Acta Bot. Neerl. 38(3), September 1989, p. 233–252

Regulation of nitrogen fixation in diazotrophs: the regulatory *nif*A gene and its characteristics

P. W. ROELVINK* and R. C. VAN DEN BOS

Department of Molecular Biology, Wageningen Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

CONTENTS

Key-words: diazotrophs, *nif*A, nitrogen fixation, regulation.

INTRODUCTION

Biological nitrogen fixation is an important link in the nitrogen cycle. The ability to convert atmospheric nitrogen to metabolically usable compounds is confined to a few classes of bacteria and blue-green algae. Nitrogen-fixing organisms can be divided into two groups. (i) The free-living species that fix nitrogen for their own purpose, e.g. *Klebsiella pneumoniae* and *Azotobacter vinelandii*. (ii) The species that depend on an intimate association with plants, e.g. the cyanobacterium *Anabaena azollae* with the waterfern *Azolla*, or on intracellular symbiosis, e.g. 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* [amongst others: pea, (*Pisum sativum* L.),

Correspondence: R. C. van den Bos.
*Present address: Department of Virology, Wageningen Agricultural University, P.O. Box 8045, 6700 EM Wageningen, The Netherlands.

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 that are 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 review 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 anearobic *K. pneumoniae* and the obligate aerobe *Rhizobium meliloti*, the symbiont of alfalfa (*M. sativa*).

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, *nif*LA. Activation of nitrogen-fixation genes in *R. meliloti* follows a path that differs in several aspects from that in *Klebsiella*. A model for this activation is emerging and will be discussed. The regulatory *nif*A gene plays a key role in all nitrogen-fixing species. We will summarize the data on the structure and function of the *nif*A gene product. A general model for the activation of nitrogen fixation genes will be discussed that, due to 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 *nif*A, 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 *fix*ABC genes that are 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. *nif*A), 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 an overview of both the similarities and differences in the nitrogen-fixing apparatus of both the free-living *K. pneumoniae* and a symbiotic nitrogen fixer such as *R. meliloti*. Reviews on nodule formation and involvement of plant genes in this process are presented elsewhere (Long 1984; Downie & Johnson 1986; Nap 1988).

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

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 into eight 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

Table 1. Genes and gene products that play a major role in nitrogen regulation of *K. pneumoniae*

Gene	Product	Function	Mode of action
*gln*D	Uridyl transferase (UTase)	'Sensor' of glutamine to 2-ketoglurate ratio	Regulates P_{II} mode of action by (de)uridylylation
*gln*B	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}
*gln*E	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
*gln*A	Glutamine synthetase (present in adenylylated form)	Catalyses conversion of glutamate and ammonia to glutamine	Deadenylylation leads to activation. Adenylylation leads to deactivation
*ntr*B	NtrB	Dephosphorylase/kinase	Together with P_{II}-UMP, NtrB activates NtrC by phosphorylation. Together with P_{II}, NtrB deactivates NtrC by dephosphorylation
*ntr*C	NtrC	Transcriptional activator	Active when phosphorylated. Inactive when dephosphorylated

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. The *gln*D product, a uridylyltransferase (UTase), responds directly to the intracellular ratio of glutamine to 2-ketoglutarate; a high ratio (Gln $\gg$ 2KG) indicates a surplus of fixed ammonia, a low ratio (2KG $\gg$ 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 *gln*B gene (Bueno *et al.* 1985). P_{II} exists in the cell as a tetramer 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 *ntr*B product, which leads to phosphorylation of NtrC (Ninfa & Magasanik 1986). The resultant NtrC–P is a DNA-binding activator that interacts with RNAP (RNA-polymerase) complexed with RpoN, a sigma factor encoded by the *rpo*N gene (also known as *ntr*A, *gln*F and *rpo*E), that recognizes *nif* and *fix* promoters. From the sensing of the nitrogen-content of the cell by the UTase and the resultant activation modifications of other proteins in the cascade, the regulatory system, that first reacted to a biochemical

