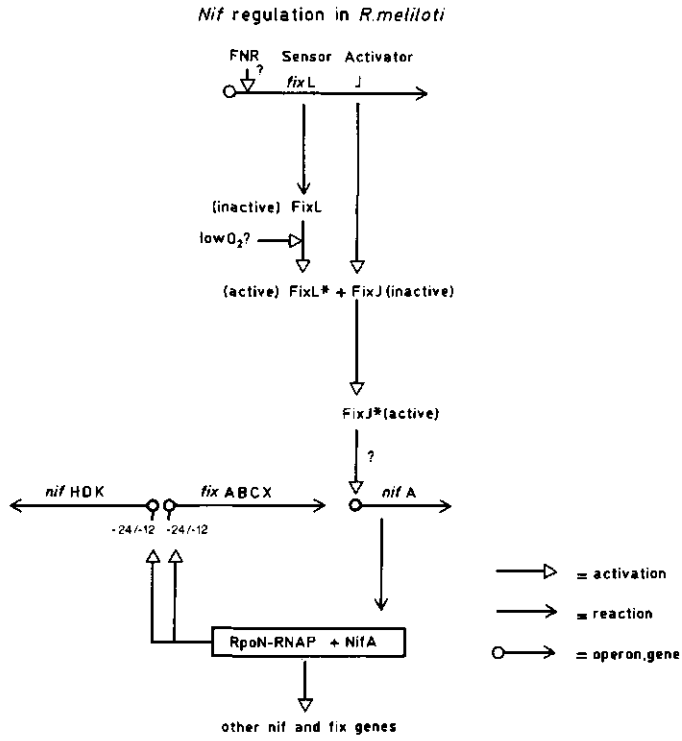

Fig. 3: Hypothetical model for activation of nifA transcription in R. meliloti by the fumarate-nitrate reduction regulator (FNR) protein. Transcription of fixLJ is activated by FNR; FixL after activation by a low oxygen concentration activates FixJ, which induces nifA transcription. NifA interacts with RpoN-RNAP in transcription of nif and fix genes.

The FixL protein has two transmembrane regions that are probably anchored in the membrane, thus exposing a region in the periplasm, which is hypothesized to react to environmental oxygen levels. A change to a microaerobic level is thought to change the conformation of the N-terminus and thereafter the C-terminus of the FixL protein. Activated FixL interacts with FixJ and activates this product. The activated FixJ then activates nifA transcription possibly by DNA-binding as evidenced by the presence of a helix-turn-helix motif thought to be crucial for such activity (Drummond et al. 1986; David et al. 1988). A DNA region, which may be the possible target site of the fixJ product has been identified. Using progressive 5'-deletions of nifA::lacZ fusions, a region between -62 and -40 relative to the point of transcription initiation was found to be essential for activation under microaerobic conditions (Virts et al. 1988). Even though the presented model is attractive and supported by experimental evidence some points remain unclear. The ability to sense oxygen in proteins depends on the presence of a sensing group (e.g. a haem group) which is usually attached to cysteine residues (see Drummond & Wootton, 1987, and references therein). The periplasmic region of FixL exposed to the environment, however, does not contain any cysteine residue (David et al. 1988). Nothing is known about the regulation of transcription of the fixLJ operon. Computer analysis of the promoter region (Roelvink, unpublished) reveals that it contains a sequence which closely resembles a target site of the fumarate-nitrate reduction regulator (FNR: see table 2) for which the term anaerobox was coined (Nees et al. (1988).

FNR is a redox-sensitive regulatory protein that senses intracellular oxygen levels. The expression of the FNR gene under anaerobic conditions is most likely regulated by Catabolite Activating Protein-cAMP (CAP-cAMP). FNR activates transcription of genes expressed under anaerobic conditions (Spiro & Guest 1987 and references therein). About 50 bp downstream of the proposed fixL anaerobox, sequences can be identified that resemble the consensus of "non-nitrogen regulated" Rhizobium promoters, TTRANN-17 bp-RARRRR (R = purine; see Ronson & Astwood, 1985). The identified sequence reads TTTACG-16 bp-AAGAGT. We hypothesize that under microaerobic conditions the FNR protein, which is capable of sensing the internal oxygen level (Spiro & Guest 1987) in concert with RNAP and the basic Rhizobium sigma factor RpoD activates transcription of the fixLJ operon.

TABLE 2

Ec narG	CTCTTGATCGTTATCAATTC	(DeBruijn et al. 1988)
	***** *****	
Ec nirB	AATTTGATTTACATCAATAA	(DeBruijn et al. 1988)
	***** *****	
Bj hemA	TCTTTGATCGGGATCAAGTT	(McClung et al. 1987)
	***** *****	
Ac nifA	AATTTGATTTACATCAAACC	(DeBruijn et al. 1988)
	***** *****	
consensus	TTGAT----ATCAA	
	***** ****	
Rm fixLJ	ACATTGATCACGGTCAATAC	(David et al. 1988)

Table 2: Comparison of a hypothetical "anaerobox" sequence of R. meliloti fixLJ with the consensus for anaeroboxes of FNR regulated genes in different organisms.
Ec= E. coli Bj= B. japonicum Ac= A. caulinodans Rm= R. meliloti
hemA= 5-aminolevulinate synthase nirB= nitrite reductase
narG= nitrate reductase

In summary it can be said that in R. meliloti, like in Klebsiella, the transcription of nifA is the result of the sensing of a signal. The difference between Klebsiella and R. meliloti is that the former, under conditions of N-limitation and low O_2, reacts to an internal biochemical signal (low fixed nitrogen), and the latter to a different internal signal, the oxygen level.

3.3. The sensor/activator model for sensing oxygen

In the model proposed by Ronson et al. (1987) for bacterial sensor/activator proteins, the sensor reacts to an environmental signal and through modification of its own protein structure (signal transduction) is switched in an "active" mode. The "active sensor" then modifies the activator, which thereby is switched to an "active" mode. The general model is based on amino acid sequence comparisons of protein pairs like A. tumefaciens VirA/VirG, R. leguminosarum DctB/DctD and E. coli PhoR/PhoB. In these cases the sensor/activator pairs react to environmental stimuli such as plant exudates, the presence of C_4-dicarboxylates and phosphate limitation respectively.

Newly identified sensor/activator pairs like FixL/FixJ, and earlier ones like NifL/NifA and NtrB/NtrC have been included in the family of protein pairs on the basis of amino acid homologies. In the preceding paragraph we offered the alternative hypothesis that in Rhizobium FNR, instead of FixL, senses the internal oxygen level. This does not imply that FixL plays no role in nitrogen fixation. It may modify FixJ activity in a way comparable to that in which NtrB modulates NtrC activity (by (de)phosphorylation). Our hypothesis thus implies that FixL/FixJ are not regular members of the sensor/activator family despite the amino acid homology. Likewise, from sequence comparisons of NifA/NtrC (Drummond et al. 1986) and NifL/NtrB (Drummond & Wootton 1987) it can be concluded that these protein pairs also differ from the sensor/activator model. NtrB, in sensu strictu, is not a sensor; it is one of the proteins in the cascade that follows the sensing of an internal signal by the product of glnD, the UTase (discussed in a preceding paragraph). At present, it is not clear whether NtrB itself phosphorylates or dephosphorylates NtrC, the DNA-binding activator (see Austin et al. 1987; MacFarlane & Merrick 1987) or that it stimulates autophosphorylation by NtrC itself, as was suggested by Drummond & Wootton (1987).

The NifL protein reacts to and may be the sensor of the internal but not external oxygen and nitrogen levels. Furthermore, deletion analysis of the K. pneumoniae nifL gene showed that NifL is only required for the inactivation (and not the activation) of its partner NifA (Arnott et al. 1988).

In summary: of the FixL/FixJ, NifL/NifA and NtrB/NtrC "sensor/activator" pairs, the sensors do not meet the demands of the model proposed by Ronson et al. (1987) in that they sense an internal stimulus (NifL), or are probably not sensing an environmental stimulus (FixL), or do not sense the internal signal itself (NtrB). Furthermore, FixJ and NifA can function independently of FixL (Kahn et al. 1988) and NifL respectively (Arnott et al. 1988). We propose to extend and modify the sensor activator model and make it fit with the data, instead of overinterpreting the data to make them fit in the model.

3.4. Nif regulation in A. caulinodans and B. japonicum

Nitrogen fixation by A. caulinodans differs in two essential aspects from that in R. meliloti. First A. caulinodans, in contrast to R. meliloti, is able to fix nitrogen at an appreciable rate in free-living conditions (de Bruijn et al. 1988), which makes it an ideal model system for studying nif-regulation in Rhizobiaceae. Second, it can induce nitrogen fixing nodules on stems as well as roots of its host Sesbania rostrata. Our understanding of the regulation of nifA expression in A. caulinodans is still rather fragmentary. The nifA promoter region contains an anaerobox (see table 2) indicating a possible influence of FNR (Ratet et al. 1988). Interestingly, an operon that may be related to the R. meliloti fixLJ operon has been identified in A. caulinodans and termed ntrYX (Pawlowski et al. 1988; De Bruijn et al. 1988). Mutations in this operon result in the lack of oxygen repression of a plasmid borne nifA::lacZ fusion (De Bruijn et al. 1988). Furthermore, the nifA promoter region contains an NtrC consensus promoter indicating possible ntr-control of nitrogen fixation (Ratet et al. 1988). NtrC mutants have a partial Nif^- phenotype (10-15% of wild type) in culture. Such mutants have a delayed Fix phenotype in planta indicating that ntr regulation of nif expression may be overridden under these circumstances. Pawlowski et al. (1987) suggested that NtrC controls nifA expression, in response to fluctuations in the concentration of "rich and poor" nitrogen sources. Apart from these three controls nifA expression also seems to be influenced by supercoiling and negative feedback by nifA itself (see De Bruijn et al. 1988).

A rather speculative model, based mainly on sequence analysis, reconciling both FNR and NtrC control of nifA expression in A. caulinodans has been proposed recently by Nees et al. (1988).
B. japonicum, the endosymbiont of soybean (Glycine max) also differs from R. meliloti in several aspects. In culture it is slow-growing and under the proper conditions will fix nitrogen, like A. caulinodans. In B. japonicum the nifA gene is preceeded by fixR in an operon (fixRnifA), which has an RpoN-dependent promoter. The upstream activator sequence (UAS) in the fixRnifA promoter region, which is essential for optimal expression of this operon, does not resemble the UAS of other nif genes regulated by NifA (Thöny et al. 1988). This finding led to the hypothesis that another, as yet unknown, regulator effectuates the expression of this operon (Hennecke et al. 1988). This regulator does not react to oxygen levels since expression of the fixRnifA operon has been observed under aerobic, microaerobic and anaerobic conditions and in root nodules (Hennecke et al. 1988).

3.5. Regulation of the expression of nif genes

3.5.1. The regulatory nifA protein

In this paragraph we will discuss structural aspects of the NifA protein. NifA genes from various diazotrophs have been cloned and the nucleotide sequences have been determined for K. pneumoniae (Buikema et al. 1985; Drummond et al. 1986), K. oxytoca (Kim et al. 1986), A. vinelandii (Bennett et al. 1988; R. meliloti (Buikema et al. 1985; Weber et al. 1985), R. leguminosarum 3855 (Grönger et al. 1987), R. leguminosarum PRE (Roelvink et al. submitted), B. japonicum (Thöny et al. 1987) and A. caulinodans ORS 571 (De Bruijn and Ratet, personal communication; Nees et al. 1988). Comparison of the derived amino acid sequences revealed several interesting features. A comparison between the NifA amino acid sequences of K. pneumoniae, R. meliloti and R. leguminosarum PRE is shown in Table 3. In a comparison between K. pneumoniae NifA, NtrC and R. meliloti NifA, Drummond et al. (1986) assigned possible functions to the NifA amino acid sequences.

TABLE 3

```
                  10        20        30        40        50        60
K.p.  MIHKSDSDTT VRRFDLSQQF TAMQRISVVL SRATEASKTL QEVLSVLHND AFMQHGMICL
                                 **  *         *         *         * *
R.l.              MIKPEARL HILYDISKEL ISSFPLDNLL KAAMNALVEH LRLRDGGIVI
                   * *   *     * *** *       *   *       * *    ** * **  *
R.m.              MRKQDKRS AEIYSISKAL MAPTRLETTL NNFVNTLSLI LRMRRGGLEI

                  70        80        90       100       110       120
K.p.  YDSQQEILSI EALQQTEDQT LPGSTQI-RY RPGEGLVGTV ------LAQG ASLVLPRVAD
        *  *                                    *                * ***
R.l.  HGSGGEPWI- ----NVRAPI GDDVRSRSLT IEQADAIDRV IASGEKHFGK NSVVLPVKVN
        * **  *       *         *          *** *                 ** *   *
R.m.  PASEGETKI- -----TAATR NSGSPSAADY TVPKAAIDQV ------MATG RLVV-PDVCN

                 130       140       150       160       170       180
K.p.  DQRFL----- ------DRLS LYDYDL-PFI AVPLMGPHSR PIGVLAAHAM ARQEERLPAC
                        *          *  * **
R.l.  RKAIGALWID FAQKSGDQDE T----LLAMI AV--------- ---------- ----------
                        **         * * *
R.m.  SELFK----- ------DQIK WRGIGPTAPI AAAVEVDHET -GGMLWFECA EESDYDYEEE

                 190       200       210       220       230       240
K.p.  TRFLEVTANL IAQTIRLMIL P------TSA AQAPQQSPRI ERPRACTPSR GFGLENMVGK
                 * * *                 *  * * *       *                 **
R.l.  ---------L IGLTCQRDRE LCSDGGAVAE EQQAGQIPKI KPKP--HPTQ LDKIDWIVGE
                 *  *    *           ** *** *                 * * * * **
R.m.  VHFLSMAANL AGRAIRLHRT ISRRERTFAE EQQEQQNSRD EQSQSSARQR LLKNDGIIGE

                 250       260       270       280       290       300
K.p.  SPAMRQIMDI IRQVSRWDTT VLVRGE-SGT GKELIANAIH HNSPRAAAAF VKFNCAALPD
                 ***         * *     ** *** *** ***  * ***   * *   **  * *****
R.l.  SPAIKRVLAT TNIVARRTSA VLLRGE-SGT GKECFARAIH ALSIRKSKAF IKLNCAALSE
                 * *     *     *      *** **  ** ******  **   * *  * * ** ** ****
R.m.  STALMTAVDT AKVMAETNSI VLL-GETGGT GKECFAKLIH QHSTRQKKPF IKFNCPALSE

                 310       320       330       340       350       360
K.p.  NLLESELFGH EKGAFTGAVR QRKGRFELAD GGTLFLDEIG ESSASFQAKL LRILQEGEME
                  ********* *********   ** ******  **** *****   *  ***** ** ***** *
R.l.  TVLESELFGH EKGAFTGALL QRAGRFELAN GGTLLLDEIG DVSPQFQAKL LRVLQEGEFE
                  *********  *********  ** **** ** ***********     * ***** *** ******
R.m.  SLLESELFGH EKGAFTGAIA QRVGRFESAN GGTLLLDEIG EIPPAFQAKL LRVIQEGEFE

                 370       380       390       400       410       420
K.p.  RVGGDETLRV NVRIIAATNR HLEEEVRLGH FREDLYYRLN VMPIALPPLR ERQEDIAELA
                 * **  ** *  ** * ***   **  *  *  ** ***** * * ** *****  *  **  **
R.l.  RLGGTKTLKV DVRVICATNK NLEVAVLRGE FRADLYYRIN VVPIILPPLR QRDGDISLLA
                 * *********  *** * ****  ** **  **  ** *******   ** *******  ***** ***
R.m.  RVGGTKTLKV DVRLIFATNK DLEMAVQNGE FREDLYYRIS GVPLILPPLR HRDGDIPLLA

                 430       440       450       460       470       480
K.p.  -HFLVRKIAH SQGRTLRISD GAIRLLMEYS WPGNVRELEN CLERSAVLSE SGLIDRU---
                   **          *        **  *     *******  * *  * * *   * *
R.l.  QVPLEQ-FNK ANDRNCDFAP SAIDILSKCA FPGNVRELDN CVQRTATLAS SNTITSSDFA
                  ** * **   * *   ***  ** ******  ********  * ** ******  * ********
R.m.  RAFL-QRFNE ENGRDLHFAP SALDILSKCK FPGNVRELEN CVRRTATLAR SKTITSSDFA

                 490       500       510       520       530       540
K.p.  ---------- ---------- ---------- ---VILFNH RDNPPKALAS -----SGPAE
                                        *  *  **     *          ** *
R.l.  CQQDQCSSAL LWK------- ----DARDGT GNGPVHSLNP RDTMLGGLGA NVGTPSGAA-
                  ** *** *   ***           **  **          *  *           *  *
R.m.  CQTDQCFSSR LWKGVHCSHG HIEIDAPAGT TPLLGAPAN- NDVPPKE--- ----PGSAGV

                 550       560       570       580       590       600
K.p.  DGWLDNSLDE RQRLIAALEK AGNVQAKAAR LLGMTPRQVA YRIQIMDITM PRL*
                  * * * *** *  * **********  ** *****  *     *
R.l.  ATIERAGLTE RDRLINAMVK AGWVQAKAAR ILGKTPRQVG YALRRHRIDV KKE*
                  *   * * ***** *    *** ****** ** ******* ******  **  *
R.m.  A----SNLIE RDRLISALEE AGWNQAKAAR ILEKTPRQVG YALRRHGVDV RKL*
                                       ****** ##^^^^#### ####
                                       helix    linker   helix
```

Table 3: Comparison of the amino acid sequences of NifA from K. pneumoniae, R. leguminosarum and R. meliloti. Organisms are abbreviated as K.p., R.l. and R.m., respectively. The different domains in the structures are indicated by letters A, C, D and E.
* : is positioned between identical amino acids.
# : DNA binding helices.
^ : linker between DNA binding helices

Domain A (see Table 3) encompasses the N-terminal part of the NifA polypeptides. Because domain A is present in K. pneumoniae NifA and lacking in NtrC, it has been suggested that it may determine a function specific to NifA like the response to the repressor NifL (Drummond et al. 1986). Evidence for a rhizobial nifL gene is lacking and therefore this suggestion is not valid for Rhizobiaceae. Domain A may be involved in regulating the activity of the NifA polypeptide (Albright et al. 1988; Beynon et al. 1988).
The homology between R. meliloti NifA and R. leguminosarum NifA is considerable: 37 identities in domain A (32% homology). Fischer et al. (1988) did not assign a domain A to the B. japonicum NifA protein, since a comparison with other Rhizobiaceae revealed only 12 identical amino acids in a stretch of 250. Domain B is missing in the comparison because it encompasses a stretch of amino acid sequence that is specific to NtrC and other regulatory proteins like E. coli PhoB, OmpR and A. tumefaciens VirG (Drummond et al. 1986; Nixon et al. 1986; Ronson et al. 1987). Domain C is short, very hydrophilic and predicted to predominantly form coils and turns. It is most likely an interdomain linker that ties two independently folding cores of tertiary structure (Drummond 1988). The central part of the NifA polypeptide, domain D, shows the greatest homology among the compared organisms (72% between Rhizobium species shown in this comparison). A B. japonicum NifA derivative, from which, beside the N-terminal part of the polypeptide, fourteen amino acids from domain D are deleted, was completely inactive, illustrating the great importance of this domain (Fischer et al. 1988).

