

Chapter 9

Nitrogen control of nitrogen fixation in free-living diazotrophs

M. J. MERRICK

*Department of Molecular Microbiology, John Innes Centre,
Norwich NR4 7UH, UK*

1. INTRODUCTION

Whilst the process of nitrogen fixation is found distributed amongst a taxonomically very diverse range of microorganisms, research over the last thirty years has, perhaps not surprisingly, revealed that the physiological constraints that these organisms face are often very similar. The ways in which free-living diazotrophs deal with these problems have been examined by physiologists, biochemists and geneticists and this research is now providing a unified picture of the exquisitely elaborate mechanisms that have evolved. Furthermore whilst each group of organisms has evolved its own particular genetic and biochemical strategies to maximise the efficiency of nitrogen fixation some common themes are now becoming clear.

The physiological issues to be addressed by a free-living diazotroph are well defined; namely to optimise nitrogen fixation whilst coping with the oxygen sensitivity of nitrogenase, to satisfy the substantial energy demands of the fixation process, to supply metals for a range of metalloenzymes, and to utilise other sources of fixed nitrogen before fixing atmospheric N₂. It is now very apparent that all of these aspects of regulation are not only inter-related but also have to be integrated with the rest of the cell's metabolism.

This chapter focuses on the mechanisms by which free-living diazotrophs regulate nitrogen fixation in response to changes in the cellular nitrogen status. In all organisms this regulation operates at the level of transcription of the nitrogen

fixation (*nif*) genes. Furthermore such regulation is usually effected at two levels: a general nitrogen control system that co-ordinates cellular nitrogen metabolism and a *nif*-specific mechanism that facilitates regulation in response to particular signals. In addition to this transcriptional control a number of organisms have evolved special mechanisms that allow very rapid short-term regulation of the activity of the nitrogenase enzyme in response to fluctuations in availability of fixed nitrogen.

2. GENERAL NITROGEN CONTROL SYSTEMS

2.1. Proteobacteria

The general nitrogen regulation (Ntr) system was discovered, and is most fully described, in the γ Proteobacteria but is now known to be present also in the α and β Proteobacteria (Merrick and Edwards, 1995). The Ntr system comprises four proteins: GlnB – a member of the P_{II} family of signal transduction proteins, GlnD – a uridylyltransferase, and a two-component regulatory pair – the sensor histidine kinase, NtrB, and the response regulator, NtrC (Fig. 1). Amongst the free-living diazotrophs the *ntr* system has been characterised, in full or in part, in *Klebsiella pneumoniae*, *Azotobacter vinelandii*, *Azospirillum brasilense*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *Azoarcus*. There have been no studies of nitrogen regulation in diazotrophic members of the δ Proteobacteria but genome sequence analysis in *Desulfovibrio vulgaris* and *Geobacter sulfurreducens* suggests that these organisms may have at least *glnB* and *glnD* genes.

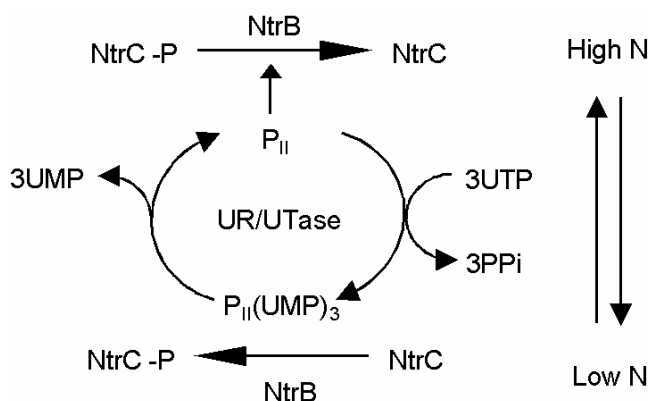


Figure 1 The nitrogen regulation (Ntr) system of enteric bacteria. The activity of the response regulator NtrC is regulated in response to the intracellular nitrogen status. UTase (*glnD* product) catalyses the uridylylation and deuridylylation of P_{II} (*glnB* product). P_{II} in turn regulates the activity of the sensor histidine kinase NtrB which catalyses the phosphorylation and dephosphorylation of NtrC.

The GlnB protein, like all P_{II} proteins, is a small trimeric protein that constitutes an intracellular sensor of nitrogen status (Arcondéguy *et al.*, 2001). The protein forms a squat barrel 30 Å high with each 12kDa monomer arranged such that a rather unstructured loop of around 18 residues, called the T-loop, extends some 13 Å above the surface of the barrel (Xu *et al.*, 1998; Carr *et al.*, 1996). A principal characteristic of the P_{II} proteins in the Proteobacteria is their ability to be switched between two forms by covalent modification of Tyr51 which lies at the apex of the T-loop.