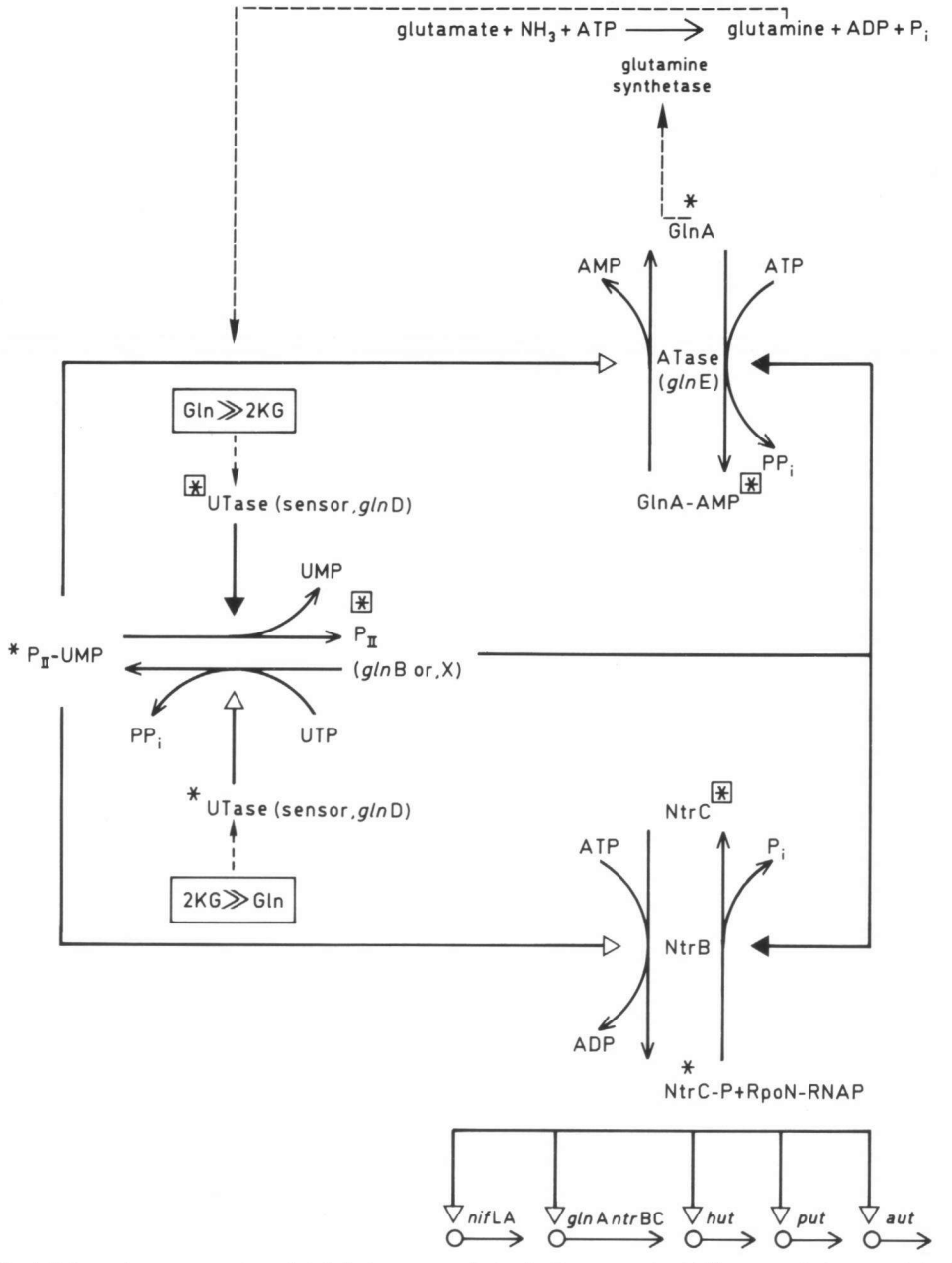


Fig. 1. Schematic representation of global nitrogen regulation in *K. pneumoniae*. Different symbols are explained in the figure. The genes and gene products involved, as well their functions, are explained in Table 1. This figure is intended to be self-explanatory; the reader is advised to follow the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

signal, now influences other operons at the transcriptional level. 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 *nif*LA. 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)

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

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. A key enzyme in this sequence is the *gln*A 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 *gln*E gene, and P_{II}-UMP. The produced glutamine increases the glutamine to 2-ketoglutarate ratio resulting in partial inhibition of the UTase (*gln*D). The UTase then 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 (*gln*E) resulting in adenylylation and inactivation of glutamine synthetase. Recent experiments with *gln*B 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, capable of reacting to shifts in the glutamine to 2-ketoglutarate ratio, is present in *Klebsiella* (Holtel & Merrick 1988). Note 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 is true for glutamine synthetase. Part 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 nitrogen 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 event of a nitrogen shortage. The interaction of NtrC–P with RpoN–RNAP leads to activation of several important genes and operons, the *gln*A*ntr*BC 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 *nif*LA operon. The two genes in the *nif*LA operon which encode 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. The *nif*LA 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 can bind specifically (Morett *et al.* 1988). The structural aspects of *nif*-specific promoters will be discussed in the section on *nif* regulation studies in *Rhizobiaceae*. NifL, in reaction to oxygen and/or ammonia, counteracts NifA activity.

REGULATION OF NITROGEN FIXATION IN *RHIZOBIACEAE*

The regulatory role of nif*A*

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 is in part hypothetical. It is based 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. Transcription of *nif* and most of the *fix* genes in NifA-dependent; mutations in *nif*A lead to a Fix$^-$ phenotype (reviewed in Gussin *et al.* 1986).

Regulation of nif*A expression in* R. meliloti

Szeto *et al.* (1984) first identified a symbiotic regulatory gene (*nif*A) in *R. meliloti* with strong homology to *E. coli ntr*C and somewhat less homology to *K. pneumoniae nif*A. Previous experiments have already shown that a Tn*5* mutation in this gene led to a Fix$^-$ phenotype and that no accumulation of NifHDK proteins was detectable (Zimmerman *et al.* 1983). Other *nif*A-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 *nif*A genes allowed the construction of *nif*A::*lac*Z transcription and translation fusions. In combination with *nif*::*lac*Z fusions, that are activated by NifA, the *nif*A 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 *nif*A::*lac*Z and *nif*H::*lac*Z fusions, together with direct mRNA measurements, revealed that *nif*A 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 that contained *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, *fix*LJ, 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 *fix*LJ 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. 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, see Fig. 3), in which FixL functions as the sensor of the oxygen level. The FixL protein has two transmembrane regions that are probably anchored in the membrane, thus exposing a region in the periplasm, which is thought 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 *nif*A 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

Table 2. Comparison of a hypothetical 'anaerobox' sequence of *R. meliloti* *fix*LJ with the consensus for anaeroboxes of FNR-regulated genes in different organisms

Ec *nar*G	CTCTTGAT CGTT ATCAATTC	(de Bruijn *et al.* 1988)
	***** *****	
Ec *nir*B	AATTTGAT TTAC ATCAATAA	(de Bruijn *et al.* 1988)
	***** *****	
Bj *hem*A	TCTTTGATCGGGATCAAGTT	(McClung *et al.* 1987)
	***** *****	
Ac *nif*A	AATTTGAT TTAC ATCAAACC	(de Bruijn *et al.* 1988)
	***** *****	
Consensus	TTGAT------ATCAA	
	***** ****	
Rm *fix*LJ	ACATTGAT CACG GTCAATAC	(David *et al.* 1988)

Ec = *E. coli*, Bj = *Bradyrhizobium japonicum*, Ac = *Azorhizobium caulinodans*, Rm = *R. meliloti*, *hem*A = 5-aminolevulinate synthase, *nir*B = nitrite reductase, *nar*G = nitrate reductase.