Furthermore, a R. meliloti NifA derivative from which both the N-terminal and C-terminal part had been deleted leaving only Domain D and the inter-domain linker (positions 479-511) intact is able to activate nifH::lacZ fusions (Albright et al. 1988). The findings for R. meliloti and B. japonicum NifA suggest that domain D interacts with the RpoN-RNAP complex. Domain E spans the C-terminal part of the NifA protein. The degree of homology between Rhizobium species in domain E is approximately 59%. Located at the very C-terminus, sequences have been identified similar to the helix-turn-helix motifs characteristic of DNA-binding proteins like activators, repressors, and transposon resolvases (Pabo & Sauer 1984). In diazotrophs the two α-helices of the DNA binding motif are separated by a linker of four amino acids. The second α-helix is the recognition helix, which makes specific contacts with the DNA-face (see Morett et al. 1988, discussed below). The importance of the DNA-binding motif has been illustrated for K. pneumoniae (Beynon et al. 1988) and B. japonicum (Fischer et al. 1988). Deletion of this motif rendered the respective NifA proteins inactive; this contrasts with the finding for R. meliloti NifA that after deletion of the part containing the helix-turn-helix motif the protein still retains a major fraction of its activity (Albright et al. 1988). A model for gene activation by NifA will be discussed below in which these seemingly contradictory findings are reconciled.

3.5.2. Domains involved in oxygen sensitivity of NifA

The full length R.meliloti NifA polypeptide is able to activate K. pneumoniae nifH::lacZ and R. meliloti nifH::lacZ fusions in an E. coli background; however, aerobic growth abolishes this activation. Deletion of domain A from NifA leads to a drastic increase in the activation of both fusions under anaerobic conditions (Beynon et al. 1988). The R. meliloti NifA protein, from which domain A has been deleted is less sensitive to oxygen than the full length NifA polypeptide, and under anaerobic conditions retains part of its capacity to activate R. meliloti nifH::lacZ fusions. These results are corroborated by Albright et al. (1988), who suggested that the N-terminal domain of R. meliloti NifA may be involved in oxygen-sensing or measurement of the redox potential.

As we will show below, another part of the NifA protein may also be involved in the oxygen sensitivity. Alternatively, the presented results suggest that a specific repressor of NifA-activity may be present (Beynon et al. 1988) possibly of a NifL-like nature (Albright et al. 1988), though such a gene has not been detected yet in Rhizobium. Deletions of the N-terminal part of the B. japonicum NifA protein have no influence on its activity. All NifA deletion derivatives remain oxygen-sensitive (Fischer et al. 1988); this implies that the N-terminus of B. japonicum NifA does not have a function in the regulation of NifA activity.

One most interesting aspect of the nifA genes cloned and tested in vivo is that all rhizobial NifA proteins are oxygen-sensitive whereas the K. pneumoniae and A. vinelandii NifA proteins are not (B. japonicum: Fischer and Hennecke, 1987; A. caulinodans: De Bruijn et al. 1988; R. meliloti: Beynon et al, 1988; R. leguminosarum biovar phaseoli: Hawkins and Johnston, 1988). An interdomain linker is present between domains D and E (see table 3) in rhizobial NifA polypeptides which is absent from K. pneumoniae NifA. It contains two conserved cysteine residues. Since cysteines are often involved in the coupling of redox-sensing groups (see Drummond & Wootton 1987), it was proposed for B. japonicum NifA that this region actually confers oxygen sensitivity upon the protein. Experiments in which both cysteines in the interdomain linker of B. japonicum NifA (corresponding to positions 491 and 496 of the R. leguminosarum NifA; table 3) and those at the end of domain D (positions 449 and 461) were individually changed into serine residues by oligonucleotide mutagenesis resulted in complete deactivation of the B. japonicum NifA polypeptide. These experiments do not prove that the cysteines are involved in oxygen sensitivity of the B. japonicum NifA protein. They do prove that the cysteines play an essential role in its functioning. Fischer et al. (1988) proposed that the NifA proteins of B. japonicum and R. meliloti contain a metal binding motif, attached to these four cysteine residues. Given the fact that all sequenced rhizobial nifA genes to date contain conserved cysteines at the same positions, it can be inferred that the NifA polypeptides of Rhizobium also contain metal binding motifs. According to a model proposed by Fischer et al. (1988) NifA exists in the cell in a non-active state.

Binding of a metal like iron in the Fe^{2+} state to the metal binding site would switch NifA in the active mode, as a result of which the DNA binding motif is positioned such that it can make DNA contacts. Under aerobic conditions Fe^{2+} is oxydized and cannot bind rendering the NifA polypeptide inactive. The positioning of the DNA binding domain resulting in the active mode is no conditio sine qua non for NifA activity. Albright et al. (1988) reported that a R. meliloti NifA protein missing the helix-turn-helix motif is active in vivo, albeit not at wild type levels. This rather surprising finding may either be a result of a gene dosage effect in these experiments or another characteristic peculiar to R. meliloti NifA (discussed below).

3.5.3. NifA targets: nif promoter elements

The RpoN-RNAP complex in concert with the NifA protein can specifically initiate transcription at nif promoters. The sequence of nif promoters has been conserved in a variety of diazotrophs (Gussin et al. 1986). Analysis of the K. pneumoniae nif promoters revealed a basic sequence which appeared to be shared with a sequence in the R. meliloti nifH promoter region (Beynon et al. 1983, Sundaresan et al. 1983, Better et al. 1983) from which a consensus CTGGYRYR-N_4-TTGCA was derived (Ausubel 1984; Y= pyrimidine, R= purine). Nif promoters are usually located around -24/-12 relative to the transcription start site, the last G of CTGG being located at -24 and the C in TTGCA at -12. The exact location of the promoters relative to the transcription start site (as calculated from the C in TTGCA) may vary from 8 - 10 bases in B. japonicum (Hennecke et al. 1988) to 15 bases in A. vinelandii (Jacobson et al. 1988), resulting in different positions of the CTGG and TTGCA promoter motifs. With untranslated 5' leaders ranging between 20 and 150 bases, the mRNAs do not differ in lengths from those reported for other bacteria (Kozak 1983). One rather extreme exception has been reported for the B. japonicum fixBCX operon where the promoter lies 700 bases upstream of the fixB gene (Gubler & Hennecke 1988a). This rather long leader has been proposed to be involved in post-transcriptional control of the fixBCX operon (Gubler & Hennecke 1988b). Analysis of the nif promoters from several diazotrophs using different mutagenesis techniques revealed that GG at -25,-24 and G at -13, are crucial to the functioning of the promoter (Gussin et al. 1986).

This finding implies that contacts are made between the RpoN-RNAP complex and both elements of the promoter. Furthermore, the crucial nucleotides are separated by exactly one helical turn and are therefore located on the same face of the DNA helix. This spacing is important, as illustrated by the fact that deletion of one base from the spacer between the two elements of the K. pneumoniae nifH promoter totally abolished activity (Buck 1986).

3.5.4. NifA targets: Upstream activator sequences

A nifH promoter cloned in a multi-copy-vector in a diazotroph inhibits nif gene expression by titrating the activating NifA protein (multicopy inhibition). This phenomenon is usually assayed by determining the acetylene reducing capacity. In early studies it was noted that multicopy inhibition was relieved by mutations in the promoter as well as by mutations in a more upstream region (Brown & Ausubel 1984). A sequence in this region with the consensus TGT-N_{10}-ACA in K. pneumoniae, A. vinelandii and Rhizobiaceae was identified, having a two-fold rotational symmetry (Buck et al. 1986); Alvarez-Morales et al. (1986) identified the same sequence upstream of the B. japonicum nifD and nifH promoters. The reported characteristics of the new element, the upstream activator sequence (UAS), are similar to those for eukaryotic enhancers. At this point it is relevant to note that most results on the UAS elements were obtained by mutational analysis of the K. pneumoniae nifH UAS in a homologous or heterologous background. The UAS resembles activator sites with the consensus TGTGT-(N_6-N_{10})ACACA recognized in other prokaryotes (Gicquel-Sanzey & Cossart 1982). The UAS is active in cis on a downstream promoter and functions independently of its orientation relative to the promoter. Placing the UAS up to 2.1 kb from the promoter reduces activity to 10% of the wild type; at this position, however, multicopy inhibition is fully lost. The optimal distance of the UAS to the promoter is between 100 and 150 bases. Enhancers can also stimulate transcription when placed downstream of a promoter. Experiments in which the UAS was placed downstream of the K. pneumoniae nifH transcription start, however, failed to show any stimulatory effect, nor was multicopy inhibition detected (Buck et al. 1987c).

3.5.5. NifA targets: Activation mechanism

As stated above, NifA is a DNA-binding protein (Drummond et al. 1986; Morett et al. 1988). This led to the hypothesis that binding of NifA to the UAS would be the first step in the activation of nif genes. This would then be followed by either sliding of NifA down to the promoter or looping out of the DNA between UAS and promoter (Buck et al. 1986). Cloning of the lac-operator sequence between the UAS and the promoter of a K. pneumoniae nifH::lacZ fusion showed no effect on β-galactosidase assays in a background where the lac repressor was overproduced. This result is an argument against the sliding model since bound repressor would have prevented a sliding NifA molecule from reaching the promoter (Buck et al. 1987a). The other possibility of looping out of the intervening DNA, as reported for several other DNA binding proteins (reviewed by Ptashne 1986) seems to offer an acceptable explanation for this result. The UAS has to be on the same DNA face as the promoter. An introduced half turn between UAS and promoter diminished activity to 10% of the wild type and relieved multicopy inhibition to a great extent. Buck et al. (1987a) suggested that half helical turns place the bound NifA protein and the downstream promoter element on opposite faces of the DNA helix, thereby not allowing optimal contacts necessary for activation. The distance between UAS and promoter is usually between 100 and 150 bases (see Gussin et al. 1986). Replacement of the UAS closer to the promoter revealed that there is also a minimum distance for the UAS to be functional. Placed at -90 the construct shows only background activity. This indicates that the size of the DNA-loop has a minimal value (Buck et al. 1987a). It was suggested that the UAS function may be to increase the effective concentration of NifA in the vicinity of the downstream promoter elements as well as to correctly orient NifA. Binding of NifA to the UAS and looping out of the intervening DNA may stabilize the downstream -24/-12 promoter RpoN-RNAP complex. This then would explain why both elements have to be in cis to titrate NifA, resulting in multicopy inhibition of nif gene expression (Buck et al. 1986).

3.5.6. Mutational analysis of the UAS

Alteration of the G or C in TGT or ACA, respectively, in the K. pneumoniae nifH UAS severely reduces activity of the nifH promoter (Buck et al. 1987b). Deletion or insertion of one base in the 10-base sequence between TGT and ACA of the motif partially relieved multicopy inhibition as indicated by an increase of acetylene reducing capacity from 0.4 to 30 % of the wild type level. Deletion of two bases resulted in a total relief of multicopy inhibition. These results illustrate the importance of the spacer length in the UAS. Again, the distance between the G in TGT and the C in ACA is exactly one helical turn. The context of the UAS is important too; bases immediately 5' and 3' to the UAS influence its activity. Based on studies of DNA binding proteins and their target sites, residues in the UAS were identified, which may make specific contacts with NifA (marked by numbers 2, 4 and 5 in Fig. 4).

```
      2 45
5'  TGTTCTGTTTCCCACA  3'
     *

3'  ACAAGACAAAGGGTGT  5'
          ** *
         54 2
```

Fig. 4: Nucleotide sequence of the K. pneumoniae nifH UAS and binding sites for NifA. Binding half sites are underlined. Numbered bases make contact with NifA. Bases marked by asterisks are protected from methylation by dimethylsulfate in the presence of NifA.

Alteration of the residues marked as 2 has a severe negative effect on promoter activity; mutations of residues 4 and 5 (both strands) have no effect (Buck et al. 1987b). Morett & Buck (1988) showed that NifA constitutively synthesized in E. coli protects four G-residues of the K. pneumoniae nifH promoter from methylation by dimethylsulfate; this indicates that these bases probably make the NifA-DNA contact.

Thus the first six 5'-bases of the UAS on both upper and lower strand probably function as half sites (see Fig. 4) each involved in binding a NifA monomer. A mutation in one half site reduces the binding of NifA monomer to this half site, resulting in a lower occupancy of the UAS. This then results in reduced activation and titration of NifA. The 10 bp spacing serves to establish optimal contacts between the bound NifA monomers, resulting in a stabilization of the binding. Spacers of 8, 9 or 11 base-pairs may not allow this, leading to lower activation of the promoter and relief of multicopy inhibition. The two NifA monomers bind to the UAS by means of the C-terminal recognition helices (Morett et al. 1988); this results in a NifA dimer, followed by looping out of the intervening DNA. Contact of the NifA dimer with the RpoN-RNAP complex then activates transcription.

3.5.7. Possible role of the UAS in modulating nif gene expression

Upstream activator sequences have been identified in a variety of diazotrophs like Klebsiella, Azotobacter and Rhizobiaceae. Alvarez-Morales et al. (1986) showed that the activity of nifD::lacZ and nifH::lacZ fusions in B. japonicum depends upon the presence of the UAS. Deletion of the R. meliloti nifH UAS however, had no effect upon activation by the constitutively expressed R. meliloti nifA in an E. coli background (Better et al. 1985). When a nifH promoter from which the UAS had been deleted was recombined back into the R. meliloti genome through marker exchange and subsequently tested for activity in planta under greenhouse conditions, wild type nitrogenase activity was found (Better et al. 1985). From these experiments it follows that R. meliloti NifA, in contrast to K. pneumoniae NifA, can be active without an UAS and may function without a DNA binding domain. Results obtained with R. meliloti NifA deletion mutants confirm this: deletion of the putative DNA binding domain results in a NifA protein still having a major fraction of its activity. Two explanations have been offered for this by Albright et al. (1988). First, DNA binding capacity is retained by NifA, despite these alterations. However, no other DNA-binding motifs have been identified in rhizobial nifA genes (Drummond et al. 1986). Second, DNA binding is not required for activation of the R. meliloti nifH promoter by R. meliloti NifA.

At this point it is relevant to note that most activation studies are performed with NifA being (constitutively) produced from multicopy plasmids. One may therefore be looking at a gene product dosage effect. As was shown for NtrC of K. pneumoniae this may make binding sites unnecessary. When tested both in vitro and in vivo, increasing the NtrC concentration can compensate for deletion of upstream binding sites (Austin et al. 1987, Reitzer et al. 1986). Albright et al. (1988) suggested that the requirement for an UAS of R. meliloti nifH may be more stringent at lower NifA concentrations. Thus, such an UAS may be crucial at a nodule development stage when the NifA concentration and activity are low e.g. during the onset of nitrogen fixation. Experimental results with low copy constructs or constructs recombined in the sym-plasmid are needed to allow a choice between these two explanations for the activity of R. meliloti NifA deletion mutants.

The analysis of UAS function in B. japonicum nif and fix genes reveals some interesting features. The fixA gene does not have a UAS, the fixBCX operon has one imperfect UAS and both nifD and nifH have two copies of the UAS (Hennecke et al. 1988). By assaying chromosomally integrated copies of nifH::lacZ, fixB::lacZ and fixA::lacZ fusions for β-galactosidase activity, it was shown that the nifH::lacZ fusion is activated three-fold over the fixB-lacZ and nine-fold over the fixA-lacZ fusions (Gubler & Hennecke 1988a). These results again show that DNA-binding by NifA is not an absolute requirement for its activity in a homologous background. The UAS serves only as an enhancer in those cases where increased mRNA and protein synthesis are required. Gubler & Hennecke (1988a) suggested that the modulation of nif and fix gene expression is brought about by the presence of one or two copies of the UAS.

The nifH promoter of R. leguminosarum PRE has one imperfect copy of the UAS (11 bp spacing) and one consensus UAS. A low copy nifH::lacZ construct having both imperfect and consensus UAS delays the onset of nitrogen fixation by three days when compared with a nifH::lacZ construct having the consensus UAS only (Chapter 5). This suggests that the modulation model may hold true also in R. leguminosarum.

4. CONCLUSIONS AND PROSPECTS

In the preceding paragraphs an attempt has been made to explain the basic mechanism of regulation of nitrogen fixation in various diazotrophs. Many of the data discussed are derived from research on the well studied K. pneumoniae for which an attractive regulatory model now exists. The onset of nitrogen fixation in K. pneumoniae, under conditions of N-limitation and low oxygen level, is a result of the biochemical sensing of the internal N-status. This leads to transcription activation of the nifLA operon that encodes the repressor NifL and the activator NifA.
In R. meliloti it is the sensing of an external signal, the oxygen level, that leads to activation of nifA transcription. No direct evidence for a NifL like repressor exists. The NifA protein of K. pneumoniae is not oxygen sensitive, whereas that of (all) rhizobial species is. A free living facultative anaerobe like K. pneumoniae should be able to react swiftly to alterations in the N-content of the cell or environmental changes. Stringent, non flexible control would serve best for this bacterium. Thus it is not surprising that structural changes in the K. pneumoniae NifA protein (e.g. deletion of the DNA binding motif) or its target site, the UAS show down phenotypes in nifH::lacZ activation studies. The endosymbiont R. meliloti fixes nitrogen in the protected environment of the nodule, and is not likely to experience any sudden shifts in environmental conditions. This allows room for a less stringent, flexible control mechanism. Thus, it is not surprising that a R. meliloti nifA mutant containing NifA with only domain D and a linker is still active, and that the presence of an UAS is not necessary for nif gene expression. Rhizobial species like B. japonicum and A. caulinodans may be intermediaries. Both species are capable of fixing nitrogen, under the proper conditions, in liquid culture.
At present it is clear that our knowledge concerning nitrogen fixation regulation in Rhizobiaceae is still rather fragmentary. It would add enormously to our knowledge if methods could be developed to purify the oxygen sensitive rhizobial NifA protein in an active state. Detailed foot-printing analysis of both UAS and nifA mutants would then be possible to elucidate the mechanism of activation.

It would be interesting to see which amino acid stretches of the central domain of NifA are making contact with RpoN-RNAP and how transcription activation is achieved. Also, the proposed contacts between the two NifA monomers, resulting in an active dimer could be studied. The purified NifA protein could also be subjected to X-ray crystallography to analyse the relevant domain structures and the nature of the proposed metal binding group. However, attempts to purify the NifA protein of K. pneumoniae sofar have not been successful because the overproduced protein precipitates with the cell membrane fraction (Tuli & Merrick 1988). No reports on the purification of rhizobial NifA proteins exist. Finally, the proposed mechanism of oxygen sensing as a stimulus for nifA transcription and subsequent transcription activation of nif and fix genes by NifA, needs further proof.

CHAPTER 3

NUCLEOTIDE SEQUENCE OF THE *NIFA* REGULATORY GENE OF *RHIZOBIUM LEGUMINOSARUM* PRE: TRANSCRIPTIONAL CONTROL SITES AND EXPRESSION IN *E. COLI*

De taak der wetenschap is, feiten voor schijn en bewijzen voor indrukken in de plaats te stellen.

John Ruskin (1819-1900)

Nucleotide sequence of the regulatory nifA gene of Rhizobium leguminosarum PRE: transcriptional control sites and expression in Escherichia coli.

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SUMMARY

We report the sequence of the regulatory nifA gene of Rhizobium leguminosarum PRE. The transcription initiation and termination sites of nifA were mapped and a potential promoter and a rho-independent terminator identified. The nifA gene has two possible translation start sites both of which are used in an E.coli background, resulting in proteins with apparent molecular weights of 58 kD and 57 kD; initiation at the second site is preferred over initiation at the first. The nifA-nifB intergenic region contains a rpoN dependent promoter for the nifB gene but no consensus upstream activator sequence (UAS).
A potential DNA-binding domain, consisting of two α-helices separated by a four amino acids linker, is located at the C-terminal end of the NifA amino acid sequence.