Both uridylylation and deuridylylation of GlnB are effected by the GlnD protein which responds to the intracellular glutamine concentration in such a way that glutamine specifically inhibits the uridylylation reaction (Jiang *et al.*, 1998). Consequently GlnB is uridylylated under nitrogen-limiting (low intracellular glutamine) conditions and de-uridylylated in nitrogen-sufficient (high intracellular glutamine) conditions. The modification state of GlnB reflects the intracellular nitrogen status and also modulates the ability of GlnB to interact with other proteins in the cell and thereby to regulate their activity.

One of these GlnB targets is the NtrB/NtrC two-component system. NtrC is a typical σ^N (σ^{54}) - dependent transcriptional activator protein that comprises a DNA-binding carboxy terminal domain, a highly conserved central domain, required for the activation of transcription, and an N-terminal domain which is characteristic of two-component response regulator proteins (Stock *et al.*, 1989). These proteins have a highly conserved tertiary structure in the N-terminal region, containing an aspartic acid residue at position 54 which, in the case of NtrC, is phosphorylated in response to low nitrogen, thereby activating the protein (Volkman *et al.*, 1995; Kern *et al.*, 1999). NtrC is a dimer in its non-phosphorylated form but for the activation of transcription to take place, oligomerisation to a tetramer or higher order oligomer must occur. Phosphorylation induces both DNA binding and the oligomerisation of NtrC and many NtrC-dependent promoters contain more than one binding site, facilitating oligomerisation (Weiss *et al.*, 1991; Weiss *et al.*, 1992; Mettke *et al.*, 1995; Wyman *et al.*, 1997).

Control of NtrC activity in response to nitrogen status is mediated by NtrB which acts as a phosphate donor to NtrC. NtrB consists of three domains: an N-terminal domain involved in signal transduction, a central phosphotransferase/phosphatase/dimerisation domain and a C-terminal kinase domain. The NtrB protein is a dimer that is autophosphorylated on a conserved histidine (His139 in *K. pneumoniae*) at the amino end of the C-terminal domain (Ninfa and Bennett, 1991). ATP binds to one subunit and phosphorylation occurs on the conserved histidine residue of the second subunit. It is NtrB that is a target for regulation by GlnB. NtrB is stimulated to dephosphorylate NtrC in the presence of GlnB and ATP: this is termed regulated phosphatase activity (Keener and Kustu, 1988; Kamberov *et al.*, 1994; Kamberov *et al.*, 1995; Liu and Magasanik, 1995). Binding of GlnB to the kinase domain of NtrB inhibits kinase activity and appears to result in an altered conformation that is transmitted to the other two domains thereby causing the central

domain to assume a conformation with potent phosphatase activity (Pioszak *et al.*, 2000).

In nitrogen excess, unmodified GlnB stimulates dephosphorylation of NtrC by NtrB and conversely in nitrogen starvation, when GlnB is modified, GlnB-UMP no longer interacts with NtrB and the kinase activity predominates so that NtrC is phosphorylated and transcriptionally active. Hence the modification of GlnB in response to the cellular nitrogen status provides the intracellular switch that in turn regulates the phosphatase and kinase activities of NtrB and hence the transcriptional activity of NtrC.

Expression of the *nif* genes has been shown to be dependent on NtrC in *K. pneumoniae* (Espin *et al.*, 1982; Merrick, 1983), *A. brasilense* (Pedrosa and Yates, 1984; Liang *et al.*, 1993), *R. capsulatus* (Kranz *et al.*, 1990; Kranz and Haselkorn, 1985; Kranz and Foster-Hartnett, 1990) and *H. seropedicae* (Persuhn *et al.*, 2000). In each of these organisms NtrC regulates expression of a *nif*-specific regulatory system (described below). The exceptions to this pattern are in *A. vinelandii*, *R. rubrum* and *G. diazotrophicus* where mutations in *ntrC* do not affect *nif* expression (Toukdarian *et al.*, 1990; Toukdarian and Kennedy, 1986; Zhang *et al.*, 1995). However NtrC is required for expression in *A. vinelandii* of *vnfH*, the structural gene for the vanadium nitrogenase Fe protein (Bali *et al.*, 1988).