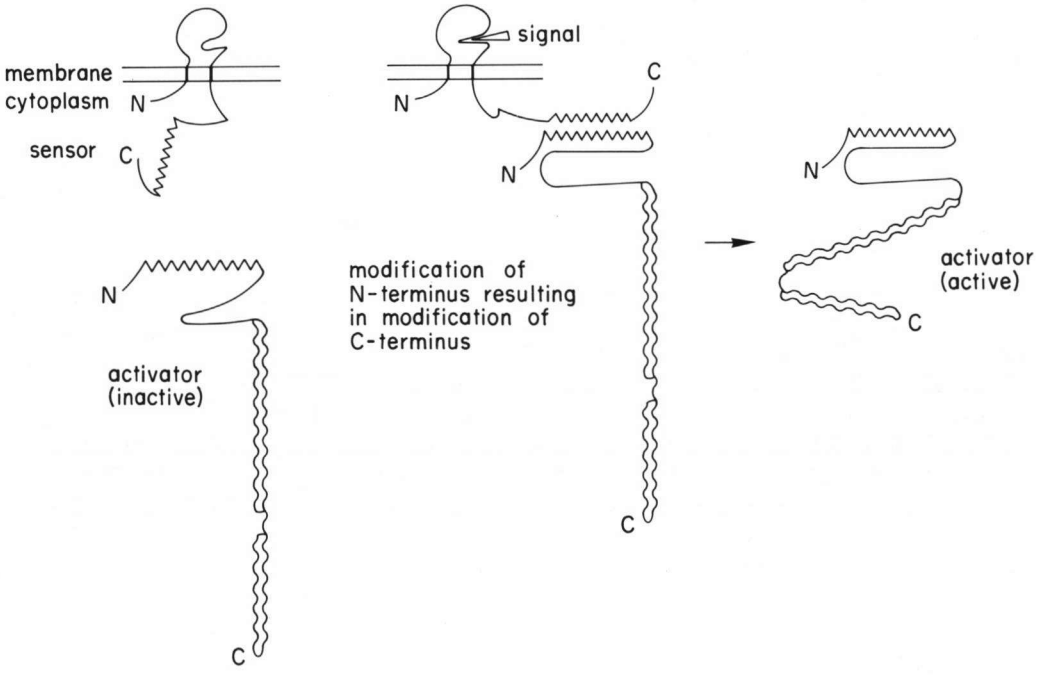


Fig. 2. Schematic representation of the sensor/activator model for bacterial regulatory protein pairs. The system consists of a sensor protein anchored in the membrane and an activator in the cytoplasm. Binding of a signal molecule to the external N-terminal part of the sensor induces a conformational 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.

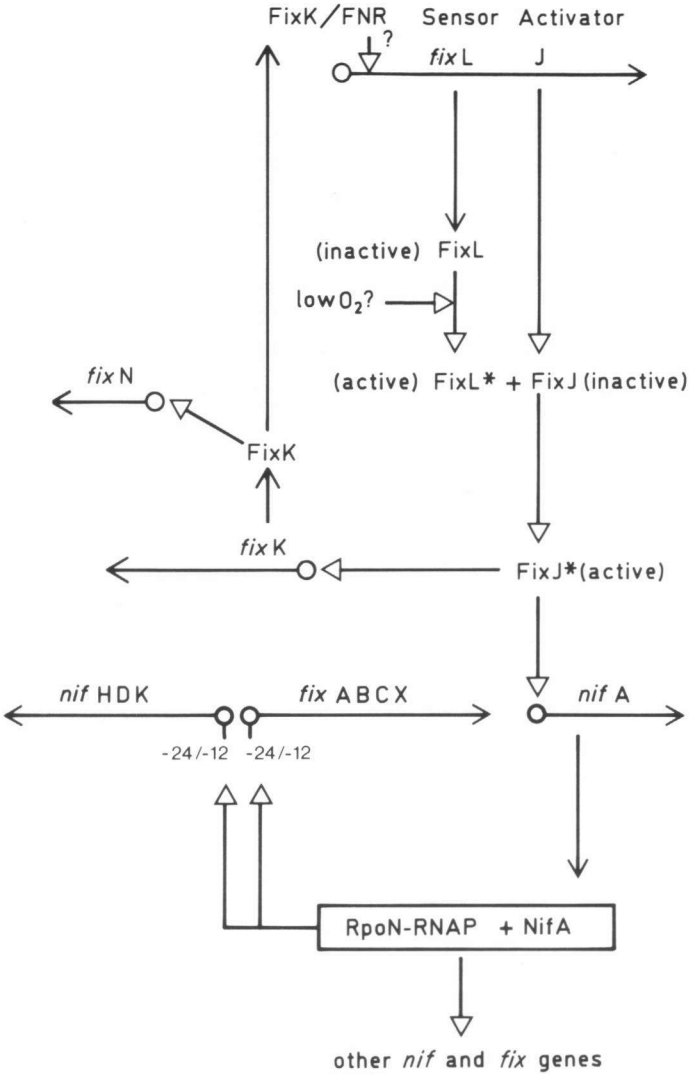


Fig. 3. Hypothetical model for activation of *nif*A transcription in *R. meliloti*. The *fix*LJ operon is positively regulated by FNR (possibly due to low oxygen). Active FixL activates the FixJ protein, which is a positive activator of *nif*A and *fix*K. NifA protein activates all the *nif* and most of the *fix* genes. FixK protein activates *fix*N. Regulation (either positive or negative) of the *fix*LJ operon by FixK is possible.