Keywords: Rhizobium leguminosarum, Nitrogen fixation, Regulation, nifA expression.

INTRODUCTION

In Rhizobium nif and fix genes are essential for nitrogen fixation in nodules formed on the roots of specific legume hosts. The expression of nif and fix genes is under the positive control of a gene that has both functional and structural homology with the nifA gene of Klebsiella pneumoniae (Downie et al. 1983; Zimmerman et al. 1983; Szeto et al. 1984; Schetgens et al. 1985; Grönger et al. 1987; Thöny et al. 1987). In K. pneumoniae it has been shown that the NifA protein is a positive regulator which, together with the sigma factor encoded by the rpoN gene, is required for activation of nif promoters (De Bruijn and Ausubel 1983; Merrick and Gibbins 1985; Hirschman et al. 1985; Hunt and Magasanik 1985; Ronson et al. 1987). Detailed comparison of the amino acid sequences of several DNA binding proteins with those of the NifA proteins of K.pneumoniae and R.meliloti have led to the hypothesis that NifA proteins have a DNA binding domain at their C-terminal end (Drummond et al. 1986). Here we report the nucleotide sequence of the R.leguminosarum PRE nifA gene and the determination of the transcription initiation and termination sites. A putative DNA binding domain was identified at the C-terminal end of the amino acid sequence of the NifA protein derived from the nucleotide sequence, similar to that of R.meliloti, Bradyrhizobium japonicum and K.pneumoniae (Drummond et al. 1986, Fischer et al. 1988). The nucleotide sequence revealed that the coding sequence contains two in phase AUG codons at the N-terminal end of its Open Reading Frame (ORF), which represent possible translation start sites. To determine which of the two is active in vivo both the wild-type nifA gene and nifA genes carrying 5' and 3' deletions were expressed in E.coli.

MATERIALS AND METHODS

General methodology

Media, concentrations of antibiotics and growth conditions were as described by Schetgens et al. (1984). DNA methodology, selection of R. leguminosarum PRE sym-plasmid clones and site-directed transposon Tn5 mutagenesis have been described by Schetgens et al. (1985). The R.leguminosarum and E.coli strains, phage and plasmids used are listed in Table 1.

Primer extension and S1-nuclease mapping

Primer extension reactions were performed essentially as described by Thöny et al. (1987), with one modification: actinomycin D was added to the reaction mixture at a final concentration of 30 µg/ml to inhibit DNA-dependent DNA polymerase activity of the reverse transcriptase. S1-nuclease protection experiments were performed as described (Greene 1987).

Sequence determination

The SmaI fragment carrying R.leguminosarum nifA (Fig. 1) was digested with TaqI, HpaII or HaeIII. The resulting fragments were cloned in M13mp18. Specific restriction fragments from the nifA containing clone pRleH12 (Schetgens et al. 1985) were cloned in both orientations in M13mp18 and M13mp19. Nucleotide sequences were determined using the dideoxy-chain termination method (Sanger et al. 1977). The data were analyzed on a VAX-computer using the Analyseq and Database programs (Staden 1980; Staden 1984).

Expression of nifA in E. coli

E.coli K38 cells from an overnight 10ml LB culture, containing pGp1-2 and a DNA fragment inserted in pT7-5 or pT7-6, grown in 25 µg/ml kanamycin (Km) and 50 µg/ml ampicillin (Amp), were diluted 1:100 in 10 ml Calf Brain Heart Infusion broth and grown in 50 µg/ml Km and 300 µg/ml Amp.

TABLE 1

Bacterial strains, plasmids and phage

Bacterial strains	Genotype or Phenotype	Source or Reference
R.leguminosarum PRE	Str^r	Lie et al. (1979)
R.leguminosarum PRE	Rif^r	Selbitschka and Lotz (1984)
E.coli strains		
JM109	Host for M13 phage vectors	Yanisch-Perron et al. (1985)
HMS174	recAI hsdR	Tabor and Richardson (1985)
K38	hfrC(λ)	Russel and Model (1985)
Phages		
M13mp18/mp19	Phage cloning vectors for sequencing purposes	Yanisch-Perron et al. (1985)
Plasmids		
pT7-5/pT7-6	Ap^r; contain T7Φ10 promoter followed by multiple cloning site (two orientations)	Tabor and Richardson (1985)
pGp1-2	Km^r; contains gene encoding T7-polymerase expressed from lambda P_L promoter, repressed by CI857ts	Tabor and Richardson (1985)
pRleH12	Ap^r; R. leguminosarum nifA on a 3.3 kb BamHI fragment in pBR322	Schetgens et al. (1985)

At $O.D._{600}$ = 0.25 the culture was centrifuged; the pellet was washed twice with 5 ml M9-medium (Maniatis et al. 1982), and centrifuged. The pellet was resuspended in 1 ml M9-medium containing 0.01% ($^{w}/v$) of each of the common amino acids except methionine. After methionine starvation at 30°C for 1 hr the culture was shifted to 42°C. After 15 min freshly prepared rifampicin in methanol was added to a final concentration of 300 µg/ml to inhibit E.coli RNA polymerase. The culture was left at 42°C for an additional 10 min, shifted to 30°C for 10 min and then pulse-labeled for 20 min at 30°C with 40 µCi ^{35}S-methionine (NEN, spec. act. $\rangle$ 800 Ci/mmol). The cells were centrifuged and washed in M9-medium to remove non-incorporated ^{35}S-methionine and centrifuged. The pellet was resuspended in sample buffer (Schetgens et al. 1985) and heated to 100°C for 3 min. The amount of ^{35}S-methionine incorporated was determined as described by Van den Bos et al. (1983). Equal amounts of acid precipitable radioactivity of different samples were loaded on a 12.5% SDS-polyacrylamide gel (Laemmli 1970). After electrophoresis the proteins were electroblotted onto a nitrocellulose filter and exposed to Kodak XAR5 film.

RESULTS

Previously the nifA gene of R.leguminosarum PRE was cloned as a 3.3 kb BamHI fragment in pBR322. Using different restriction enzymes a physical map of this fragment was made (Fig. 1) and by Tn5-mutagenesis it was shown that the complete nifA gene is located on a 2.2 kb SmaI fragment (Schetgens et al. 1985).

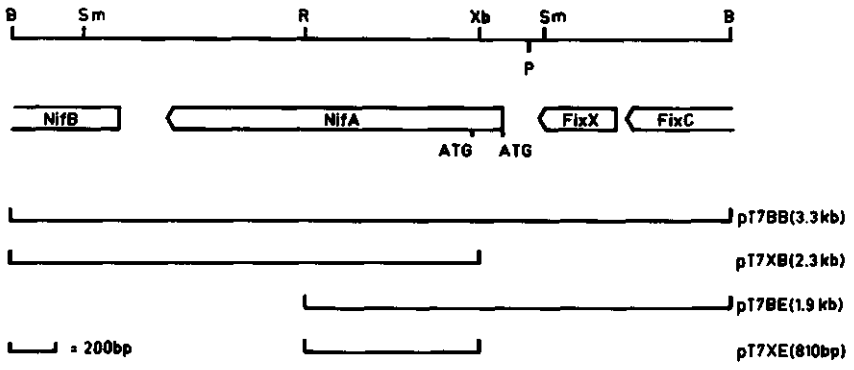


Figure 1
Physical map of the 3.3 kb BamH1 fragment from pRleH12 encoding the nifA gene of R. leguminosarum PRE (Schetgens et al. 1985). B, BamHI; Sm, SmaI; R, EcoRI; Xb, XbaI, P indicates the nifA promoter. The coding sequences for NifB, NifA, FixX and FixC are shown. Also shown are fragments used in the expression experiments (see Fig. 4).

This SmaI fragment was sequenced and in Fig. 2 2074 bp of the sequence are shown, starting with the SmaI site in fixX (Grönger et al. 1987). The 10 C-terminal amino acids of this putative ferredoxin gene are shown.

Figure 2

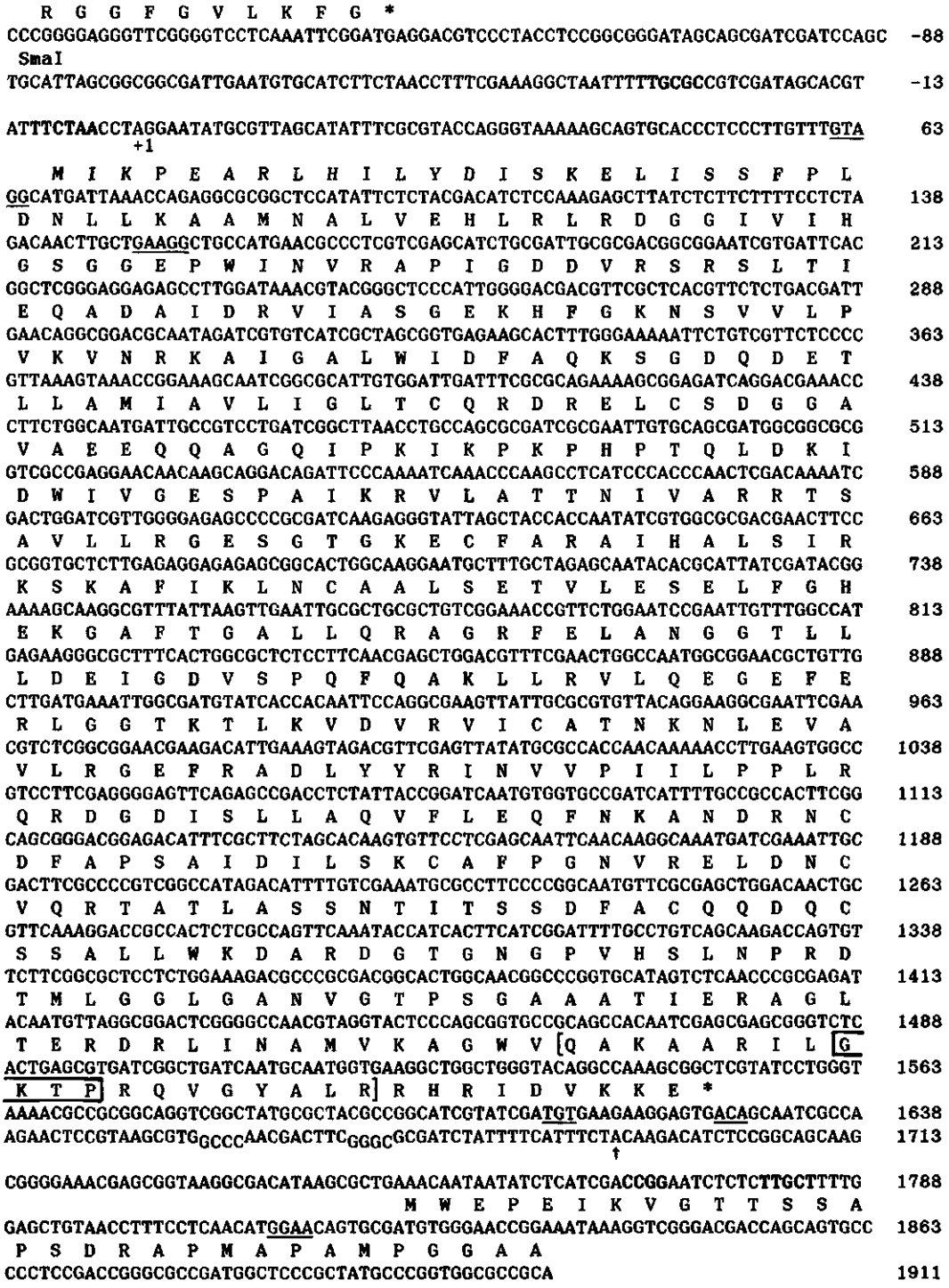

R G G F G V L K F G *
CCCGGGGAGGGTTCGGGGTCCTCAAATTCGGATGAGGACGTCCCTACCTCCGGCGGGATAGCAGCGATCGATCCAGC -88
SmaI
TGCATTAGCGGCGGCGATTGAATGTGCATCTTCTAACCTTTCGAAAGGCTAATTTTTGCGCCGTCGATAGCACGT -13
ATTTCTAACCTAGGAATATGCGTTAGCATATTTCGCGTACCAGGGTAAAAAGCAGTGCACCCTCCCTTGTTTGTA 63
+1
M I K P E A R L H I L Y D I S K E L I S S F P L
GGCATGATTAAACCAGAGGCGCGGCTCCATATTCTCTACGACATCTCCAAAGAGCTTATCTCTTCTTTTCCTCTA 138
D N L L K A A M N A L V E H L R L R D G G I V I H
GACAACTTGCTGAAGGCTGCCATGAACGCCCTCGTCGAGCATCTGCGATTGCGCGACGGCGGAATCGTGATTCAC 213
G S G G E P W I N V R A P I G D D V R S R S L T I
GGCTCGGGAGGAGAGCCTTGGATAAACGTACGGGCTCCCATTGGGGACGACGTTCGCTCACGTTCTCTGACGATT 288
E Q A D A I D R V I A S G E K H F G K N S V V L P
GAACAGGCGGACGCAATAGATCGTGTCATCGCTAGCGGTGAGAAGCACTTTGGGAAAAATTCTGTCGTTCTCCCC 363
V K V N R K A I G A L W I D F A Q K S G D Q D E T
GTTAAAGTAAACCGGAAAGCAATCGGCGCATTGTGGATTGATTTCGCGCAGAAAAGCGGAGATCAGGACGAAACC 438
L L A M I A V L I G L T C Q R D R E L C S D G G A
CTTCTGGCAATGATTGCCGTCCTGATCGGCTTAACCTGCCAGCGCGATCGCGAATTGTGCAGCGATGGCGGCGCG 513
V A E E Q Q A G Q I P K I K P K P H P T Q L D K I
GTCGCCGAGGAACAACAAGCAGGACAGATTCCCAAAATCAAACCCAAGCCTCATCCCACCCAACTCGACAAAATC 588
D W I V G E S P A I K R V L A T T N I V A R R T S
GACTGGATCGTTGGGGAGAGCCCCGCGATCAAGAGGGTATTAGCTACCACCAATATCGTGGCGCGACGAACTTCC 663
A V L L R G E S G T G K E C F A R A I H A L S I R
GCGGTGCTCTTGAGAGGAGAGAGCGGCACTGGCAAGGAATGCTTTGCTAGAGCAATACACGCATTATCGATACGG 738
K S K A F I K L N C A A L S E T V L E S E L F G H
AAAAGCAAGGCGTTTATTAAGTTGAATTGCGCTGCGCTGTCGGAAACCGTTCTGGAATCCGAATTGTTTGGCCAT 813
E K G A F T G A L L Q R A G R F E L A N G G T L L
GAGAAGGGCGCTTTCACTGGCGCTCTCCTTCAACGAGCTGGACGTTTCGAACTGGCCAATGGCGGAACGCTGTTG 888
L D E I G D V S P Q F Q A K L L R V L Q E G E F E
CTTGATGAAATTGGCGATGTATCACCACAATTCCAGGCGAAGTTATTGCGCGTGTTACAGGAAGGCGAATTCGAA 963
R L G G T K T L K V D V R V I C A T N K N L E V A
CGTCTCGGCGGAACGAAGACATTGAAAGTAGACGTTCGAGTTATATGCGCCACCAACAAAAACCTTGAAGTGGCC 1038
V L R G E F R A D L Y Y R I N V V P I I L P P L R
GTCCTTCGAGGGGAGTTCAGAGCCGACCTCTATTACCGGATCAATGTGGTGCCGATCATTTTGCCGCCACTTCGG 1113
Q R D G D I S L L A Q V F L E Q F N K A N D R N C
CAGCGGGACGGAGACATTTCGCTTCTAGCACAAGTGTTCCTCGAGCAATTCAACAAGGCAAATGATCGAAATTGC 1188
D F A P S A I D I L S K C A F P G N V R E L D N C
GACTTCGCCCCGTCGGCCATAGACATTTTGTCGAAATGCGCCTTCCCCGGCAATGTTCGCGAGCTGGACAACTGC 1263
V Q R T A T L A S S N T I T S S D F A C Q Q D Q C
GTTCAAAGGACCGCCACTCTCGCCAGTTCAAATACCATCACTTCATCGGATTTTGCCTGTCAGCAAGACCAGTGT 1338
S S A L L W K D A R D G T G N G P V H S L N P R D
TCTTCGGCGCTCCTCTGGAAAGACGCCCGCGACGGCACTGGCAACGGCCCGGTGCATAGTCTCAACCCGCGAGAT 1413
T M L G G L G A N V G T P S G A A A T I E R A G L
ACAATGTTAGGCGGACTCGGGGCCAACGTAGGTACTCCCAGCGGTGCCGCAGCCACAATCGAGCGAGCGGGTCTC 1488
T E R D R L I N A M V K A G W V [Q A K A A R I L G
ACTGAGCGTGATCGGCTGATCAATGCAATGGTGAAGGCTGGCTGGGTACAGGCCAAAGCGGCTCGTATCCTGGGT 1563
K T P] R Q V G Y A L R] R H R I D V K K E *
AAAACGCCGCGGCAGGTCGGCTATGCGCTACGCCGGCATCGTATCGATGTGAAGAAGGAGTGACAGCAATCGCCA 1638
AGAACTCCGTAAGCGTG$_{GCCC}$AACGACTTC$_{GGGC}$GCGATCTATTTTCATTTCTACAAGACATCTCCGGCAGCAAG 1713
CGGGGAAACGAGCGGTAAGGCGACATAAGCGCTGAAACAATAATATCTCATCGACCGGAATCTCTCTTGCTTTTG 1788
M W E P E I K V G T T S S A
GAGCTGTAACCTTTCCTCAACATGGAACAGTGCGATGTGGGAACCGGAAATAAAGGTCGGGACGACCAGCAGTGCC 1863
P S D R A P M A P A M P G G A A
CCCTCCGACCGGGCGCCGATGGCTCCCGCTATGCCCGGTGGCGCCGCA 1911

Figure 2

The nucleotide and deduced amino acid sequence of the R. leguminosarum PRE nifA gene; possible promoter elements are shown in bold print. Ribosome binding sites and the nifB upstream activator sequence are underlined. The transcription initiation site of nifA is at +1; the terminator is shown in subscript. The site of transcription termination is marked by an arrow. The putative DNA-binding domain starts with Q at position 1537 and ends with R at 1596. The helix-linker-helix motif is placed between brackets. The linker is boxed.

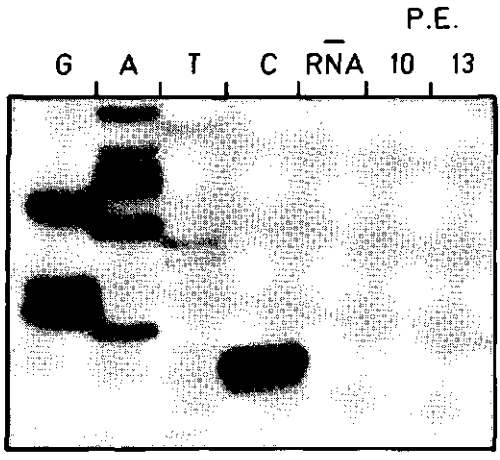
The position indicated as +1 is the site of transcription initiation (see below). An open reading frame (ORF) starting with ATG at position 67 is preceded by GTAGG which may represent a ribosome binding site (RBS) at a distance of one base. In the same reading frame an ATG (position 160) is present representing a second possible start codon preceded by a possible RBS (GAAGG) at a distance of five bases. In Fig. 2 also a putative rho-independent transcription terminator (positions 1656-1672; ΔG^{37}= -5.0 Kcal/mole) is indicated (Platt 1986). The ATG at position 1823 is the start of another ORF, presumably nifB (Buikema et al. 1987). The start codon is preceded by a RBS (GGAA) at a distance of seven bases. Between positions 1768-1784 sequences characteristic for the rpoN-dependent nifB promoter are marked (Ausubel 1984).