may be the possible target site of the *fix*J product, has been identified. Using progressive 5′-deletions of *nif*A::*lac*Z 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). Little is known about the regulation of

transcription of the *fix*LJ operon. Computer analysis of the promoter region by the present authors revealed that it contains a sequence that 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. FNR activates transcription of genes expressed under anaerobic conditions (Spiro & Guest 1987 and references therein). About 50 bp downstream of the proposed *fix*L 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 *fix*LJ operon.

Recently, Batut *et al.* (1989) identified a novel *fix* gene (*fix*K). The FixK protein shows considerable homology with the *E. coli* FNR protein and activates expression of the *fix*N gene (David *et al.* 1988) possibly by binding to a FNR consensus sequence in the promoter upstream region of this gene. The *fix*K gene may encode the FNR-like protein predicted by our hypothesis. Possibly FixK, through feedback, negatively regulates expression of *fix*LJ. The expression of *fix*K is regulated by the *fix*LJ operon (Batut *et al.* 1989). The *fix*LJ operon may be regulated positively by a second FNR-like protein.

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

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 to an 'active' mode. The 'active sensor' then modifies the activator, which is then also switched to an 'active' mode.

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 their 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 *gln*D, 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 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 nif*L gene showed that NifL is only required for the inactivation (and not the activation) of its partner NifA

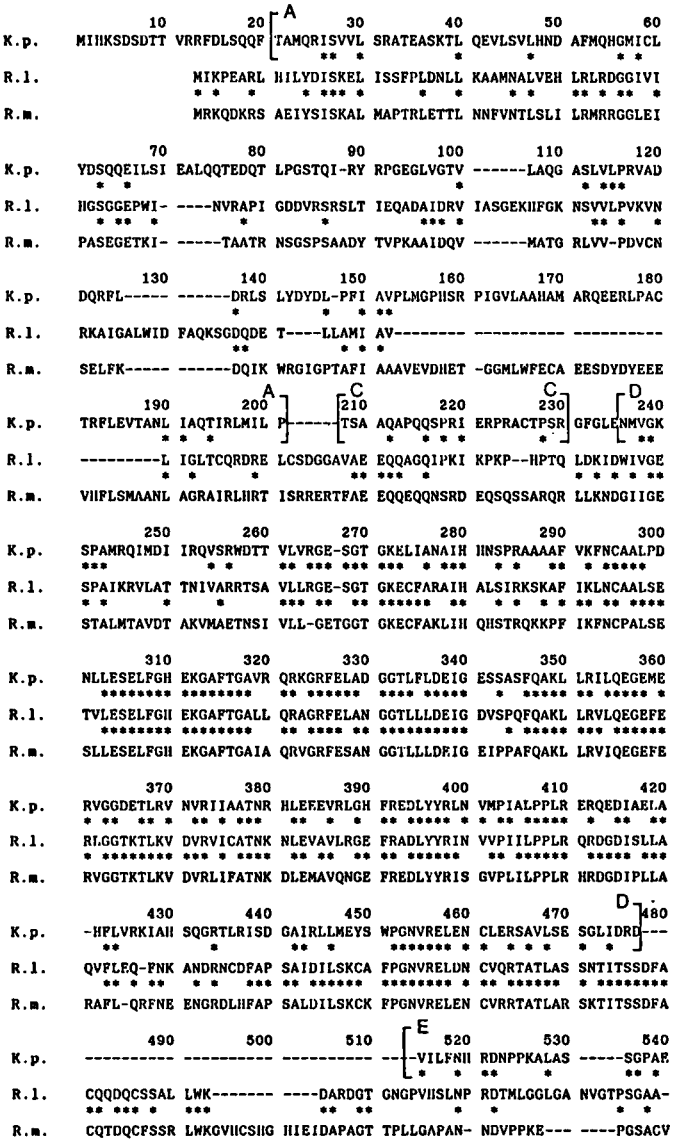
(Arnott *et al.* 1988). In summary: of the FixL/FixJ, NifL/NifA and NtrB/NtrC 'sensor/activator' pairs, the sensors do not meet the demands of the model proposed by Ronson *et al.* (1987) in that they sense an internal stimulus (NifL), or probably do not sense an environmental stimulus (FixL), or do 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). In order to include these phenomena the 'sensor/activator' model should be extended.

Regulation of the expression of nif *genes*

The regulatory nif*A protein.* In this paragraph we will discuss structural aspects of the NifA protein. *Nif*A 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 (P.W. Roelvink *et al.* submitted), *B. japonicum* (Thöny *et al.* 1987) and *A. caulinodans* ORS 571 (F.J. De Bruijn & P. 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 (Drummond *et al.* 1986). 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 *nif*L 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 because 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 form coils and turns predominantly. It is most likely to be 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, 14 amino acids from domain D are deleted, was completely inactive, illustrating the importance of this domain (Fischer *et al.* 1988). Furthermore a *R. meliloti* NifA derivative, from which both the NH_2-terminal and COOH-terminal part had been deleted leaving only domain D and the interdomain linker (positions 479–511) intact, is able to activate *nif*H::*lac*Z 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

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

C-terminus, sequences have been identified similar to the helix–turn–helix motifs characteristic of DNA-binding proteins such as 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.

Domains involved in oxygen sensitivity of NifA. The full length *R. meliloti* NifA polypeptide is able to activate *K. pneumoniae nif*H::*lac*Z and *R. meliloti nif*H::*lac*Z 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. 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 nif*H::*lac*Z 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 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 *nif*A 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 & Hennecke 1987; *A. caulinodans*: de Bruijn *et al.* 1988; *R. meliloti*: Beynon *et al.* 1988; *R. leguminosarum* biovar *phaseoli*: Hawkins & 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. As 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 *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 (Fischer *et al.* 1988). 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 *nif*A 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 to the active mode, as a result of which the DNA-binding motif positions such that it can make DNA contacts. Under aerobic conditions Fe^{2+} is oxidized and cannot bind rendering the NifA polypeptide inactive. The positioning of the DNA-binding domain resulting in the active mode is not an indispensable condition 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).

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 nif*H promoter region (Better *et al.* 1983; Beynon *et al.* 1983; Sundaresan *et al.* 1983) from which a consensus CTGGYRYR-N4-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 to 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 length from those reported for other bacteria (Kozak 1983). One rather extreme exception has been reported for the *B. japonicum fix*BCX operon where the promoter lies 700 bases upstream of the *fix*B gene (Gubler & Hennecke 1988a). This rather long leader has been proposed to be involved in post-transcriptional control of the *fix*BCX 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 nif*H promoter totally abolished activity (Buck 1986).

NifA targets: upstream activator sequences (UAS). A *nif*H promoter cloned in a multicopy-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-N10-ACA in *K. pneumoniae, A. vinelandii* and *Rhizobiaceae* was identified with a twofold rotational symmetry (Buck *et al.* 1986); Alvarez-Morales *et al.* (1986) identified the same sequence upstream of the *B. japonicum nif*D and *nif*H 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 nif*H UAS in a homologous or heterologous background. The UAS resembles activator sites with the consensus TGTGT-(N6-N10)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 nif*H transcription start, however, failed to show any stimulatory effect, nor was multicopy inhibition detected (Buck *et al.*1987c).

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 nif*H::*lac*Z 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 as 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 of this result. The UAS has to be on the same DNA face as the promoter. An introduced half turn between the 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 preventing the 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 downsteam −24/−12 promoter RpoN–RNAP complex. This would then explain why both elements have to be *in cis* to titrate NifA, resulting in multicopy inhibition of *nif* gene expression (Buck *et al.* 1986).

Mutational analysis of the UAS. Alteration of the G to C in TGT or ACA, respectively, in the *K. pneumoniae nif*H UAS severely reduces activity of the *nif*H promoter (Buck *et al.* 1987b). Deletion or insertion of 1 base in the 10-base sequence, between TGT and ACA of the motif, partially relieved multicopy inhibition as indicated by an increase in the 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

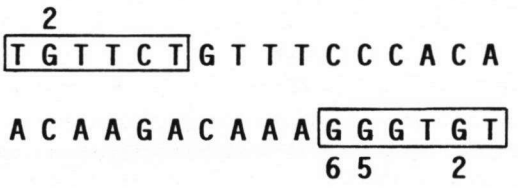


Fig. 4. Nucleotide sequence of the *K. pneumoniae nif*H UAS and binding sites for NifA. Binding half sites are boxed. Numbered bases make contact with NifA.

their target sites, residues in the UAS were identified, which may make specific contacts with NifA. Alteration of the residues marked as 2 has a severe negative effect on promoter activity; surprisingly mutations of residues 5 and 6 had no effect (Buck *et al.* 1987b). Morett *et al.* (1988) showed that NifA constitutively synthesized in *E. coli* protects four G-residues (indicated by numbers in Fig. 4) of the *K. pneumoniae nif*H promoter from methylation by dimethylsulphate; this indicates that these bases probably make the NifA–DNA contact. Thus the first six 5′ bases of the UAS, on both the upper and lower strands, 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 the 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 basepairs 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 the NifA dimer, followed by looping out of the intervening DNA. Contact of the NifA dimer with the RpoN–RNAP complex then activates transcription.

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 *nif*D::*lac*Z and *nif*H::*lac*Z fusions in *B. japonicum* depends upon the presence of the UAS. Deletion of the *R. meliloti nif*H UAS, however, had no effect upon activation by the constitutively expressed *R. meliloti nif*A in an *E. coli* background (Better *et al.* 1985). When a *nif*H promoter from which the UAS had been deleted was recombined with 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 a 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 retaining 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 *nif*A genes (Drummond *et al.* 1986). Second, DNA binding is not required for activation of the *R. meliloti nif*H 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 faced with a gene-product dosage effect. As 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 & Magasanik, 1986). Albright *et al.* (1988) suggested that the requirement for a UAS of *R. meliloti nif*H may be more stringent at lower NifA concentrations. Thus, such a 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 *fix*A genes does not have a UAS, the *fix*BCX operon has one imperfect UAS and both *nif*D and *nif*H have two copies of the UAS (Hennecke *et al.* 1988). By assaying chromosomally integrated copies of *nif*H::*lac*Z, *fix*B::*lac*Z and *fix*A::*lac*Z fusions for β-galactosidase activity, it was shown that the *nif*H::*lac*Z fusion is activated threefold over the *fix*B–*lac*Z and ninefold over the *fix*A–*lac*Z 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 *nif*H promoter of *R. leguminosarum* PRE has one imperfect copy of the UAS (11 bp spacing) and one consensus UAS. A low copy *nif*H::*lac*Z construct, with both imperfect and consensus UAS, delays the onset of nitrogen fixation by 3 days when compared with a *nif*H::*lac*Z construct with the consensus UAS only (Roelvink, 1989). This suggests that the modulation model may also hold true in *R. leguminosarum*.