The overall homology between the amino acid sequences of the R.leguminosarum PRE and R.meliloti nifA gene products (Buikema et al. 1985; Weber et al. 1985, not shown) is 53%. A similar comparison between R. meliloti and K.pneumoniae (Drummond et al. 1986) shows 39% homology. The amino acid sequence of the R.leguminosarum NifA protein reported here differs in 30 amino acids, at several positions in the sequence, from that reported for R.leguminosarum strain 3855 (Grönger et al. 1987). This may be explained by strain-specific differences, or in some cases by possible errors in the R.leguminosarum 3855 nifA sequence. The amino acids at positions 1350-1401 in Fig. 2 e.g. match better with those of R.meliloti and K.pneumoniae in corresponding positions, than those in R.leguminosarum 3855 (due to C-T or G-A changes in the DNA sequence of the latter).

The NifA proteins of both R.leguminosarum PRE and R.leguminosarum 3855 contain a sequence at the C-terminal end, resembling (in 14 of the 20 amino acids) the helix-turn-helix motif QAKAARLLGMTPRQVAYRIQ of the putative DNA-binding domain of the K.pneumoniae NifA protein (Drummond et al. 1986). The turn of the motif, starting with Gly and ending with Pro is boxed in Fig. 2.

Mapping of the nifA promoter and terminator

The transcription start of nifA was mapped by primer extension using bacteroid RNA isolated from 10 and 13 days old nodules, on which a 16-mer complementary to the nucleotides 76 to 61 of the nifA sequence (Fig. 2) was extended. The products of the reactions were analyzed on a sequence gel next to a sequencing ladder of the promoter region sequenced with the same 16-mer (Fig. 3).



Mapping of the 5'end of the R.leguminosarum PRE nifA gene. The sequence ladder shown spans the nucleotides between -13 and +4 (Fig. 2) and is derived from the SmaI-XbaI promoter fragment (Fig. 1), sequenced with the 16-mer used in the primer extension experiment. The lane marked P.E.13 shows the product of the extension on bacteroid RNA from 13 days old nodules. No extension product was present when bacteroid RNA from 10 days old nodules was used (P.E. 10). The lane marked "-RNA" shows a control in which no RNA was added.

In the lane containing the extended product from RNA of bacteroids isolated from 13 days old nodules (P.E. 13) one band is visible. The C-nucleotide with which it co-migrates is the complementary base of the G residue marked +1 in Fig. 2, which is taken as the site of transcription initiation. No extension product from 10 days RNA (P.E. 10) is present indicating that transcription of nifA is not yet detectable in 10 days old nodules. S1-nuclease mapping confirmed the data of the primer extension experiment (not shown).
The transcription termination site of nifA was mapped by S1-nuclease protection. When a single-stranded DNA fragment complementary to the nucleotides 1607-1764 (Fig. 2) was hybridized against RNA from bacteroids of 13 days old nodules a 86 bp fragment was protected against S1-nuclease digestion showing that the site of transcription termination lies at position 1692, one base downstream of the T-stretch of the proposed rho-independent terminator.

Expression of R.leguminosarum nifA in E.coli

Translation of nifA from the two possible start codons at positions 67 or 160 would lead to a 56.5 kD (519 amino acids) or 53.0 kD (488 amino acids) polypeptide respectively. To determine which of the two potential starts is actually used the 3.3 kb BamHI fragment (Fig. 1) was cloned in pT7-5, downstream of the T7 promoter (pT7BB). Expression of nifA yielded two insert encoded polypeptides with sizes of 58 and 57 kD (Fig. 4, lane 3). Expression of this fragment in the reverse orientation (pT7BBREV; Fig. 4, lane 2) showed only background. Thus both AUG codons appear to be used as translation starts of nifA in E.coli. A 2.3 kb XbaI-BamHI fragment (Fig. 1) containing the second but not the first AUG codon (pT7XB) produced only the smaller of the two insert encoded proteins (Fig. 4, lane 4). Deletions in the nifA gene were constructed to analyze the proteins at an improved resolution and to determine whether a preference for one of the two translation initiation sites exists. Plasmid pT7BE contains a 1.9 kb BamHI-EcoRI fragment from nifA with both AUG codons and produces two proteins with sizes of 34 and 32 kD (Fig. 4, lane 5). Plasmid pT7XE contains a 810 bp XbaI-EcoRI fragment and has the second AUG codon only.

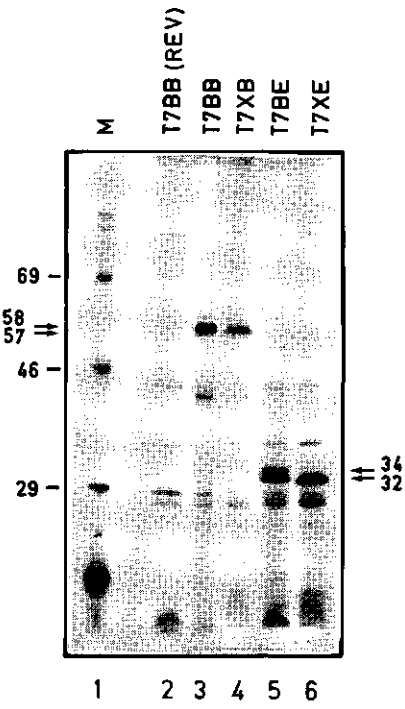


Figure 4

Autoradiograph of ^{35}S-methionine labeled polypeptides expressed from nifA constructs in E.coli (The constructs are shown in Fig. 1).
Lane 1: size markers. Lane 2: T7BBREV; BamHI fragment (reversed orientation). Lane 3: T7BB; BamHI fragment; two translation starts of nifA. Lane 4: T7XB; XbaI-BamHI fragment; first translation start of nifA deleted. Lane 5: T7BE; BamHI-EcoRI fragment; two translation starts, deletion of 3' end. Lane 6: T7XE; XbaI-EcoRI fragment: first translation start and 3' end deleted.

This results in the synthesis of the 32 kD product only (Fig. 4, lane 6). The 34 kD polypeptide contains three methionine residues, the 32 kD polypeptide only two. Yet, the smaller polypeptide gives rise to a more intense band (Fig. 4, lane 5). We therefore conclude that, in E. coli, preference exists for the second translation initiation site.

DISCUSSION

The R.leguminosarum PRE nifA gene has an ORF corresponding to a 519 amino acids polypeptide. Upon expression of this nifA gene in E.coli 2 polypeptides of 58 kD and 57 kD respectively were synthesized. The first ATG in the ORF is preceded by a possible RBS (GTAGG) at 1 base distance, the second by a possible RBS (GAAGG) at a distance of 5 bases, which makes both ATG's potential translation initiation sites. In E.coli an AUG codon preceded by a RBS at a distance of 5-10 bases is preferred in translation initiation to one with a RBS at a shorter distance (Stormo et al. 1982). In Rhizobiaceae it has been shown for the vir genes of Agrobacterium tumefaciens (Das et al. 1986) and the nod genes of R.meliloti (Török et al. 1984) that the AUG initiation codon is preceded by a RBS at 5-10 bases distance. This suggests that the distance between the RBS and the potential AUG initiation codon may be similarly important in Rhizobium as in E.coli. Our experimental results agree with this as we found that the second AUG codon of the ORF in the R.leguminosarum PRE nifA gene is used more frequently than the first one. The R.meliloti nifA ORF also has 2 AUG codons preceded by putative RBS's at 1 and 5 bases respectively (Buikema et al. 1985; Weber et al. 1985).

Recently, using R. meliloti nifA constructs in which the translation initiation rate at the first AUG codon was greatly enhanced by the introduction of a synthetic RBS, Beynon et al. (1988) concluded that only the full-length NifA protein of R.meliloti is capable of activating fusions of K.pneumoniae nifH::lacZ in E.coli. No activity was observed, however, when the original nifA gene was tested. Given the fact that translation initiation of Rhizobium mRNA may occur in E. coli, as we showed for R. leguminosarum nifA, this is a remarkable result. Hypothesizing that most prokaryotes use a translation initiation pathway that is conceptually similar to that in E.coli (Gold 1988), the present experiments suggest that the second translation start will also be more frequently used than the first, when the R. leguminosarum nifA (and probably also R. meliloti) nifA genes are expressed in a homologous background. The results of Beynon et al. (1988) would indicate then that production of the non-active form of the NifA protein is preferred over the active form.

We consider this to be highly unlikely, and suggest that the results presented by Beynon et al. (1988) are not conclusive as to the size of the functional protein in a Rhizobium background.

The putative DNA-binding motif of NifA is strongly conserved between K. pneumoniae and Rhizobium species and has the characteristics of a helix-turn-helix structure (Drummond et al. 1986).

The turn of the DNA binding motif is usually marked by a Gly or a Glu residue (Pabo and Sauer 1984). In Klebsiella and Rhizobium this residue is followed by 3 residues the last of which is Pro, which disrupts the α-helix (Stryer 1988). We conclude that the DNA-binding motif of Klebsiella and Rhizobium species consist of two α-helices separated by a linker of 4 amino acids. The nifA sequence (Fig. 2) is followed by 30 5'terminal codons of the nifB gene; the derived amino acid sequence shares 90% homology with the corrected version of R.leguminosarum FixZ (=NifB, Buikema et al. 1987). The proposed translational start is 72 bases upstream of that reported for R.leguminosarum 3855. Presumably this difference is due to frame shift errors in the latter sequence (Grönger et al. 1987).

The transcriptional start of R.leguminosarum PRE nifA was identified by primer extension and S1-nuclease protection. Comparison of a possible promoter sequence upstream of this transcriptional start (see Fig. 2) with the consensus of "non-nitrogen" regulated Rhizobium promoters TTRANN-17bp-RARRRR (R= purine, Ronson and Astwood 1985) reveals that the "-35" region of the promoter matches in 3 out of 4 positions. The "-10" region differs from the consensus in that it contains only 2 purines instead of 6. Other exceptions to the consensus, however, have been reported (Ronson and Astwood 1985).

The nifA ORF is followed by an intergenic region containing a putative rho-independent terminator, the features of which are consistent with the consensus rules (Platt 1986). Mapping by the S1-nuclease protection method showed that termination at this site indeed takes place. At positions 1768 to 1784 (Fig. 2) the sequence CCGGN$_8$TTGCT represents the probable promoter for nifB, the ORF of which starts at position 1823. This promoter differs from the consensus CTGGN$_8$TTGCA for rpoN dependent promoters (Ausubel 1984) in two positions. However, the crucial nucleotides GG at -25/-24 and GC at -13/-12 are present (Gussin et al. 1986).

It is doubtful whether the sequence TGT-N_{12}-ACA (positions 1611-1628) represents a functional upstream activator sequence (UAS) for nifB since the spacing is 12 bases instead of 10 as in the consensus (Buck et al. 1986). Studies on the UAS for K.pneumoniae nifH have shown that this spacing is critical (Buck et al. 1987). We suggest that transcription of the nifB gene may be largely due to readthrough from nifA and that the nifA terminator is only partly functional as such.

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CHAPTER 4

COMPLEMENTATION OF *RHIZOBIUM LEGUMINOSARUM* PRE *NIFA* MUTANTS: THE *NIFA* GENE IS ONE OPERON WITH *NIFB*

The suppression of uncomfortable ideas may be common in religion and politics, but it is not the path to knowledge; it has no place in the endeavor of science. We do not know in advance who will discover fundamental new insights

Carl Sagan Cosmos

Complementation of R. leguminosarum PRE nifA mutants: the nifA gene is in one operon with nifB

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Summary

We have analyzed the R. leguminosarum nifA-nifB intergenic region and found that it contains a ρ-independent terminator for the nifA gene. Furthermore, on the basis of sequence homology, a promoter for the nifB gene was identified. Using constructs in which the nifA terminator was cloned between the Tet promoter and lacZ, we show that this terminator allows 85% readthrough. However, RNA::DNA hybridisations studies show that in bacteroids the nifA gene is transcribed at a level twice that of nifB. We conclude that nifA and nifB are in one operon. This is corroborated by complementation studies in which a plasmid having the DNA fragment encoding the nifA gene only, does not complement a Rhizobium nifA::Tn5 mutant, whereas a plasmid having a DNA fragment spanning nifA, nifB and a putative *ferredoxin like gene*, does.

Introduction

The diazotroph R. leguminosarum can fix atmospheric nitrogen in symbiosis with its host plant, Pisum sativum L. Nitrogen fixation is catalysed by the products of the nif and fix genes. Nif genes are homologues of nitrogen fixation genes previously identified in the free living nitrogen fixer Klebsiella pneumoniae. Fix genes are only found in other diazotrophs and are also essential to the nitrogen fixation process. The product of the nifA regulatory gene activates the expression of genes involved in nitrogen fixation. The nif and fix genes have promoter elements that can be activated by NifA. Most nif and fix genes have an element with twofold rotational symmetry, the upstream activator sequence (UAS), at a distance of 100 to 150 bases upstream of the transcription start site. The sequence of this UAS, TGT-N_{10}-ACA, resembles that of the target sites of DNA binding proteins (Gicquel-Sanzey and Cossart 1982). The NifA protein has a helix-turn-helix motif at its C-terminus, characteristic of DNA-binding proteins (Drummond et al. 1986). Buck et al. (1987) postulated that the UAS is the target site for NifA. All nif and fix genes have promoter elements located at -24/-12 relative to the transcription start matching the consensus NNGGYRYR-N_4-TTG$\frac{C}{A}$N (Ausubel 1984, this thesis Chapter 5). This promoter element functions as the recognition site for the core of RNA-polymerase complexed with RpoN, the sigma factor (also known as NtrA) that confers specificity for this promoter on the core, as evidenced by footprinting studies (Kustu et al. 1986). Activation of nif and fix genes is thought to involve binding of NifA to the UAS, subsequent looping out of DNA between UAS and downstream promoter elements and a hitherto not understood interaction between NifA and RpoN-RNAP resulting in transcription activation of the nitrogen fixation genes (Buck et al. 1987a). NifA genes have been cloned from several rhizobial strains (see Gussin et al. 1986). In R. leguminosarum and R. meliloti nifA is located downstream of the fixABCX operon (Schetgens et al. 1985, Earl et al. 1987). Analysis of nifA transcription in R. meliloti revealed that 50% of nifA transcription originates from the upstream fixA promoter (Kim et al. 1986).

Termination of nifA transcription in R. leguminosarum PRE occurs at a ρ-independent terminator located in the nifA-nifB intergenic region (This thesis, chapter 3). The nifB gene, located downstream of this terminator, has a putative RpoN-dependent nif-promoter that contains the nucleotides crucial to the activity of nif promoters but it does not perfectly match with the consensus (see Gussin et al. 1986). Recently, Hawkins and Johnston (1988) presented a study on the mel gene of R. leguminosarum biovar phaseoli, which encodes the enzyme tyrosinase involved in the synthesis of the dye melanin. The mel gene also has a RpoN-dependent promoter. Mutations in the nifA regulatory locus caused a Mel^-, Fix^- phenotype. The Mel^- phenotype was restored by introduction of the R. leguminosarum or K. pneumoniae nifA gene. However, the Fix^- phenotype of the nifA mutant was not restored. This failure to complement the Fix^- phenotype was also noted in a R. leguminosarum PRE nifA::Tn5 mutant (Roelvink et al. 1988). In this paper we present an analysis of the nifA-nifB intergenic region which focuses on the role of the nifA terminator in transcription termination. We present data on the strength of this terminator and conclude that nifB transcription is fully due to readthrough from nifA. Furthermore, we show that a nifA::Tn5 mutant can be complemented by introducing a DNA construct having both nifA and nifB. These results indicate that nifA and nifB are in one operon.

Materials and methods

Bacterial strains, phage and plasmids used are listed in Table 1.

Bacterial strains

R. leguminosarum PRE	Wild type; Str^R	Lie et al., 1979
R. leguminosarum 2107	*nif*A::Tn*5*	Schetgens et al., 1985
E. coli		
KMBL 1164	*pro*, *thi*, Δ*lac*	Van de Putte, Leiden
MM 294	*endo*I, *hsd*R, *hsd*M, *pro*	Ruvkun and Ausubel, 1981
JM 109	host for M13 phage	Yanisch-Perron et al., 1985
Phages		
M13mp18	Phage cloning vectors used for sequencing	Yanisch-Perron et al., 1985
M13AH27	M13MP18 with the *R. leguminosarum* PRE *nif*A terminator as TaqI fragment inserted in the AccI site	Roelvink et al., 1988
M13AT24	As M13AT24, reversed orientation	Roelvink et al., 1988
Plasmids		
pRleH12	Contains 3.3 kb BamHI fragment containing *R. leguminosarum* *nif*A in pBR322; Ap^R	Schetgens et al., 1985

pRleH61	Contains 8.7 kb EcoRI fragment with nifB and downstream genes in pBR322; Ap^R	This work
pRK404	Wide host range cloning vector; Tc^R	Ditta et al., 1985
pRK2013	Complementation of tra genes in conjugations; Km^R	Figurski and Helinski, 1979
pMP220	Low copy wide host range transcriptional lacZ fusion vector; Tc^R	Spaink et al., 1986
pBR322	Cloning vector. Ap^R Tc^R	Bolivar et al., 1977
pRK404B	BamHI fragment from pRleH12 in BamHI site of pRK404; R. leguminosarum nifA; Tc^R	This work
pRK404XB	As pRK404B. Contains 4.3 XhoI fragment from pRleH61 with nifB; Tc^R	This work

DNA methodology and cloning of R. leguminosarum PRE symbiotic genes have been described (Schetgens et al., 1985). Bacteroid isolation was performed as described by Katinakis et al. (1988). RNA isolation was as described by Krol et al. (1981). Before 5'-^{32}P- labelling the RNA was heated at 100^{o} for 1 min to destroy secondary structure. 5'-^{32}P-RNA labelling was as described by Maniatis et al.(1982). β-galactosidase assays were performed according to standard procedures (Miller 1972).

Results and discussion

The sequence of the nifA-nifB intergenic region

Previously, we have determined the sequence of the regulatory nifA locus of R. leguminosarum PRE (Roelvink et al. 1988) and observed that the nifA-nifB intergenic region contains putative regulatory sequences as shown in Fig. 1.

```
 SstII            NifA>>>              TaqI
 P  R  Q  V  G  Y  A  L  R  R  H  R  I  D  V  K  K  E  *
CCGCGGCAGGTCGGCTATGCGCTACGCCGGCATCGTATCGATGTGAAGAAGGAGTGACAG    60

CAATCGCCAAGAACTCCGTAAGCGTG(GCCC)AACGACTTC(GGGC)GCGATCTATTTTCATTT  120

CTACAAGACATCTCCGGCAGCAAGCGGGGAAACGAGCGGTAAGGCGACATAAGCGCTGAA  180
  ↑
           TaqI
ACAATAATATCTCATCGACCGGAATCTCTCTTGCTTTTGGAGCTGTAACCTTTCCTCAAC  240

                     NifB>>>
  RBS          M  W  E  P  E  I  K  V  G  T  T  S  S  A  P  S
ATGGAACAGTGCGATGTGGGAACCGGAAATAAAGGTCGGGACGACCAGCAGTGCCCCCTC  300

  D  R  A  P  M  A  P  A  M  P  G  G  A  A
CGACCGGGCGCCGATGGCTCCCGCTATGCCCGGTGGCGCCGCA                   343
```

Fig. 1: Nucleotide sequence of the R. leguminosarum nifA-nifB intergenic region. The 18 C-terminal amino acids of the NifA open reading frame are shown. A pseudo UAS at 42/59 and a putative nifB promoter at 199/215 are underlined. The nucleotides in the stem of the *ρ*-independent terminator are shown in undercast. The transcription termination point at 123 is indicated by an arrow. The NifB open reading frame starts at 254, preceded by a ribosome binding site (RBS). The TaqI sites used in cloning the fragment containing the terminator are indicated.