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 nitrogen limitation and low oxygen level, is a result of the biochemical sensing of the *internal* nitrogen status. This leads to transcription activation of the *nif*LA 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 the activation of *nif*A 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 nitrogen 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, result in down-phenotypes in *nif*H::*lac*Z activation studies. The endosymbiont *R. meliloti* fixes nitrogen in the protected environment of the nodule, and is unlikely 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 nif*A mutant containing NifA with only domain D and a linker is still active, and that the presence of a 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 in liquid culture, under the proper conditions.

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 *nif*A 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 make contact with RpoN–RNAP and how transcription activation is achieved. In addition, 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*, so far, 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 *nif*A transcription and subsequent transcription activation of *nif* and *fix* genes by NifA, needs further proof.

ACKNOWLEDGEMENTS

The authors would like to thank Professor A. van Kammen and Dr R. van Veen for critically reading the various versions of the manuscript. Piet Madern is thanked for the artwork and Gré Heitkönig and Marie-José van Neerven for typing the manuscript.

REFERENCES

Albright, L., Huala, E., Gu, Q. & Ausubel, F.M. (1988): Regulation of *R. meliloti* NifA function. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 345–349. Gustav Fischer, Stuttgart.

Alvarez-Morales, A., Betancourt-Morales, M., Kaluza, K. & Hennecke, H. (1986): Activation of the *Bradyrhizobium japonicum nif*H and *nif*DK operons is dependent on promoter-upstream DNA sequences. *Nucl. Acids Res.* **14:** 4207–4227.

Arnold, W., Rump, A., Klipp, W., Priefer, U.B. & Pühler, A. (1988): Nucleotide sequence of a 24,206 basepair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. In: Bothe, H., de Bruijn, F.J. and Newton W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 303. Gustav Fischer, Stuttgart.

Arnott, M., Sidoti, C., Hill, S., Kennedy, C. & Merrick, M. (1988): Deletion analysis of the *Klebsiella pneumoniae nif*L gene. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 304. Gustav Fischer, Stuttgart.

Austin, S., Henderson, N. & Dixon, R. (1987): Requirements for transcriptional activation *in vitro* of the nitrogen-regulated *gln*A and *nif*LA promoters from *Klebsiella pneumoniae*: dependence on activator concentration. *Mol. Microbiol.* **1:** 92–100.

Ausubel, F.M. (1984): Regulation of nitrogen fixation genes. *Cell* **37:** 5–6.

Batut, J., Daveran-Mingot, M.-L., David, M., Jacobs, J., Garnerone, A.M. & Kahn, D. (1989): *fix*K, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. *EMBO J.* **8:** 1279–1286.

Bennett, L.T., Cannon, F. & Dean, D. (1988): Nucleotide sequence and mutagenesis of the *nif*A gene from *Azotobacter vinelandii*. *Mol. Microbiol.* **2:** 315–321.

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

—, Lewis, B., Corbin, D., Ditta, G. & Helinski, D.R. (1983): Structural relationships among *Rhizobium meliloti* symbiotic promoters. *Cell* **35:** 479–485.

Beynon, J., Cannon, M., Buchanan-Wollaston, V. & Cannon, F. (1983): The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. *Cell* **34:** 665–671.

—, Williams, M.K. & Cannon, F.C. (1988): Expression and functional analysis of the *Rhizobium meliloti nif*A gene. *EMBO J.* **7:** 7–14.

Brown, S.E. & Ausubel, F.M. (1984): Mutations affecting regulation of the *Klebsiella pneumoniae nif*H (nitrogenase reductase) promoter. *J. Bacteriol.* **157:** 143–147.

de Bruijn, F.J., Pawlowski, K., Ratet, P. *et al.* (1988): Molecular genetics of nitrogen fixation by *Azorhizobium caulinodans* ORS571, the diazotrophic stem nodulating symbiont of *Sesbania rostrata*. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 351–355. Gustav Fischer, Stuttgart.

— (1986): Deletion analysis of the *Klebsiella pneumoniae* nitrogenase promoter: Importance of spacing between conserved sequences around positions −12 and −24 for activation by the *nif*A and *ntr*C (*gln*G) products. *J. Bacteriol.* **166:** 545–551.

—, Cannon, W. & Woodcock, J. (1987a): Transcriptional activation of the *Klebsiella pneumoniae* nitrogenase promoter may involve DNA loop formation. *Mol. Microbiol.* **1:** 243–249.

—, — & — (1987b): Mutational analysis of upstream sequences required for transcriptional activation of the *Klebsiella pneumoniae nif*H promoter. *Nucl. Acids Res.* **15:** 9945–9956.

—, Woodcock, J., Cannon, W., Mitchenall, L. & Drummond, M. (1987c): Positional requirements for the function of *nif*-specific upstream activator sequences. *Mol. Gen. Genet.* **210:** 140–144.

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

Bueno, R., Pahel, G. & Magasanik, B. (1985): Role of *gln*B and *gln*D gene products in regulation of the *gln*ALG operon of *Escherichia coli*. *J. Bacteriol.* **164:** 816–822.

Buikema, W.J., Szeto, W.W., Lemley, P.V., Orme-Johnson, W.H. & Ausubel, F.M. (1985): Nitrogen-fixation specific regulatory genes of *Klebsiella pneumoniae* and *Rhizobium meliloti* share homology with the general nitrogen regulatory gene *ntr*C of *K. pneumoniae*. *Nucl. Acids Res.* **13:** 4539–4555.

David, M., Daveran, M.-L., Batut, J. *et al.* (1988): Cascade regulation of *nif* gene expression in *Rhizobium meliloti*. *Cell* **54:** 671–683.

—, Domergue, O., Pognonec, P. & Kahn, D. (1987): Transcription patterns of *Rhizobium meliloti* symbiotic plasmid pSym: Identification of *nif*A-independent *fix* genes. *J. Bacteriol.* **169:** 2239–2244.

Ditta, G., Virts, E., Palomares, A. & Choong-Hyun, Kim (1987): The *nif*A gene of *Rhizobium meliloti* is oxygen-regulated. *J. Bacteriol.* **169:** 3217–3223.

Dixon, R.A. (1987): Genetic regulation of nitrogen fixation. In: Cole, J.A. and Ferguson, S. (eds): *The Nitrogen and Sulphur Cycles*. 417–438. Cambridge University Press, Cambridge.

Downie, J.A. & Johnston, A.W.B. (1986): Nodulation of legumes by *Rhizobium*: the recognized root? *Cell* **47:** 153–154.

Drummond, M. (1988): Probing the domain structure of the NifA and NtrC from *Klebsiella pneumoniae* by protein engineering. In: Bothe, H., de Bruijn, F.J. and Newton W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 307. Gustav Fischer, Stuttgart.

—, Whitty, P. & Wootton, J. (1986): Sequence and domain relationships of *ntr*C and *nif*A from *Klebsiella pneumoniae*: homologies to other regulatory proteins. *EMBO J.* **5:** 441–447.

— & Wootton, J. (1987): Sequence of *nif*L from *Klebsiella pneumoniae*: mode of action and relationship to two families of regulatory proteins. *Mol. Microbiol.* **1:** 37–44.

Fischer, H.-M., Bruderer, T. & Hennecke, H. (1988): Essential and non essential domains in the *Bradyrhizobium japonicum nif*A protein: Identification of indispensable cysteine residues potentially involved in redox reactivity and/or metal binding. *Nucl. Acids Res.* **16:** 2207–2223.

— & Hennecke, H. (1987): Direct response of *Bradyrhizobium japonicum nif*A-mediated *nif* gene regulation to cellular oxygen status. *Mol. Gen. Genet.* **209:** 621–626.

Gicquel-Sanzey, B. & Cossart, P. (1982): Homologies between different procaryotic DNA-binding regulatory proteins and between their sites of action. *EMBO J.* **5:** 591–595.

Grönger, P., Manian, S.S., Reiländer, H., O'Connell, M., Priefer, U.B. & Pühler, A. (1987): Organization and partial sequence of a DNA region of the *Rhizobium leguminosarum* symbiotic plasmid pRL6JI containing the genes *fix*ABC, *nif*A, *nif*B and a novel open reading frame: *Nucl. Acids Res.* **15:** 31–56.

Gubler, M. & Hennecke, H. (1988a): Regulation of the *fix*A gene and the *fix*BC operon in *Bradyrhizobium japonicum*. *J. Bacteriol.* **170:** 1205–1214.

— & — (1986b): Regulation of the *fix*A gene and the *fix*BCX operon in *Bradyrhizobium japonicum*: fine tuning by upstream activator sequences (UASs) and posttranscriptional control. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 368. Gustav Fischer, Stuttgart.

Gussin, G.N., Ronson, C.W. & Ausubel, F.M. (1986): Regulation of nitrogen fixation genes. *Ann. Rev. Genet.* **20:** 567–591.

Hawkins, F.K.L. & Johnston, A.W.B. (1988): Transcription of a *Rhizobium leguminosarum* biovar *phaseoli* gene needed for melanin synthesis is activated by *nif*A of *Rhizobium* and *Klebsiella pneumoniae*. *Mol. Microbiol.* **2:** 331–339.

Hennecke, H., Fischer, H-M., Gubler, M. *et al.* (1988): Regulation of *nif* and *fix* genes in *Bradyrhizobium japonicum* occurs by a cascade of two consecutive gene activation steps of which the second one is oxygen sensitive. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen*

Fixation: Hundred Years After. 293–302. Gustav Fischer, Stuttgart.

Holtel, A. & Merrick, M. (1988): Identification of the *Klebsiella pneumoniae glnB* gene: Nucleotide sequence of wild type and mutant alleles. *Mol. Gen. Genet.* **215:** 134–138.

Jacobson, M.J., Newton, W.E., Weiss, M.C. & Dean, D.R. (1988): Differential accumulation of the *nif* structural gene mRNAs and gene products in *Azotobacter vinelandii*. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 321. Gustav Fischer, Stuttgart.

Kahn, H., David, M., Batut, J. *et al.* (1988): Cascade activation of *nif* genes in *Rhizobium meliloti*. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 357–361. Gustav Fischer, Stuttgart.

Kim, Y-M., Kyung-Joon, A., Beppu, T. & Uozumi, T. (1986): Nucleotide sequence of the *nifLA* operon of *Klebsiella oxytoca* NG13 and characterization of the gene products. *Mol. Gen. Genet.* **205:** 253–259.

Kozak, M. (1983): Comparison of initiation of protein synthesis in procaryotes, eucaryotes and organelles. *Microbiol. Rev.* **47:** 1–45.