The sequence begins with the 18 C-terminal amino acids of the regulatory NifA protein. A possible upstream activator sequence (UAS) for nifB is present at positions 42 through 59. This sequence, however, differs from the consensus, TGT-N_{10}-ACA (Buck et al., 1986), in that it has a spacing of 12 instead of 10 bases and Buck et al. (1987b) showed that insertions of two bases in the spacer render the K. pneumoniae nifH UAS inactive. If this holds true also for the upstream activator sequences in Rhizobium, it is doubtful that the identified sequence will be functional. We designate this sequence as a pseudo UAS.

Downstream of the pseudo UAS a possible ρ-independent terminator is present, at positions 87 through 123, consisting of a 4 bp stem and a 9 base loop. This sequence meets the consensus for bacterial ρ-independent terminators formulated by Brendel et al. (1986) in that the stem contains a CGGG stretch and is followed by a DNA stretch rich in T-residues (110-120). The four nucleotides TCTG frequently found following the T-stretch are replaced here by TCTA (120-123). S_1-nuclease mapping indicated that the transcription termination point lies at position 123 (Roelvink et al., 1988, this thesis, Chapter 3). The stability of this stem loop structure was calculated from a nearest neighbour model (Freier et al. 1986). The ΔG^{37} of -5.0 Kcal/mole indicates that the nifA mRNA may fold to form a terminator structure. The ΔG^{37} value in itself, however, does not allow precise predictions as to the efficiency at which such a secondary structure will function as a terminator. Arndt and Chamberlin (1988) determined the termination efficiency of the T7 terminator, the trpA+ and the rrnBt1 terminators by using steady state transcription assays. At calculated ΔG^{37} values of -15 kcal/mole, -23 kcal/mole and -24 kcal/mole respectively these structures terminate transcription with efficiencies of 94, 77 and 71%. Thus, no correlation between release of the RNA polymerase and the predicted stem-loop stability is apparent.

Positions 199-215 contain a sequence resembling a RpoN dependent promoter for the nifB gene. This sequence (CCGGAATC-N_4-TTGCT) has the nucleotides GG at -25/-24 and GC at -13/-12 shown to be crucial to the activity of RpoN dependent promoters. It differs, however in four nucleotides from the consensus CTGG-YRYRN$_4$-TTGCA for such promoters. The nucleotides at -23 to -20 are RRYY (R=purine, Y=pyrimidine) rather than YRYR.

The other two differences between the putative nifB promoter and the consensus also occur in the same positions in other nif promoters of several Rhizobium species and these do not appear to influence promoter function (see e.g. Hennecke et al., 1988). Downstream of the putative nifB promoter, at positions 243-246, a ribosome binding site (GGAA) the nifB start codon and 30 N-terminal amino acids for the nifB gene are indicated.

Complementation of a R. leguminosarum nifA::Tn5 mutant

Previously it has been reported that the K. pneumoniae nifA gene constitutively expressed from the cat-promoter cannot complement Rhizobium nifA::Tn5 mutants to a restoration of the Fix$^-$ phenotype (Hawkins and Johnston 1988, Roelvink et al., 1988). We suggested that this phenomenon results from the failure of the K. pneumoniae NifA protein to interact with the Rhizobium RNA-polymerase-RpoN complex. To test this hypothesis the nifA gene from R. leguminosarum PRE on a 3.3 kb BamHI fragment from pRleH12 (Schetgens et al., 1985) was cloned in pRK404 (Ditta et al., 1985): the resulting construct pRK404B (see Fig 2. for cloning scheme) was conjugated into a R. leguminosarum nifA::Tn5 mutant and the resulting transconjugant was inoculated on pea plants.

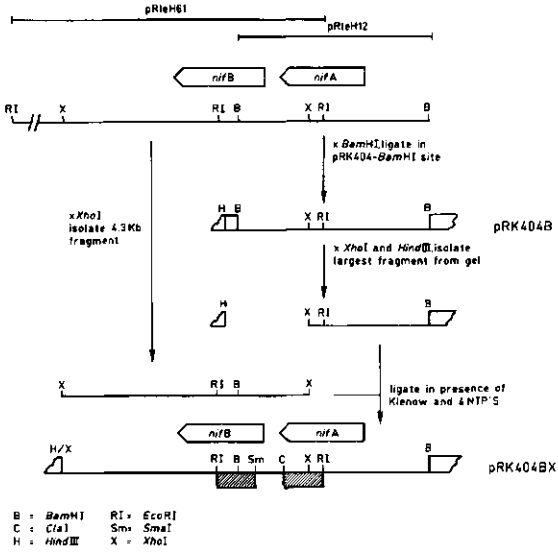

Fig. 2: Cloning strategy for constructs used in the complementation of R. leguminosarum nifA::Tn5 mutants. To construct pRK404B a 3.3 Kb BamHI fragment from pRleH12 was cloned in pRK404. This construct was digested to completion with XhoI and HindIII and the fragments were separated on gel. The largest fragment, containing the vector and a part of the insert encoding nifA was isolated from gel and ligated to a 4.3 Kb XhoI fragment from pRleH61, resulting in pRK404BX. The fragments specific for nifB or nifA used in the RNA::DNA hybridisations studies, are indicated by hatched bars.

After 21 days no restoration of the Fix^- phenotype was observed, which invalidates the interaction hypothesis. We then cloned a 4.3 kb XhoI fragment carrying nifB into pRK404B (see Fig. 2) resulting in pRK404BX. The resulting construct contains nifA, nifB and a recently identified gene encoding a ferredoxin like protein (fixY) downstream of nifB, which lacks a promoter (Klipp et al., 1988). This construct when conjugated into a R. leguminosarum nifA::Tn5 mutant fully restored the Fix^- phenotype of the mutant to Fix^+. This result can be explained by assuming that the failure to complement a R. leguminosarum nifA::Tn5 mutant by a cloned nifA gene is caused by a polar effect of the transposon on the transcription of genes downstream of nifA, in this case nifB and fixY. Construct pRK404XB has nifA, nifB and fixY in one contiguous fragment and thus complements the Fix^- phenotype. This suggests that the nifA terminator does not fully terminate transcription and that the nifB promoter is not fully functional. We conclude that nifA, nifB and fixY are in one operon.

Determination of nifA and nifB transcription levels

The results of the experiments described above indicate that nifB transcription largely if not fully depends on readthrough from the nifA promoter. We therefore decided to test the strength of the ρ-independent terminator between nifA and nifB (indicated in Fig. 1). The clones M13AH27 and M13AT24 contain a 161 bp TaqI fragment carrying the nifA terminator (see Fig. 1) in two orientations.

A 180 bp BamHI-SphI fragment was excised from each clone and recloned in pBR322 (Bolivar et al.,1977) to replace a 185 bp BamHI-SphI fragment that encodes part of the Tet gene. From the resulting pBR322 recombinants, a 560 bp EcoRI-SphI fragment carrying the Tet promoter, the first 100 N-terminal amino acids of the Tet gene and the nifA terminator, in correct and reversed orientation, was excised and cloned in the transcriptional lacZ fusion vector pMP220 (Spaink et al., 1986). These constructs are shown in Fig. 3.

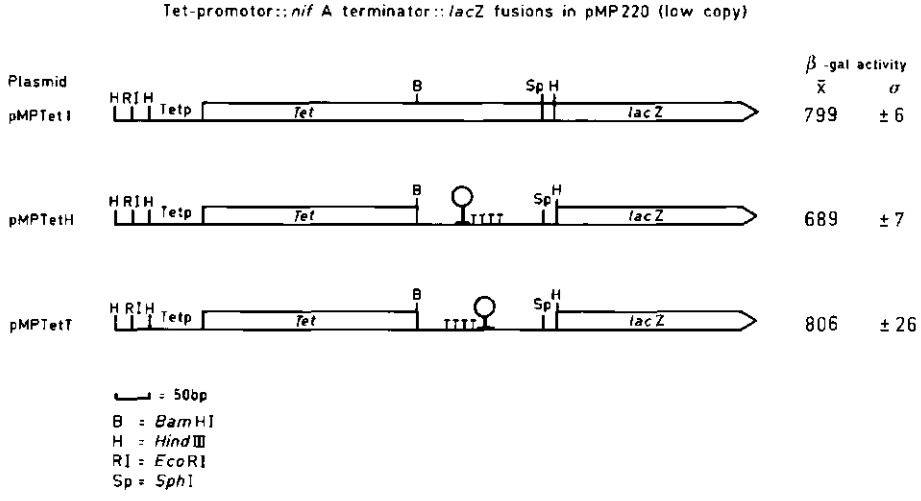

Fig. 3: Structure of constructs used to evaluate the functionality of the nifA terminator.

pMPTet1: containing the Tet-promoter (Tetp) and 170 N-terminal amino acids of the Tet-gene fused to E. coli lacZ in pMP220

pMPTetH: containing the Tet-promoter and 100 N-terminal amino acids of the Tet-gene, and the nifA terminator in the correct orientation fused to E. coli lacZ in pMP220

pMPTetT: as pMPTetH but terminator in reversed orientation.

The β-galactosidase activities shown are averages of 10 measurements; σ is the standard deviation.

The rationale of the experiment to measure nifA terminator strength is that a construct containing this terminator in the proper orientation downstream of the Tet promoter (pMPTetH: Fig. 3) should show significantly lower levels of β-galactosidase activity, as compared with the wild type construct. The analysis showed that the nifA terminator only inhibits 15% of lacZ transcription from the Tet promoter; if the terminator is placed in the inverse orientation (pMPTetT: Fig. 3) no inhibitory effect upon transcription is apparent. If the same goes for the transcription of nifA in vivo, it would mean that 85% of the transcription from the nifA promoter reads through into the downstream nifB gene. With the nifA transcription put at 100% and nifB transcription at 85%, this implies that nifA and nifB should be transcribed at comparable levels in R. leguminosarum bacteroids. To measure the transcription levels of both genes, DNA fragments specific for each (indicated in Fig. 2: a 640 bp EcoRI-ClaI fragment specific for nifA and a 650 bp EcoRI-SmaI fragment, specific for nifB) were selected. One microgram of each of these fragments was bound to a nitrocellulose filter and hybridized with 5'-^{32}P labelled total bacteroid RNA from 17 day old nodules. The bacteroid RNA was heated before 5'-^{32}P-labelling to destroy RNA secondary structure and expose hidden breaks. Therefore the probe supposedly will contain non-specific mRNA fragments. After extensive washing, the bound activity was determined by scintillation counting. The nifA filter contained 125 cpm of bound activity and the nifB filter 54 cpm. These results suggest that the nifA gene is transcribed at a level of at least twice that of nifB. The identified ρ-independent nifA terminator terminates transcription in Rhizobium bacteroids at a level several times higher than that found when tested in an E. coli background, where the construct is on a small low copy number plasmid (10.5 Kb). In Rhizobium the nifA terminator is on the large Sym-plasmid (400 kb). Small plasmids are usually more supercoiled than larger ones (Drlica, 1984). Gubler and Hennecke (1988) reported that nifH::lacZ, fixA::lacZ and fixB::lacZ fusions, when chromosomally integrated in B. japonicum could be activated at substantially higher levels than the plasmid borne fusions. Furthermore, the expression of genes has been shown to be influenced either positively of negatively by superhelicity (Drlica, 1984). We suggest that the partial functioning of the nifA terminator in E. coli is due to the influence of superhelicity on the plasmid used.

CHAPTER 5

THE *NIFH* PROMOTER REGION OF *R. LEGUMINOSARUM* P13: DNA SEQUENCE ANALYSIS AND PROMOTER ELEMENTS INVOLVED IN ACTIVATION BY NIFA PROTEIN

In our own epoch such a thing would be unthinkable. For almost every modern Western man has his inventive faculty to some extent developed; the Western man invents machines as naturally as the Polynesian islander swims. Give a Western man a job of work and he immediately begins devising a machine that would do it for him; give him a machine and he thinks of ways of improving it

George Orwell: The Road to Wigan Pier

The *nifH* promoter region of *R. leguminosarum* P13: DNA-sequence analysis and promoter elements involved in the activation by NifA protein.

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Summary

We have determined the nucleotide sequence of the R. leguminosarum nifH promoter region. This promoter region contains a consensus promoter, a consensus upstream activator sequence (UAS), a pseudo promoter and a pseudo UAS. The consensus promoter was mapped by primer extension. This promoter differs from the consensus in one of the four supposedly invariant nucleotides. The pseudo promoter and pseudo UAS do not function when activated by Klebsiella NifA in E. coli. When studied in Rhizobium background, with low copy number nifH::lacZ fusions, it appeared that the construct having the pseudo UAS as well as the consensus UAS delays the onset of nitrogen fixation. Studies with nifH::lacZ fusions on multicopy plasmids show that partial deletion of the consensus UAS does not alter the ability of these constructs to inhibit nitrogen fixation.

Introduction

The soil bacterium Rhizobium leguminosarum PRE induces root nodules on the pea (Pisum sativum L.). During endosymbiosis the bacteroid, a specialized form of the bacterium, fixes nitrogen into ammonia, which is excreted and made available to the host plant. Two specialized groups of genes, the nif and the fix genes are involved in nitrogen fixation. Nif genes have a homologue in the free living nitrogen fixing Klebsiella pneumoniae, fix genes do not. Most of the nif and fix genes are activated by the regulatory nifA gene (Gussin et al., 1986) acting in concert with RNA-polymerase (RNAP) and RpoN, a sigma factor encoded by the rpoN gene (Ronson et al., 1987). The promoters of nif and fix genes have a characteristic consensus sequence CTGGYRYRN$_4$TTGCA (Y=pyrimidine, R=purine, Ausubel, 1984, Gussin et al., 1986). An upstream activator sequence (UAS) with the consensus TGT-N$_{10}$-ACA is present in most cases. The product of the nifA gene has a DNA binding domain at its C-terminus and as suggested by Buck et al. (1986, 1987) binds to the UAS (Morett et al., 1988). Binding of NifA to the UAS is followed by looping out of the intervening DNA as a result of which NifA makes contact with the RpoN-RNAP complex leading to transcription activation from the nif and fix promoters. We have determined the nucleotide sequence of the nifH promoter region of R. leguminosarum P13 and studied the mechanism of transcription activation from this promoter. For this purpose we have cloned the intact nifH promoter region and promoter regions containing different deletions in both low and high copy number transcriptional lacZ fusion vectors (Spaink et al., 1986) having a wide host range. The function of the different nifH promoter elements was evaluated from studies on the activation of nifH::lacZ by K. pneumoniae NifA in an Escherichia coli background. The functionality of these promoter elements was also analyzed in a Rhizobium background. To this end multicopy inhibition defined as the capture of an activator protein by a promoter region cloned in a multicopy vector, resulting in a Fix$^-$ phenotype in plant, was studied using multicopy derivatives of the different nifH::lacZ fusions. Remarkably, deletion of the UAS, that binds NifA, does not lead to relief of multicopy inhibition, indicating that the NifA protein may also use the promoter as it target site.

Materials and Methods

(a) Bacterial strains and plasmids are listed in Table 1

Bacterial strains	Genotype	Source
R. leguminosarum P13	Wild type; Rif^R	Selbitschka and Lotz, 1984
E. coli strains		
KMBL 1164	Δ(*lac*-*pro*AB)	Van de Putte, Leiden
DH5αF'	Host for pTZ18R and pTZ19R cloning and sequencing vectors	Pharmacia, Uppsala, Sweden
TH1	Δ*lac*U169; Δ*gln*F	Ow and Ausubel, 1983
Plasmids		
pGB5	Amp^R; contains *nif*HD from *R. leguminosarum* PRE in pSUP201	Schetgens et al., 1984
pTZ18R/pTZ19R	Amp^R; Cloning and sequencing vectors	Pharmacia, Uppsala
M13mp18/mp19	Phage cloning and sequencing vectors	Yanisch-Perron et al., 1985
pMP220	Tc^R; IncP1; low copy promoter probe vector	Spaink et al., 1986
pMP190	Cam^R, Str^R; IncQ; high copy promoter probe vector	Spaink et al., 1986
pRK 2013	Km^R; contains *tra*-genes for mobilisation during conjugation	Figurski and Helinski, 1979
pWK131	Cam^R; constitutively expresses *K. pneumoniae* *nif*A from cat-promoter in *E. coli*.	Pühler et al., 1983

pMH1	Amp^R; 524 bp Hpa1-Acc1 *nifH* promoter fragment from *R. leguminosarum* PRE in pTZ19R	This work
pMH1Δ constructs	Amp^R; series of *nifH* promoter region deletions in pTZ18R	This work
pMPΔ1.6L etc	Tc^R; pMH1Δ constructs fused to *lacZ* in pMP220 (low copy number)	This work
pMPΔ1.6H etc.	Cam^R; pMH1Δ constructs fused to *lacZ* in pMP190 (high copy number)	This work
pTZAPB1	Amp^R; constitutive *Tet* promoter *nifA* fusion in pTZ18R (both translation starts)	This work
pTZAPB2	Amp^R; as pTZAPB1 (only the second translation start)	This work
pTZES1	Amp^R: pTZAPB1 recloned in pTZ18R to generate cloning sites	This work
pTZES1ΔSst	Amp^R: pTZAPB1 recloned in pTZ18R to generate cloning sites. DNA binding domain deleted	This work
pTZES2	Amp^R: pTZAPB2 recloned in pTZ18R to generate cloning sites.	This work
pTZES2ΔSst	Amp^R; pTZAPB2 recloned in pTZ18R to generate cloning sites. DNA binding domain deleted	This work

pMPES1	Tc^R; Fusion from pTZES1 cloned in pMP220	This work
pMPES1ΔSst	Tc^R; Fusion from pTZESΔSst cloned in pMP220	This work
pMPES2	Tc^R; Fusion from pTZES2 cloned in pMP220	This work
pMPES2ΔSst	Tc^R; Fusion from pTZES2ΔSst	This work

Media for growth of Escherichia coli (LB) and R. leguminosarum strains (TY) were as described before (Schetgens et al., 1984). M13-phages were selected on minimal A-medium (Miller, 1972) supplemented with glucose (final concentration 20mM), $MgSO_4$ (2mM) and vitamine B1 (0.001%), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 32μg/ml) and isopropyl-β-D-thio-galactopyranoside (IPTG, 20 μg/ml). E. coli DH5αF' strains containing pTZ18R or pTZ19R cloning and sequencing vectors and derivatives were grown in Calf Brain Heart Infusion Broth (Gibco). Beta-galactosidase assays were performed on bacteria grown in NFDM (Cannon et al., 1974). Antibiotics were used in the following concentrations (in μg/ml): tetracycline (Tc) 10, ampicilline (Amp) 50 (200 for pTZ18R/pTZ19R derivatives), chloramphenicol (Cam) 25, kanamycin (Km) 12.5.