Long, S. (1984): Genetics of *Rhizobium* nodulation. In: Kosuge, T. and Nester, E. (eds): *Plant–Microbe Interactions*. 265–306. MacMillan Publishing Co. Inc., New York.

McClung, C.R., Sommerville, J.E., Guerinot, M.L. & Chelm, B.K. (1987): Structure of the *Bradyrhizobium japonicum* gene *hemA* encoding 5-aminolevulinic acid synthase. *Gene* **54:** 133–139.

MacFarlane, S.A. & Merrick, M.J. (1987): Analysis of the *Klebsiella pneumoniae ntrB* gene by site directed *in vitro* mutagenesis. *Mol. Microbiol.* **1:** 133–142.

Magasanik, B. (1982): Genetic control of nitrogen assimilation in bacteria. *Ann. Rev. Genet.* **16:** 135–168.

Merrick, M. (1987): Regulation of nitrogen assimilation by bacteria. In: Cole, J.A. and Ferguson, S. (eds): *The Nitrogen and Sulphur Cycles*. 331–361. Cambridge University Press, Cambridge.

— (1988): Organisation and regulation of nitrogen fixation genes in *Klebsiella* and *Azotobacter*. In: Bothe, H., de Bruijn, F.J. and Newton, W.E. (eds): *Nitrogen Fixation: Hundred Years After*. 293–302. Gustav Fischer, Stuttgart.

Morett, E., Cannon, W. & Buck, M. (1988): The DNA-binding domain of the transcriptional activator protein NifA resides in its carboxy terminus, recognises the upstream activator sequences of *nif* promoters and can be separated from the positive control function of NifA. *Nucl. Acids Res.* **16:** 11469–11488.

Nap, J.P. (1988): *Nodulins in root nodule development*. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

Nees, D.W., Stein, P.A. & Ludwig, R.A. (1988): The *Azorhizobium caulinodans nifA* gene: identication of upstream-activating sequences including a new element, the 'anaerobox'. *Nucl. Acids Res.* **16:** 9839–9853.

Ninfa, A. & Magasanik, B. (1986): Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *gln* ALG operon in *Escherichia coli*. *Proc. Natl. Acad. Sci., USA* **83:** 5909–5913.

Nixon, B.T., Ronson, C.W. & Ausubel, F.M. (1986): Two component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation genes *ntrB* and *ntrC*. *Proc. Natl. Acad. Sci., USA* **83:** 7850–7854.

Pabo, C.R. & Sauer, R.T. (1984): Protein–DNA recognition. *Ann. Rev. Biochem.* **53:** 293–321.

Pawlowski, K., Ratet, P., Schell, J. & de Bruijn, F.J. (1987): Cloning and characterization of *nifA* and *ntrC* genes of the stem nodulating bacterium ORS571, the nitrogen fixing symbiont of *Sesbania rostrata*: Regulation of nitrogen fixation (*nif*) genes in the free living versus symbiotic state. *Mol. Gen. Genet.* **206:** 207–219.

Ptashne, M. (1986): Gene regulation by proteins acting nearby and at a distance. *Nature* **322:** 697–701.

Reitzer, L.J. & Magasanik, B. (1986): Transcription of *glnA* in *Escherichia coli* is simulated by activator bound to sites far from the promoter. *Cell* **45:** 785–792.

Roelvink, P.W. (1989): *Characterization of the* nif*A regulatory gene of* Rhizobium leguminosarum PRE. Ph.D. Thesis, Wageningen Agricultural University, The Netherlands.

Ronson, C.W. & Astwood, P.M. (1985): Genes involved in the carbon metabolism of bacteroids. In: Evans, H.J., Buttomley, P.J. and Newton, W.E. (eds): *Nitrogen Fixation Research Progress*. 201–207. M. Nijhoff, Dordrecht.

—, —, Nixon, B.T. & Ausubel, F.M. (1987): Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* **49:** 579–581.

Schetgens, Th.M.P., Hontelez, J.G.J., van den Bos, R.C. & van Kammen, A. (1985): Identification and phenotypical characterization of a cluster of *fix* genes, including a *nif* regulatory gene, from *Rhizobium leguminosarum* PRE. *Mol. Gen. Genet.* **200:** 368–374.

Spiro, S. & Guest, J.R. (1987): Activation of the *lac*-operon of *Escherichia coli* by a mutant FNR protein. *Mol. Microbiol.* **1:** 53–58.

Sundaresan, V., Jones, J.D.G., Ow, D.W. & Ausubel, F.M. (1983): *Klebsiella pneumoniae nifA* product activates the *Rhizobium meliloti* nitrogenase promoter. *Nature* **301:** 728–732.

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

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

Tuli, R. & Merrick, M.J. (1988): Over-production and characterization of the *nif*A gene product of *Klebsiella pneumoniae*—the transcriptional activator of *nif* gene expression. *J. Gen. Microbiol.* **134:** 425–432.

Virts, E.L., Stanfield, S.W., Helinski, D.R. & Ditta, G. (1988): Common regulatory elements control symbiotic and microaerobic induction of *nif*A in *Rhizobium meliloti. Proc. Natl. Acad. Sci., USA* **85:** 3062–3065.

Weber, G., Reiländer, H. & Pühler, A. (1985): Mapping and expression of a regulatory nitrogen fixation gene (*fix*D) of *Rhizobium meliloti. EMBO J.* **4:** 2751–2756.

Zimmerman, J.L., Szeto, W.W. & Ausubel, F.M. (1983): Molecular characterization of *Tn*5-induced symbiotic (fix$^-$) mutants of *Rhizobium meliloti. J. Bacteriol.* **156:** 1025–1035.