(b) Restriction enzymes, DNA polymerase I (Klenow fragment), Bal31 nuclease, and T4-DNA ligase were obtained from Boehringer (Mannheim, FRG) and used according to the manufacturer's instructions.

(c) Methods

Recombinant DNA technologies, such as restriction enzyme digestions, ligation, transformation and Bal31 nuclease deletions were as described (Maniatis et al. 1982). The nifH nucleotide sequence (of fragments cloned in M13mp18) and the 5'-end points of deletions of fragments cloned in pTZ18R were determined by the dideoxy chain termination method using both the universal primer for M13 and the reversed sequencing primer (Messing, 1983) for single stranded DNA produced from pTZ vectors.

Primer extension experiments were performed as described by Thöny et al., 1987 with one modification: actinomycin D was added to a final concentration of 30 µg/ml to inhibit DNA dependent DNA polymerase activity of the reverse transcriptase. Bacteroids were isolated from root nodules according to the procedure described by Katinakis et al. (1988). Isolation of total RNA from bacteroids was as described (Krol et al., 1980). Beta-galactosidase assays (Miller, 1972) were routinely performed in triplicate. Transfer of plasmid constructs from E. coli to R. leguminosarum by conjugation was as described by Schetgens et al. (1984).

Results and discussion

(a) Cloning, sequencing and structural analysis of the R. leguminosarum nifH promoter region

With a view to determine the sequence of the R. leguminosarum PRE nifH promoter region, a detailed restriction map of pGB5 was made, a recombinant plasmid that contains the nifH and nifD genes (Schetgens et al., 1984).

Figure 1:Nucleotide sequence of the R. leguminosarum PRE nifH promoter region

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  Hpa1                *Δ1.6                    *Δ1.7
 GTTAACCCTATGCGGGCACCAGACGCGGCATGGCGCTTGTCATCGCCGGAGACAGCGCT  -305

         *Δ2.5              *Δ2.7    *Δ2.6
TTGCGGCAAGTGATCAGAATTTGTTACGGCCGGTCTGGTGATGGCTGCTCTAACAAAGTC  -245

CTAGAAGCTACGCATGGATAAATGAGACATCCTGCCGGTTGGTCAGATATTGCACAAACG  -185

                                          *Δ3.2             *Δ3.7
GCCACCATTCATTGGCCCTCAGAGGGTGACAATCTATTTCGACATTGTCATCACCTTTGT  -125
                                                            TGT
   *Δ3.6
CGGTTACTTGACAAGCGTTTGTGTTCTCAATCCCGTATTTTACGCCCACATAGCTGGAAT  - 65
---N10----ACA

CGCGGCGAATTATTGTTCGTCAGCTCAATCGGCCGCTTGGCACGAATCTTGAGAGCTATT  -  5
                                     CTGGYRYR-N4-TTGCA
    +1
GAGAGGCAGCGGAACGGCCGCCGCATTCCGTTGCGGGTAACCAAATTGCTTCGAACACAT  + 55
    ↑

  RBS          M  A  A  L  R  Q  I  A  F  Y  G  K  G  G  I
GAAGGAACGCCAAGCATGGCAGCTCTGCGTCAGATCGCATTCTATGGAAAAGGCGGAATT  +115
                                          Acc1
 G  K  S  T  T  S  Q  N  T  L  A  A  L  V  D
GGCAAGTCCACTACGTCCCAAAACACGCTGGCCGCCCTTGTCGAC                 +161
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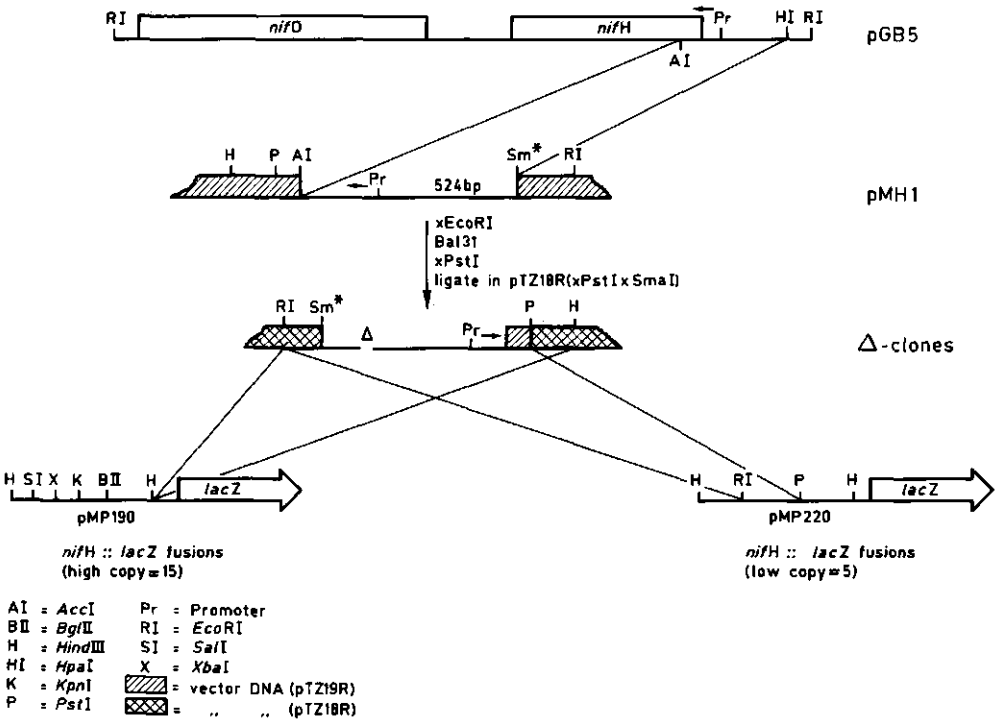
Fig. 1: Nucleotide sequence of a 524 bp HpaI-AccI fragment from pGB5 containing the nifH promoter region of R. leguminosarum P13. UAS (-127/-112) and promoter sequences (-25/-11) are indicated by the consensus below the respective sequences in which they appear. A pseudo promoter (-206/-191) and a pseudo UAS (-326/-310) are underlined. The end-points of the various deletions generated by Bal31 digestion, running from the HpaI site at -363, are marked by an asterisk above the first nucleotide of the remaining sequence. The transcription start site is indicated by an arrow and marked +1. RBS= ribosome binding site.

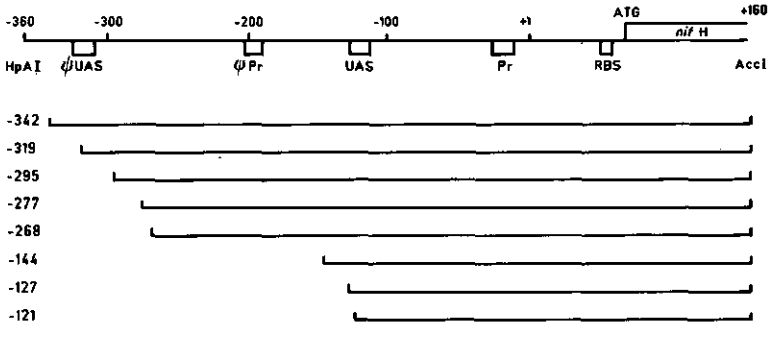
The sequence of a 524 bp HpaI-AccI fragment, most likely to contain the nifH promoter region, since the AccI site is located in the N-terminal part of the nifH gene (unpublished observation) was determined (Fig. 1). Indeed, a purine rich stretch, with a ribosome binding site (RBS), at +56 through +62 and an AUG codon at +70 form the translation initiation site for the nifH open reading frame (ORF). The sequence indicated at -25 through -11 differs in three bases from the consensus CTGGYRYRN$_4$TTGCA (Ausubel, 1984, Gussin et al., 1986) for RpoN dependent promoters. One difference, at position -12, concerns a nucleotide (C in the consensus) supposed to be invariant in such promoters. Primer extension experiments indicated that the G-residue at +1 is the major transcriptional initiation site (not shown), which confirms the functionality of the identified promoter. At -127 through -112 a sequence consistent with the consensus for an UAS (TGT-N$_{10}$-ACA; the N$_{10}$ is known as the spacer, Buck et al. 1986) is present. At -206 through -191 another sequence resembling a RpoN-dependent nif promoter is present, but the spacer between the two promoter elements (in the consensus the two promoter elements are CTGG and TTGCA, the spacer between them being 8 basepairs) is one base shorter than in the consensus. Buck (1986) showed that the K. pneumoniae nifH promoter with a 7 in stead of 8 base spacer is not functional; therefore we consider this sequence as a pseudo promoter. Upstream of this pseudo promoter a second UAS-like sequence is present between positions -326 and -310. This sequence differs from the consensus (Buck et al., 1986) in that the spacing is 11 in stead of 10 bases; we designate this sequence as a pseudo UAS.

(b) The effect of deletions in the nifH promoter upstream region on the activation by K. pneumoniae NifA in E. coli

To determine the possible functions of the (pseudo) UAS and (pseudo) promoter sequences, Bal31 nuclease deletions were generated in the region upstream of the nifH promoter (Fig. 2). To this end the 524 bp HpaI-AccI fragment from pGB5 was cloned in pTZ19R digested with SmaI and AccI resulting in recombinant plasmid pMH1. This plasmid was used to generate deletions in the nifH promoter upstream region, by Bal31 digestion. The fragments carrying deletions were transcriptionally fused to lacZ. This procedure (see Fig. 2) resulted in fusions with deletion endpoints as indicated in Figs. 1 and 3. The nifH::lacZ fusions in the low copy number vector pMP220 were transformed into E. coli KMBL 1164 containing the recombinant plasmid pWK131 (Pühler et al., 1983) that constitutively expresses K. pneumoniae nifA. After overnight growth of the cells at 30°C the β-galactosidase activity was assayed (Fig. 3). Deletion of the pseudo UAS and the pseudo promoter (pMPΔ1.6L through pMPΔ3.6L) does not decrease the β-galactosidase activity indicating that these elements do not contribute to the activation of the promoter. Deletion of the upstream promoter DNA including the first base of the UAS as in pMPΔ3.7L, or six bases of the UAS, as in pMPΔ3.6L, abolishes activation by K. pneumoniae NifA. When the constructs were tested in E. coli TH1 that has a deletion of the ntrA gene that encodes the sigma factor specifically recognising nif and fix promoters, no activation of the R. leguminosarum nifH promoter was observed. This indicates that the β-galactosidase activity measured is dependent upon the presence of the ntrA gene product. From these experiments we conclude that the pseudo UAS and the pseudo promoter are not functional in nifH activation. The UAS at -127/-112 and the promoter at -25/-11 are functional promoter elements.



pMH1Δconstructs



plasmids	β-galactosidase
pMP220	67
pMPd 1.6 L	1277
" 1.7 L	1364
" 2.5 L	1398
" 2.7 L	2319
" 2.6 L	3183
" 3.2 L	2446
" 3.7 L	57
" 3.6 L	59

Fig. 2: Cloning strategy of the DNA fragments containing various deletions in the region upstream of the R. leguminosarum nifH promoter. Recombinant plasmid pMH1 was constructed by cloning a 524 bp HpaI-AccI fragment from pGB5 into pTZ19R digested with SmaI and AccI. To generate deletions, plasmid pMH1 was digested with EcoRI and the linearized DNA was subjected to Bal31 nuclease digestions for 1, 2 or 3 min. After inactivation of the nuclease, the DNA was digested with PstI and separated on gel. The resulting diffuse band of nifH deletion fragments was excised from the gel and the isolated DNA was recloned in pTZ18R digested with SmaI and PstI. The various deletion end points were determined by sequencing and are indicated in Fig 1. Transcriptional nifH::lacZ fusions were created by cloning the EcoRI-PstI fragments from the pTZ18 plasmids vectors containing the deletion fragments into the low copy number vector pMP220, or by cloning the EcoRI-HindIII fragments into the high copy number vector pMP190 (Spaink et al., 1986).

Fig. 3: Activation of the R. leguminosarum nifH promoter in E. coli by K. pneumoniae NifA. The nifH promoter region is represented graphically. The positions of the pseudo UAS, pseudo promoter, UAS, promoter, transcription start site, RBS and start codon are indicated. The end points of the Bal31 generated deletions are indicated (see also Fig. 1) followed by the corresponding plasmid codes and the assayed β-galactosidase activities (in Miller units, Miller, 1972).

(c) Functional analysis of the nifH promoter region in R. leguminosarum

The experiments described above show that the heterologous K. pneumoniae NifA can activate the nifH promoter of R. leguminosarum in an E. coli background. and that a UAS is essential for promoter activity. In diazotrophs like Rhizobium and Klebsiella the presence of multiple copies of plasmids carrying a nif promoter and, in cis, the UAS will result in capturing of the NifA protein. This limits the amount of activator protein available for activation of the nif and fix genes which may result in a decrease or inhibition of nitrogen fixation, as assayed by acetylene reduction.

This phenomenon is known as multicopy inhibition (reviewed in Gussin et al., 1986). We used multicopy inhibition as a means to evaluate the functionality of the R. leguminosarum nifH promoter elements. To this end both the different low and high copy number transcriptional nifH::lacZ fusions were conjugated into R. leguminosarum P13 and the resulting strains were inoculated on pea plants. At days 21 and 24 after inoculation acetylene reduction assays were performed (Table 2).

Relative acetylene reducting capacity (units.mg^{-1}nodule.h^{-1})

	Low copy number	
	day 21	day 24
pMP220	100	100
pMPΔ1.6L	21	95
pMPΔ1.7L	97	99
pMPΔ2.6L	105	122
pMPΔ3.2L	130	139
	High copy number	
	day 21	day 24
pMP190	100	100
pMPΔ1.6H	3	1
pMPΔ3.2H	3	3
pMPΔ3.6H	3	ND

Table 2: Relative acetylene reducting capacity of P. sativum inoculated with R. leguminosarum P13 carrying different nifH::lacZ fusions. The activity was measured as units.mg^{-1}nodule.h^{-1}. The strains harboring the vectors, either pMP220 or pMP190, were taken as wild type controls (100%) and experiments were performed triplicate. The relative acetylene reduction activities for the low copy constructs are averages plus or minus 4% (standard deviations). The values obtained with the high copy constructs equal background levels.

The presence of the various low copy number constructs in R. leguminosarum had no influence on nodule morphology. The nodules were reddish and appeared on the main and lateral roots in approx. the same numbers as after nodulation with a wild type strain harboring the vector pMP220 only. None of the low copy number constructs, containing the nifH promoter region (except pMPΔ1.6L) appeared to influence the level of acetylene reduction when compared to the control strain having the pMP220 vector. The construct pMPΔ1.6L, which has both the pseudo UAS and the consensus UAS, caused a fivefold decrease of acetylene reducing capacity at 21 days. This decrease was absent at 24 days after inoculation in three separate experiments. Nodules formed on plants inoculated with R. leguminosarum P13 harboring the multicopy number vector pMP190 (copy number 15 , H. Spaink, pers. comm.) have the same appearance as those induced by a wild type R. leguminosarum P13 strain. Nodules induced on the main root by R. leguminosarum P13 strains harboring the multicopy inhibiting plasmids pMPΔ1.6H, pMPΔ3.2H, pMPΔ3.6H or pMPΔ3.7H are smaller than wild type nodules, are greenish white in appearance and have little or no leghemoglobin. They resemble nodules induced by R. leguminosarum nifA::Tn5 mutants and senesce fast. On the lateral roots appeared, in addition, reddish looking nodules. Measurements at day 28 or later showed nitrogen fixation levels up to 45% of that of the wild type. This can be attributed to the presence of the reddish nodules on the very ends of the lateral roots. Bacteria were isolated from these reddish nodules and plated on TY-medium containing the antibiotics to select for Rhizobium (Rif) and the pMP190 recombinant plasmid (Str). As a control bacteria isolated from two greenish white nodules were tested in the same way. Whereas approximately 90% of the bacteria from the greenish white nodules contained the multicopy plasmid, those from the reddish nodules appeared to have lost it. The results in table 2 indicate that of the low copy number constructs, only pMPΔ1.6L delays the onset of nitrogen fixation. This is probably due to the fact that it has two UAS's, one imperfect and one perfect. An imperfect UAS, which has an 11 bp instead of a 10 bp spacer as in the consensus can apparently still bind NifA protein. The cumulative binding of NifA during the onset of nitrogen fixation results in the decrease in acetylene reduction capacity as found for pMPΔ1.6L. The conclusion that an imperfect UAS can still bind NifA is in agreement with the results of Buck et al. (1987b).

They showed that a multicopy vector carrying the K. pneumoniae nifH promoter region with a consensus UAS inhibits acetylene reduction to 0.4% of the wild type level; a construct with an imperfect UAS having a 11 bp spacer limits the decrease to 30%. We conclude that the onset of nitrogen fixation is delayed because of the capturing of NifA by both the imperfect and the consensus UAS. The presence of fifteen plasmid borne copies of the promoter region containing an UAS leads to a total lack of nitrogen fixation. An additional effect caused by the presence of the pseudo UAS, as was found for the low copy construct pMPΔ1.6L, is not measurable.
Interestingly, multicopy constructs containing the nifH promoter and a partially deleted UAS, as in pMPΔ3.6H and pMPΔ3.7H, still cause multicopy inhibition. Apparently, the presence of a RpoN dependent promoter suffices to cause multicopy inhibition. Beta-galactosidase assays should show significant differences in the levels of lacZ activity (see Gubler and Hennecke, 1988) between the constructs having the imperfect as well as the consensus UAS and those having only the consensus UAS. However, bacteroids isolated from the nodules of plants inoculated with the R. leguminosarum strains harboring the nifH::lacZ deletion fusions showed β-galactosidase activity only little over background (data not shown).

(d) Can multicopy inhibition be relieved by overexpression of NifA?

From the results described above it appears that multicopy plasmids containing a nifH promoter region inhibit nitrogen fixation. Overproduction of the R. leguminosarum NifA protein might lead to relief of this multicopy inhibition. In order to overproduce the NifA protein it is important to consider the different translational start sites of the nifA gene that can be used. Roelvink et al. (1988) showed that the R. leguminosarum nifA gene has two translation initiation sites which are both active in E. coli. The first one leads to a 519 amino acids polypeptide; the second, which is preferred in E. coli, results in a 488 amino acids polypeptide. The R. meliloti nifA (Buikema et al., 1985, Weber et al., 1985) and Bradyrhizobium japonicum nifA genes (Thöny et al., 1987) also both have two translational start sites. Recently, Beynon et al. (1988) showed that only the full length R. meliloti NifA protein is capable of activating nifH::lacZ fusions in E. coli.

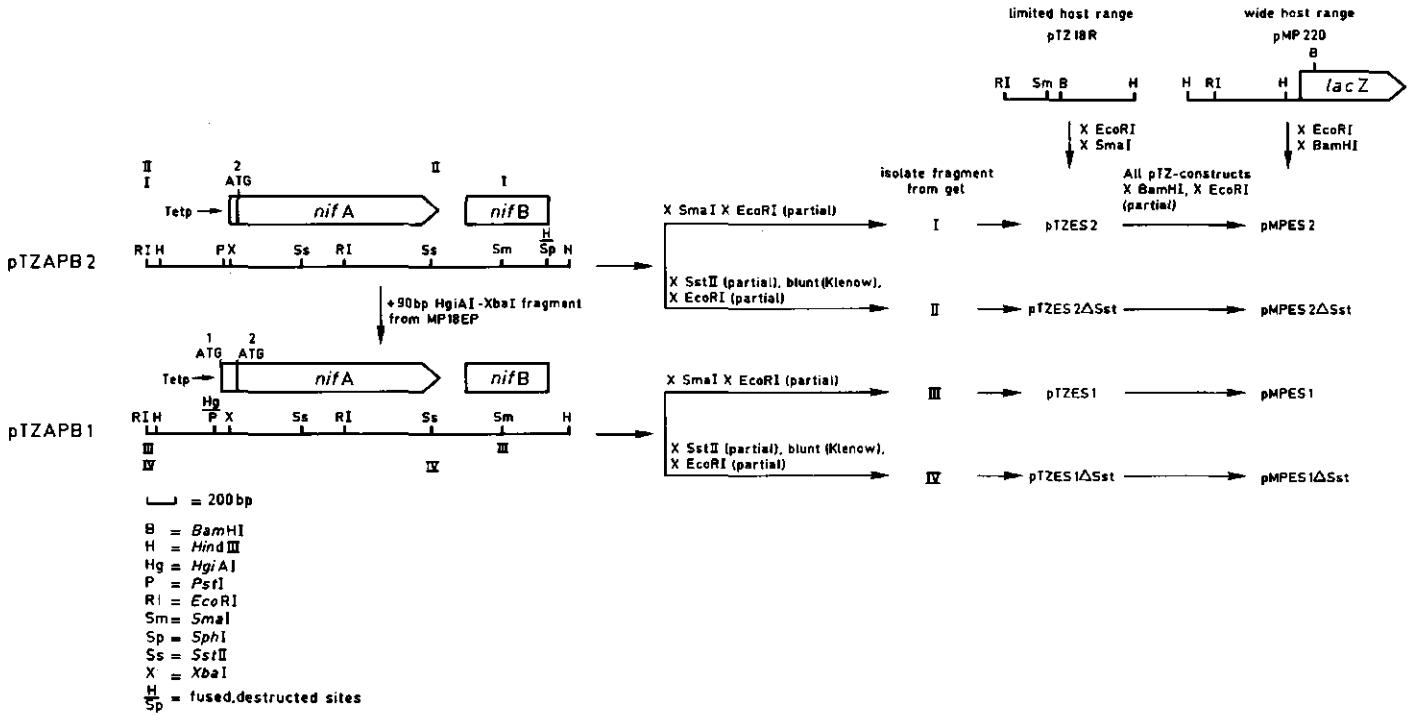

limited host range
pTZ 18R
wide host range
pMP 220
B
RI Sm B H
H RI H lacZ
X EcoRI
X SmaI
X EcoRI
X BamHI
isolate fragment from gel
All pTZ-constructs X BamHI, X EcoRI (partial)
pTZAPB2
Tetp
nifA
nifB
RI H P X Ss RI Ss Sm H Sp H
X SmaI X EcoRI (partial)
I
pTZES2
pMPES2
X SstII (partial), blunt (Klenow), X EcoRI (partial)
II
pTZES2ΔSst
pMPES2ΔSst
+90bp HgiAI-XbaI fragment from MP18EP
pTZAPB1
ATG
Hg P X Ss RI Ss Sm H
III
pTZES1
pMPES1
IV
pTZES1ΔSst
pMPES1ΔSst
= 200bp
B = BamHI
H = HindIII
Hg = HgiAI
P = PstI
RI = EcoRI
Sm = SmaI
Sp = SphI
Ss = SstII
X = XbaI
H/Sp = fused, destructed sites

Fig. 4: Cloning strategy of expression plasmids containing transcription fusions of the Tet-promoter with various fragments of R. leguminosarum nifA. A 2.25 Kb XbaI-BamHI fragment from pRleH12 containing nifA from the second translation start was subcloned in pMP220 (x XbaI and BglII) to obtain pMP220XB (not shown). The Tet-promoter fragment from pBR322 on a 185 bp EcoRI-EcoRV fragment was cloned into pTZ18R (x EcoRI and HincII) to obtain pTZTet. This plasmid was digested with SphI, blunt ended with Klenow, and digested with PstI. Into this plasmid a 2.3 Kb PstI-HindIII fragment from pMP220XB was ligated, resulting in pTZAPB2, containing a fusion of the Tet-promoter to the nifA ORF starting from the second translation start site. To generate pTZAPB1, containing a fusion of the Tet-promoter with the complete nifA ORF, a 90 bp HgiAI-XbaI fragment from the nifA promoter region (from MP18EP, which contains a 385 bp SmaI-AvaI fragment with the nifA promoter region) was cloned in pTZAPB2 digested with PstI and XbaI. Both pTZAPB1 and pTZAPB2 were digested completely with SmaI and partially with EcoRI. Fragments I (2.2Kb) and III (2.3 Kb) were isolated from the gel and ligated into pTZ18R (x EcoRI and SmaI) resulting in pTZES1 and pTZES2. After partially digesting pTZAPB1 and pTZAPB2 with SstII, blunt ending with Klenow, and partial digestion with EcoRI, fragments II (1.7 Kb) and IV (1.8 Kb) were purified by gel electrophoresis. These two fragments containing the Tet::nifA fusions but lacking the DNA recognition helix of the DNA binding domain were also cloned in pTZ18R (X EcoRI and SmaI) resulting in pTZES1ΔSst and pTZES2ΔSst, respectively. The four constructs were digested completely with BamHI, and partially with EcoRI. The fragments containing the Tet::nifA fusions were isolated from the gel and cloned in pMP220 (x EcoRI and BamHI) resulting in the four

Likewise, Kwiatkowski et al. (1988) showed that the full length B. japonicum NifA protein activates nifH::lacZ fusions to a level four times higher than that by a NifA protein arising from the second translation start. Furthermore, Albright et al. (1988) reported that a R. meliloti NifA protein lacking the C-terminal DNA binding domain is active in vivo. We therefore decided to test whether the same is also true for R. leguminosarum.

To determine which of the two possible R. leguminosarum NifA proteins is more active in a homologous background in overcoming multicopy inhibition, and to establish whether the presence of the DNA binding domain is a prerequisite for this activity , four fusions of the constitutive Tet promoter with nifA were constructed: the Tet promoter fused to either the first or the second translation start; and, in addition, fusions that contain the nifA sequence minus the DNA-binding domain. The cloning strategy is explained in Fig. 4. The four resulting fusions were cloned in pMP220 (copy number 5) and conjugated into R. leguminosarum P13 containing the multicopy inhibiting plasmid pMPΔ1.6H. The resulting transconjugants were inoculated on plants. At days 21 and 22 acetylene reducing capacity was assayed: at both times all plants were Fix^-. The nodules of these plants were greenish white and did not appear to contain leghemoglobin. Remarkably, at day 25, all tested plants were Fix^+ at levels up to 75% of the wild type control. Examination of the plants revealed that two types of nodules were present: greenish white nodules typical of strains harboring a multicopy inhibiting plasmid, and reddish nodules on the lateral roots. Several reddish nodules and a few greenish white ones were picked from 25 days old plants, crushed and a few microliters of the suspension were plated on TY-medium containing selective antibiotics. It appeared that more than 90% of the bacteria isolated from the greenish white nodules still contained the multicopy plasmid. Less than one percent contained both plasmids. Of the bacteria isolated from the reddish nodules approx. 5% contained the multicopy inhibiting plasmid and approx 15% the low copy Tet::nifA constructs. Less than one percent contained both plasmids. We conclude that the observed Fix^+ phenotype is due to the loss of the multicopy inhibiting plasmid.

(e) Conclusions

We have analyzed the R. leguminosarum PRE nifH promoter region and identified a promoter TTGGYRYRN$_4$TTGAG which differs from the consensus CTGGYRYRN$_4$TTGCA (Ausubel, 1984 indicated in Fig. 1) in three bases. Primer extension experiments confirmed the functionality of this promoter. Four nucleotides were suggested to be invariant in the consensus promoter i.e. GG at -25/-24 and GC at -13/-12 (see Gussin et al., 1986). We found on the other hand an A at position -12 for the R. leguminosarum nifH promoter. This is however not the only exception to the rule, for both the R. trifolii nifH promoter (Watson and Schofield, 1983) and the R. phaseoli nifH promoter (Quinto et al., 1985) contain an A at this position. Furthermore, a replacement of the C-residue at -12 by an A in the K. pneumoniae nifH promoter had no influence upon the activity (Buck et al., 1985). The identified promoter furthers differs from the consensus in positions -11 and -27; variations at these positions do not influence the activity of the promoter (see Alvarez Morales and Hennecke, 1985, Gussin et al., 1986). We propose to redefine the consensus for Rhizobium nif and fix promoters as NNGGYRYRN$_4$TTG$\frac{C}{A}$N.

The functionality of both the R. leguminosarum pseudo UAS and the UAS was evaluated in a heterologous E. coli and a homologous Rhizobium background. The pseudo UAS and pseudo promoter do not appear to contribute to the activity of the nifH promoter when activated by K. pneumoniae NifA in an E. coli background (Fig. 3). Deletion of the DNA sequence including the first base of the UAS (position -127, Fig. 1) abolishes activity. Mutational analysis of the K. pneumoniae nifH promoter UAS has shown that the 5' and 3' context influence its function (Buck et al., 1987b). This, and the partial deletion of the UAS, may explain the detrimental effect upon activity found here.

The presence of multicopy plasmids containing nif promoter regions results in the inhibition of nitrogen fixation. Such multiple copies of the nif promoter region capture NifA activator protein with the UAS resulting in a shortage of regulator available for activation of the nif and fix genes.

Insertion of one base in the ten base spacer the K. pneumoniae nifH UAS relieves this multicopy inhibition. A wild type construct with a consensus UAS causes a decrease of acetylene reduction to 0.4% of the wild type level. A construct with an UAS with a 11 bp spacer limits the decrease to 30%. This shows that such an imperfect UAS can still bind NifA (Buck et al., 1987b). Hennecke et al. (1988) suggested that the presence of UAS's modulates the expression of nif and the fix genes. Thus, a gene driven by a promoter with two UAS copies is expressed at a higher level than genes driven by a promoter with one UAS copy or a promoter preceded by an imperfect UAS. The term "fine tuning" was coined for this phenomenon. Nodules appearing on plants inoculated with R. leguminosarum P13 containing the low copy number construct pMPΔ1.6L, that has a pseudo UAS as well as a consensus UAS, show a five-fold reduction of acetylene reducing capacity. This reduction is most likely explained by the cumulative capturing of NifA by the pseudo and the consensus UAS. During the onset of nitrogen fixation the available amount of NifA may be limited. Capture of NifA by the UAS's limits the amount available for the activation of nif and fix genes, the expression of which is thus delayed. At a later stage of development the amounts of NifA increase to such levels that capturing by the UAS's does not influence the expression of these genes anymore. We have been unable to study activation of plasmid borne nifH::lacZ fusions by measuring β-galactosidase levels. Gubler and Hennecke (1988) measured the β-galactosidase activity of chromosomally integrated as well as plasmid borne nifH::lacZ, fixA::lacZ and fixB::lacZ constructs. They conclude that the integrated fusions could be activated to appreciably higher levels than the plasmid borne ones and suggested that this effect was due to some constraint at the level of the plasmid used like supercoiling. It is remarkable that the pMPΔ3.7H and pMPΔ3.6H constructs that have deletions including one and six bases of the consensus UAS respectively, still retain their inhibiting capacities. This is in sharp contrast with results obtained for K. pneumoniae nifH, where deletion of the UAS results in a complete relief of multicopy inhibition (Buck et al., 1987). Our present result may be interpreted in two ways. First, the presence of half of a functional site, defined by Buck et al. (1987b) as the first six bases on either upper or lower strand of the twofold rotational symmetrical UAS suffices for the capturing of NifA and the resulting multicopy inhibition.

This suggestion is however refuted by the observation that altering the sequence of one of the two half sites or the spacing in the UAS of the K. pneumoniae nifH promoter greatly reduces the ability to cause multicopy inhibition (Buck et al., 1987b). According to a model proposed by Buck et al (1987a,b) each half site binds a NifA monomer, which results in a dimer NifA stabilizing the binding. After looping out of the DNA between UAS and promoter the NifA dimer contacts the RpoN-RNA polymerase complex and is then capable of activating transcription. A second explanation is that for the activation of transcription at the R. leguminosarum PRE nifH promoter binding to the UAS is not necessary and that direct complex formation between RpoN-RNAP and NifA followed by interaction at the promoter may result in transcription activation. This suggestion is supported by the finding that a R. meliloti nifH gene driven by a promoter without an UAS after recombination into the Sym-plasmid can be activated to wild type levels in planta (Better et al., 1985). For reasons stated above, it seems unlikely that the pMPA3.6H and pMPA3.7H constructs, with partly deleted UAS's can still bind NifA protein. These constructs do have an intact promoter where transcription initiation by the RpoN-RNAP-NifA complex may take place. We therefore favour the second explanation for the inhibitory effect of these constructs. We tested the possibility to relieve multicopy inhibition by conjugating a plasmid carrying a fusion of the constutive Tet-promoter with nifA into R. leguminosarum P13 harbouring a multicopy nif inhibiting plasmid but these attempts were not succesful. Plants inoculated with the P13 strain harboring both plasmids exhibit two types of nodules: greenish white ones, typical of strains harboring multicopy inhibiting plasmids, in which most bacteria have lost the Tet::nifA fusion plasmid, and reddish ones, in which the multicopy plasmid or both plasmids are lost. This plasmid loss most probably explains the restoration of nitrogen fixing capacity of nodules induced by these strains. Clearly, the presence of the two plasmids, when not under selective pressure results in plasmid instability and loss. This is surprising since these plasmids belong to different incompatibility groups: pMP220 to the IncP1 group and pMP190 to the IncQ group. At present we have no explanation for this plasmid instability. By placing the Tet::nifA fusions in the same vector as the nifH::lacZ fusion it may become possible to study the effect of constitutively produced NifA on multicopy inhibition.

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Summary

This thesis describes the characterization of the nifA regulatory gene of the pea endosymbiont Rhizobium leguminosarum PRE.

Chapter 1 gives a general overview on the regulation of nitrogen fixation in diazotrophs, with special focus on the regulatory NifA protein. The regulation of genes involved in nitrogen fixation in two bacteria is discussed in detail: the free living Klebsiella pneumoniae and the endosymbiont of alfalfa R. meliloti. Major differences exist between these organisms where the onset of nitrogen fixation is concerned. K. pneumoniae has a general nitrogen regulatory circuitry which senses an internal biochemical signal i.e. the level of available ammonia as defined by the glutamine to 2-ketoglutarate ratio, a high ratio indicating a surplus, a low ratio a deficit. Sensing of a N-deficit results is translated, through a chain reaction of protein modifications, into activation of the regulatory NtrC product by phosphorylation. The resulting NtrC-P activates transcription of the regulatory nifLA operon, which encodes the inhibitor NifL and the activator NifA. The Klebsiella NifA thereupon activates transcription of the genes involved in nitrogen fixation. In a recently published paper David et al. (1988) suggest that the onset of nitrogen fixation in R. meliloti starts with the sensing of the external oxygen level. The FixL protein is hypothesized to sense a decrease in oxygen level. This protein is thus activated and in turn activates the FixJ protein, which directly or indirectly activates transcription of the nifA gene. The Rhizobium NifA protein activates transcription of the nitrogen fixation genes. In this overview we hypothesize that the oxygen sensing protein FNR instead of FixL senses the internal oxygen level. FNR then activates transcription of the fixLJ operon. The FixL protein may be a moderator of the activity of FixJ, comparable to the role of NtrB in activating NtrC. To date all rhizobial NifA proteins, in contrast to Klebsiella NifA, were shown to be oxygen sensitive. The structural analysis of the NifA protein is described and possible functions ascribed to domains identified in this protein are discussed.

A model for NifA activity emerging from data presented for K. pneumoniae is

discussed. At present a complete model cannot be presented for Rhizobiaceae. The similarities and differences between the models for K. pneumoniae and Rhizobium species are discussed.

In **Chapter 2** the DNA sequence and deduced amino acid sequence of R. leguminosarum PRE are presented. The amino acid sequence differs in 30 amino acids from that published for R. leguminosarum 3855 (Grönger et al., 1987). A possible explanation for this difference is discussed. The NifA Open Reading Frame (ORF) reveals two potential translation start sites, which in a heterologous E. coli background appear to be used both. The second translation start, which leads to a 488 amino acids, 53 kD protein, is preferred over the first, which leads to a 519 amino acids, 56.1 kD protein. The R. meliloti (Weber et al., 1985, Buikema et al., 1985) and B. japonicum nifA genes (Thöny et al., 1987) also have two translation start sites. It was shown for R. meliloti NifA (Beynon et al., 1988) the full length protein is the active form in an E. coli background. It is discussed that a translational preference for the second translational start site, leading to the inactive protein, as was found in pulse labeling experiments in E. coli may also exist in Rhizobium. We therefore suggest that the experiments presented by Beynon et al. (1988) are not conclusive as to the size of the functional protein in a Rhizobium background. Primer extension experiments and S_1-nuclease protection were used to identify the putative nifA promoter. A transcription terminator was identified by S1-nuclease protection.

Chapter 3 deals with a phenomenon reported by Hawkins and Johnston (1988) and Roelvink et al. (1988). A nifA::Tn5 mutant can not be complemented by a plasmid having only the nifA coding DNA fragment. A detailed analysis of the nifA-nifB intergenic region is presented. The nifA gene has a transcriptional terminator typical of bacterial genes (Brendel et al., 1986) consisting of a four GC basepairs stem and a nine base loop followed by a thymidine rich DNA stretch. This terminator was mapped by S1-nuclease protection. The nifB gene has a RpoN dependent promoter, having all nucleotides thought to be crucial to its activity. The nifA terminator was fused to the Tet-promoter and this fusion was cloned in a low copy

transcriptional lacZ vector. The results show that the nifA terminator allows 85% readthrough. RNA::DNA hybridisation studies show that the nifA gene is transcribed at a level twice of that of nifB. By using a plasmid, which has a DNA region encompassing nifA, nifB and a ferredoxin like gene downstream of nifB (Grönger et al., 1988, Klipp et al., 1988) it was shown that nifA::Tn5 mutants can be fully complemented. Taken together these findings suggest that the nifA and the nifB gene are in one operon. The failure of plasmids having the nifA encoding DNA fragment alone to complement a nifA::Tn5 mutant results from a polar effect of the Tn5 transposon on nifB transcription.

Chapter 4 deals with the nifH promoter region of R. leguminosarum PRE as one of the target sites of the NifA protein. We determined the nucleotide sequence of this region and identified a pseudo upstream activator sequence (UAS), a pseudo promoter, a consensus UAS and a consensus promoter. The promoter, mapped by primer extension experiments, differs from the consensus in one of the nucleotides thought to be invariant (see Gussin et al., 1986). The function of the nifH promoter elements was tested in a heterologous E. coli and a homologous Rhizobium background. Fusions of the nifH promoter region to lacZ, and fusions of deleted nifH promoter regions to lacZ, were used in activation studies by K. pneumoniae NifA in E. coli. Both high and low copy (deletion) nifH::lacZ fusions were conjugated to Rhizobium. The activation study in an E. coli background showed that the pseudo UAS and the pseudo promoter are not involved in the function of the promoter. A different result was obtained with low copy nifH::lacZ constructs in a Rhizobium background. The construct having both pseudo and consensus UAS, when compared with a construct having the consensus UAS only, seems to delay the onset of nitrogen fixation by three days. We suggest that this indicates that the presence of one or more UAS's modulates the expression of nif and fix genes, as was suggested for UAS's of B. japonicum nif and fix genes (Gubler and Hennecke, 1988). A nif promoter region holding a UAS, when cloned in a multi copy vector, can inhibit nitrogen fixation by capturing the NifA activator needed for expression of nif and fix genes. A multicopy inhibition study with (deleted) nifH::lacZ fusions led to a surprising finding: deletion of part of the consensus UAS

on the multicopy plasmid did not result in inhibition of nitrogen fixation. The relevance of this finding is discussed. We conclude that R. leguminosarum nifH can function without an UAS as was found for R. meliloti nifH in planta (Better et al., 1985). We suggest that NifA may form a complex with RpoN-RNAP that can bind directly at the promoter to activate transcription.

Samenvatting

In dit proefschrift wordt het regulerende nifA gen van de symbiontische stikstoffixerende bacterie Rhizobium leguminosarum beschreven.

Hoofdstuk 1 geeft een algemeen overzicht over de regulatie van stikstofbinding in diazotrofe bacteriën (diazo=stikstof troof=eten). Hierin beperk ik mij tot de beschrijving van de twee meest bestudeerde stikstoffixerende bacteriën: enerzijds de vrijlevende Klebsiella pneumoniae, anderzijds Rhizobium meliloti, die met luzerne (Medicago sativa L.) in symbiose stikstof fixeert. Tussen beide organismen blijken grote verschillen aanwijsbaar als het gaat om de signalen die leiden tot de start van de stikstoffixatie. K. pneumoniae heeft een algemeen regulerend systeem dat reageert op een intern biochemisch signaal, in dit geval de hoeveelheid gebonden stikstof in de cel. Dit blijkt te kunnen worden gemeten door een regulerend systeem dat de verhouding tussen glutamine en 2-ketoglutarate meet. Een hoge waarde geeft een overmaat stikstof aan, een lage verhouding een tekort. Nadat een tekort is vastgesteld, wordt dit signaal via een keten van reacties vertaald in de activering van een regulatoreiwit NtrC. Deze activering bestaat uit de koppeling van een fosfaatgroep aan het eiwit en resulteert in de vorming van NtrC-P dat dan de transcriptie (overschrijving van DNA in RNA, die wordt gevolgd door translatie = vertaling in eiwit) activeert van de twee genen, nifL en nifA, die zeer nauw betrokken zijn bij de activering van de stikstofbindingsgenen. Het NifL eiwit is een remmer van de transcriptie van stikstofbindingsgenen nodig om snelle veranderingen in milieu en leefomstandigheden van de K. pneumoniae bacterie (toename van de hoeveelheid gebonden stikstof, plotselinge daling van zuurstofgehalte) te vertalen in een noodremprocedure op de stikstofbinding. Klebsiella NifA is een eiwit dat de transcriptie van de stikstofbindingsgenen kan activeren, dat niet gevoelig is voor zuurstof (het enzymcomplex dat stikstof bindt, nitrogenase, is dat wel). De start van de stikstofbinding in R. meliloti vindt plaats als de bacteroid, die zich in de wortelknol bevindt, een daling van het zuurstofpercentage van het externe milieu waarneemt. Dit leidt tot de productie en omvorming van een FixJ eiwit, dat direct of indirect (deze stap is nog niet vastgesteld) de

transcriptie van het nifA gen activeert. Het Rhizobium NifA eiwit is een zuurstofgevoelige activator (dit dus in tegenstelling tot K. pneumoniae NifA) die de transcriptie van stikstofbindingsgenen activeert. Het overzicht in hoofdstuk 1 bevat een analyse van de structuur van het NifA eiwit, dat diverse "domeinen" bevat waaraan functies zijn toegekend. Op dit moment bestaat alleen voor K. pneumoniae een regulatiemodel. Hierin wordt uitgelegd hoe het NifA eiwit transcriptie vanaf de promoter (de aan-en uitschakeleenheid) van de stikstofbindingsgenen activeert. Een dergelijk model kan niet worden gepresenteerd voor Rhizobiaceae (de bacteriën uit de Rhizobium familie). Het is zeker dat een dergelijk regulatiemodel op enkele essentiële punten zal afwijken van dat voor K. pneumoniae.

In **Hoofdstuk 2** zijn de nucleotidenvolgorde van R. leguminosarum nifA en de daarvan afgeleide aminozuurvolgorde weergegeven. Het open leesraam van R. leguminosarum NifA heeft twee translatie starts die bij analyse in E. coli beide blijken te worden gebruikt. Het belang van deze waarneming wordt geanalyseerd. Start op het eerste methionine codon levert een eiwit op van 519 aminozuren (56 kD) en start op het tweede een eiwit van 488 aminozuren (53 kD). Het verschijnsel van twee translatiestarts doet zich ook voor bij R. meliloti. Gedetailleerd onderzoek heeft geleid tot de conclusie dat start op de eerste methionine, de actiefste vorm van het eiwit oplevert. Hoe is dan te verklaren dat in E. coli de vertalingsvoorkeur uitgaat naar start op de tweede methionine, wat een minder actieve vorm oplevert? Of duidt het gevonden verschijnsel op een complex regulerend systeem voor NifA activiteit? Het is nog niet mogelijk een pasklaar antwoord te geven.
Analyse van de nifA promoter regio leidde tot de identificatie van een promoter voor het nifA gen. Ook werd een transcriptie terminator geïdentifitificeerd, die nader werd geanalyseerd.

Hoofdstuk 3 bevat een analyse van een Rhizobium mutant, waarin het regulerend nifA gen is uitgeschakeld. Het gevolg hiervan is dat planten die met deze bacterie worden beënt, knolletjes gaan vormen waarin bacteroiden voorkomen die niet meer tot stikstofbinding in staat zijn. Een dergelijke plant maakt na drie weken een zeer schrale indruk. In de praktijk zou het mogelijk moeten zijn om, door aanbieding van een DNA fragment waarop het

nifA gen ligt, herstel van het vermogen tot stikstofbinding te bewerkstelligen; dit blijkt echter niet op te gaan. Dit hoofdstuk bevat een analyse van de DNA regio tussen nifA en het daaropvolgende nifB gen. NifA heeft zoals beschreven een transcriptie terminator. Het nifB gen heeft zijn eigen gereguleerde promoter. Om vast te stellen hoe goed de nifA terminator functioneert werden fusies in elkaar gezet van de Tet promoter gevolgd door de nifA terminator en daarachter lacZ, dat als reporter gen dient (een reporter gen is een gen, waarvan het product of de activiteit van dat product op biochemische wijze valt te ontdekken en in getallen om te zetten). Het idee hierachter is, dat als de terminator functioneert, dit zal resulteren in de afwezigheid van β-galactosidase enzym. Dit eiwit zet de kleurloze stof ONPG om in een gele stof, waarvan de concentratie op biochemische wijze is te bepalen. Het bleek de nifA terminator nauwelijks functioneert en 85% doorlezing toestaat. Hieruit volgt dus dat de nifA en nifB genproducten kennelijk ongeveer gelijkelijk aanwezig zullen zijn in stikstofbindende bacteroide. Uit verdere studies waarbij het RNA van de bacteroiden tegen het DNA werd gehybridiseerd, bleek dat het boodschapper RNA van nifA twee maal zoveel aanwezig is als dat van nifB. Terugredenerend zou dit moeten betekenen dat de nifA terminator een doorlezing van 50% te zien had moeten geven bij de lacZ studie in E. coli. Kennelijk functioneert de nifA terminator in bacteroiden dus beter dan in E. coli. Deze resultaten suggereren dat de transcriptie van nifB afhankelijk is van doorlezing vanaf de nifA promoter.
Het bleek wel mogelijk om een nifA mutant te complementeren door het DNA fragment waarop zowel nifA als nifB liggen gecodeerd aan te bieden op een vector. De conclusie hieruit is dat nifA en nifB, die allebei de structuren hebben die het mogelijk moeten maken om onafhankelijk van elkaar te functioneren, toch in één operon liggen, en gezamenlijk worden afgelezen.

Hoofdstuk 4 bevat een analyse van een van de doelgebieden van het NifA eiwit: het nifH promoter gebied. NifH codeert voor een eiwit van het nitrogenasecomplex, het enzym dat atmosferische stikstof omzet in ammonia. De nucleotidenvolgorde van het nifH promotergebied werd bepaald. In deze nifH regio werden een pseudo upstream activator sequentie (UAS, dit is de DNA sequentie waarvan is vastgesteld dat het NifA eiwit eraan bindt) en een

pseudo promoter geïdentificeerd. Het nifH promoter gebied bevat een consensus UAS (zo'n DNA structuur komt ook voor in andere stikstofbindende bacteriën; het is als het ware een sequentie die deze bacteriën als gemeenschappelijk kenmerk hebben) en een promoter geïdentificeerd. De promoter wijkt af in een nucleotide, dat werd geacht invariant te zijn. Het bleek dat meer van dergelijke afwijkingen zijn gerapporteerd, maar dat deze niet zwaar genoeg bij de bepaling van de consensus promoter hebben meegewogen. Daarom wordt voorgesteld de algemene consensus voor dergelijke promoters bij te stellen. De functie van de nifH promoterelementen werd bestudeerd in een heterologe (E. coli) of homologe achtergrond (Rhizobium). K. pneumoniae NifA werd daarbij gebruikt om de activering van nifH::lacZ fusies te bestuderen in een E. coli achtergrond. De studie leidde tot de conclusie dat bij de activering door K. pneumoniae NifA de pseudo UAS en de pseudo promoter geen rol spelen. De geïdentificeerde consensus promoter is dus de functionele.

De nifH::lacZ fusies, en fusies waarbij van het nifH promoter gebied een steeds groter wordend stukje werd verwijderd, werden verwijderd gekruist naar de homologe R. leguminosarum achtergrond. De studie met lage copie fusies (dat zijn vectoren waarin de nifH::lacZ fusie in is gezet. Deze vectoren hebben als eigenschap dat ze in Rhizobium in aantallen tot 5 copieën kunnen voorkomen) leverde als resultaat op dat een constructie, die zowel de pseudo UAS als de UAS bevatte, vergeleken met een die alleen de UAS bevatte, een vertraging van de start van de stikstofbinding van drie dagen veroorzaakte. Dit bracht ons tot de conclusie dat de aanwezigheid van de pseudo UAS wel effect heeft. Dit verschijnsel wijst erop dat, zoals ook werd gesuggereerd voor de B. japonicum stikstofbindingsgenen, een fijnregelmechaniek bestaat. De aanwezigheid van twee, een of zelfs geen UAS zou het expressieniveau van stikstoffixatiegenen kunnen beïnvloeden (Hoe meer copieën van de UAS hoe hoger de expressie). NifH::lacZ fusies, indien aanwezig in veel copieën (meer dan tien) kunnen leiden tot remming van de stikstofbinding. Dit wordt veroorzaakt door het "vangen" het regulatoreiwit, waardoor geen regulator meer beschikbaar is voor de stikstofbindingsgenen resulterend in een totaal onvermogen tot stikstofbinding. Het is bekend dat de UAS, waaraan het NifA eiwit bindt verantwoordelijk is voor dit effect. Verwijdering van de UAS uit een nifH::lacZ fusie zou dan ook

moeten leiden tot normale stikstofbinding. Dit bleek echter niet het geval te zijn in een R. leguminosarum achtergrond. Iets dergelijks werd ook gevonden voor de nifH promoter van R. meliloti. Een verklaring is dat NifA met RpoN-RNAP (een complex van eiwitten, dat ervoor zorgt dat de promoter als zodanig wordt herkend, en dat het gen wordt overgeschreven in RNA) een complex vormt. Dit bindt dan rechtstreeks aan de promoter, wat leidt tot transcriptie activering.

NAWOORD

"Waarom promoveer je eigenlijk?", vroeg de stamgast met een ietwat door drank ontregelde stem. "Daar hebben we het al eens over gehad, weet je wel. Toen vlogen de ovenschalen bijna door de lucht!" Tja, waarom? Een promotie onderzoek biedt je de kans om experimenteel bezig te zijn. Als je dat, zoals ik, graag wilt, is het unieke gelegenheid. Na een aantal jaren schrijf je je resultaten op, werk je je conclusies uit en zet je de discussie op papier. Proefschrift! Als het geheel voldoet aan de eisen van de beoordelende vakmensen, komt het tot een openbare verdediging tegen hun bedenkingen, die soms op de inhoud, soms op de vormgeving betrekking hebben. Dat is dan heel wat slapeloze nachten verder. Wie zou iets kunnen toevoegen aan hetgeen Erasmus, die op een der eerste pagina's is aangehaald, heeft opgeschreven? Uitgaande van zijn analyse, die overigens soms wat aangezet overkomt, wil ik op deze plaats geen uitputtende analyse geven van de gevoelsmatige kant van de zaak. Ik laat het in het midden of promotie onderzoek een gang naar Canossa (voor beide partijen) of een uitvoering van Bordewijk's Karakter is. Promotie is tenslotte omzien in rok. In stijl. Daarom zal de lijst dankbetuigingen niet korter zijn dan anders. Dank aan de promoter, Ab van Kammen, voor de gebleken belangstelling en de actieve bemoeienis met de definitieve inhoud van het proefschrift. Rommert van den Bos, co-promoter, dank voor de vele uren die we spraken over van alles en nog wat, waaruit jouw bewogenheid bij het maatschappelijke gebeuren bleek. Jouw belangstelling voor het onderzoek daalde zelden, en je was altijd bereid de ontwerpteksten van dit boekwerkje te lezen, en het wanordelijk mozaiek van zinnen en paragrafen tot een leesbaar geheel te herschikken. Samen met Ab deed je dat zo vaardig, dat de resultaten van jullie werk overbrengt wat ik wil zeggen op die manier dat ik het zou hebben gezegd als ik dat had gekund.

Jan Hontelez, clowneske analytische steunpilaar, dank voor je collegialiteit, het afronden van alweer een ultiem experiment. René Klein-Lankhorst, altijd in voor een humoristische benadering van de onderzoeksproblematiek, die net iets andere kijk op onderzoek (Groningse?), ideeën.

Elk onderzoek valt of staat met de inbreng van doctoraal studenten, die gelukkig allemaal hebben begrepen dat zij in de eerste plaats aanwezig waren om iets te leren en pas in de tweede plaats om iets te presteren.

Dank dus aan **Frank van der Wilk**, expert, naar later bleek, in het werken bij kunstlicht. **Thijs Broos**, de eerste student die ik wekelijks voorzag van verse peterselie, omdat een nachtelijke escapade (alweer) bij de Shoarma-tent was geëindigd. **Connie van Oers**, die de basis heeft gelegd, opnieuw, voor het sequence werk aan nifH. **Lilian Colbers**, vergeefs op zoek naar NtrC, heb je toch alle ins en outs van het cloneren en hybridiseren onder de knie gekregen. **Mariët van der Werf**, begeleid door Jan Hontelez leverde jij 't fraaie plaatje af van de twee NifA eiwitten. **John Stigter**, handyman bij uitstek, via Harvard uiteindelijk op het Max Planck instituut terechtgekomen, waar je je vele ideeën kwijt kunt. **Michiel Hermsen**, bijna ouwe hap, jij hebt met jouw experimenten Hoofdstuk 5 van zijn ruggegraat voorzien. Jouw bereidheid op, zelfs na afloop van je studietijd, toch nog die laatste clonering te doen, heb ik zeer kunnen waarderen.
Een goede sfeer tijdens het werk is van levensbelang voor een ieder. Vandaar dat ik iedereen, zonder uitzonderingen, of hoewel, **Ben Scheres** en **Jan Verver** in het speciaal, wil bedanken.
Gré en **Marie José** worden gewaardeerd voor type en tekst(ver)werkzaamheden. Voor zover ik zelf het verwerken van de tekst ter hand nam, worden zij bedankt voor tips betreffende de werking van het Océ-kreng. **Piet**, bedankt voor het tekenwerk, je vermogen om toch nog maar weer die paar kleine wijzigingen die echt, ik zweer het je, niet waren opgevallen, aan te brengen. **Peter**, bedankt voor jouw fotografische inspanningen. Ben ik iemand vergeten? **Ton** G. Chauffeur van het eerste uur. Kenner van verkeer(d) en hinderlijk weggedrag bij uitstek. Bezitter van een encyclopedisch geheugen van oude moppen. Dank voor die jaren van rijden. Je soepelheid. Nog iemand vergeten? Nee toch? Ja dan. Sorry! **Vrienden en vriendinnen**, bedankt voor jullie belangstelling, ook al draafde ik, ik weet het wel, zo nu en dan eens te ver door.

Beknopte levensbeschrijving

Ik ben geboren op 5 augustus 1956 om vijf voor zeven 's ochtends in de stad Kampen. Na een onbewogen jeugd, afgesloten met het examen middelbare school (Almere College) in 1974, vertrok ik naar Utrecht. Leraar worden, dat leek me wel wat. In 1979 deed ik dossierexamen op de nieuwe leraren-opleiding in de vakken Biologie en Scheikunde. Ik had in de jaren daaraan voorafgaand ontdekt dat het leraarsvak het toch niet helemaal was, en 'sluisde' in op de Rijksuniversiteit te Utrecht. In juni 1979 maakte ik in het kader daarvan kennis met 550 plantesoorten (in het Latijn wel te verstaan) in het Zeeuwse, het Gelderse, het Texelse en het Limburgse. Twijfel. Gelukkig waren de andere cursessen wel interessant. In oktober 1980 begon ik met de doctoraalvakken. Het eerste hoofdvak deed ik bij de vakgroep Algemene Plantkunde (**Prof. dr J. van Die**) onder begeleiding van **dr. Henri W. Groeneveld**. Secundaire plantestoffen. Een grote groep stoffen, die ondermeer de biochemicaliën omvat waarmee Moeder Natuur chemisch oorlog voert. Ik deed een wat onschuldiger, maar daarom niet minder interessant onderwerp: Biosynthese van sterolen. Ik combineerde dit studieonderwerp met een deeltijdbaan als vaste medewerker bij het Utrechts Universiteitsblad. Een tweede hoofdvak deed ik in Leiden bij de vakgroep Moleculaire Plantkunde (**Prof. dr Rob A. Schilperoort**) onder begeleiding van **dr. Andre Hoekema** (dr 'Hook'). Het onderzoek had betrekking op Agrobacterium tumefaciens (vrij vertaald: de bacterie die tumoren veroorzaakt; op planten wel te verstaan). Het derde vak van mijn doctoraalstudie had ook betrekking op een planteziekten veroorzakende bacterie: Pseudomonas syringae pathovar phaseolicola. Deze bacterie veroorzaakt de vetvlekkenziekte bij de boon. Ik schreef over deze bacterie, begeleid door **Prof. dr K. Verhoeff**, een scriptie. Op 9 april 1984 deed ik doctoraal examen. In driedelig, op blauw/oranje gymschoenen. Van april 1984 tot december 1984 werkte ik, gefinancierd door de firma Centrascience bij de Vakgroep Moleculaire Plantkunde te Leiden. In december 1984 werd ik aangesteld als wetenschappelijk assistent, in dienst van de Landbouwuniversiteit, bij de vakgroep Moleculaire Biologie (**Prof. dr A. van Kammen**; begeleiding: **dr R.C. van den Bos**) en verrichtte daar tot januari 1989 het in dit proefschrift beschreven onderzoek. Sinds 1 februari 1989 ben ik verbonden aan diezelfde Landbouwuniversiteit. Ik werk als moleculair bioloog bij de vakgroep Virologie in het kader van het 'Risk Assessment' programma van de EEG.