Members of the α , β , γ Proteobacteria (and probably the δ Proteobacteria) encode two or more P_{II} -like proteins. Typically these are a GlnB protein and one or more homologues designated GlnK, the structural genes for which are almost invariably linked to a second gene (*amtB*) that encodes a high-affinity ammonium transporter (Thomas *et al.*, 2000; Arcondéguy *et al.*, 2001). Expression of *glnK* is regulated by NtrC such that GlnK levels are markedly elevated under nitrogen limitation (Atkinson and Ninfa, 1998). Recent studies indicate that in *Escherichia coli* GlnK is necessary to control the levels of NtrC-P during nitrogen starvation when the fixed levels of GlnB are insufficient to provide this control (Atkinson *et al.*, 2002b; Atkinson *et al.*, 2002a; Blauwkamp and Ninfa, 2002a; Blauwkamp and Ninfa, 2002b). GlnK also regulates the activity of AmtB and may therefore play a part in controlling the intracellular ammonium pool (Coutts *et al.*, 2002). The roles of the P_{II} proteins extend to a wide variety of aspects of nitrogen metabolism (Arcondéguy *et al.*, 2001) and (as discussed later) they play a critical role in *nif*-specific gene regulation in a number of organisms.

Nitrogen and carbon metabolism must invariably be kept in balance and in *R. capsulatus* and in *Rhodobacter sphaeroides* they are co-ordinated through the actions of the RegB-RegA two-component system. This control system was originally discovered through its role in anaerobic activation of the *puf*, *puc* and *puh* photosynthetic gene operons in *R. capsulatus* (Sganga and Bauer, 1992). In *R. sphaeroides* the RegB-RegA system is required for positive regulation of the *cbbI* and *cbbII* encode the enzymes of the Calvin cycle CO₂ fixation pathway (Qian and Tabita, 1996). The precise signal to which RegB responds is unknown but the current model favours a response to redox state rather than to molecular oxygen, and studies in *R. sphaeroides* suggest that the signal originates from the *cbb₃*-type oxidase (O'Gara *et al.*, 1998).

That this system is also involved in regulation of nitrogen fixation became apparent when it was found that in a *cbb* mutant of *R. sphaeroides* nitrogenase synthesis is derepressed in the presence of ammonium and that this derepression requires RegB (Qian and Tabita, 1996). In *R. capsulatus*, RegA acts as a coactivator, together with NtrC, of *nifA2* expression but the precise mechanism of this co-activation is not known (Elsen *et al.*, 2000). The derepression of nitrogenase in a *cbb* mutant appears to serve as a means of controlling redox balance, because the hydrogenase activity of nitrogenase allows removal of excess reducing equivalents in the absence of the ability to use CO₂ as an electron acceptor. In *R. sphaeroides* the regulatory circuits controlled by NtrBC and RegBA are tightly integrated. Both *glnB* and *glnK* are normally regulated in response to nitrogen source; *glnB* expression being threefold higher in glutamate than ammonium whilst *glnK* is induced more than seventyfold. However a mutant that is devoid of a functional reductive pentose pathway fails to express *glnB* and shows constitutive expression of *glnK* (Qian and Tabita, 1998). The factor(s) responsible for this control are presently unknown.

RegBA homologues are present in a number of α -Proteobacteria including *Bradyrhizobium japonicum* where the RegA homologue, RegR, activates transcription of the *fixR-nifA* operon (Bauer *et al.*, 1998). *R. capsulatus regA* can complement a *B. japonicum regR* mutant confirming that the two genes are functionally similar (Emmerich *et al.*, 2000).

2.2. Gram-positive bacteria

There is very little information concerning nitrogen control amongst the diazotrophic Gram-positive organisms. Global regulation of nitrogen metabolism in *Bacillus subtilis* is mediated by the transcription factor TnrA (Wray *et al.*, 2000; Fisher, 1999) but whether this system is present in the diazotrophic *Bacillus* species is unknown. A number of *nif* genes have been cloned from *Clostridium pasteurianum* including six copies of *nifH* homologues (Wang *et al.*, 1988). Under nitrogen-fixing conditions transcripts of most of these genes are present suggesting that they are functional. Sequences identical to or very similar to the consensus *Escherichia coli* promoter are found in the -35 and -10 regions and a common upstream sequence (ATCAATAT-N₆₋₁₀-ATGGATTC) is present around position -100 but the role of this sequence is not known (Wang *et al.*, 1988).

Homologues of the standard *ntr* genes found in the Proteobacteria are not present in the genera *Bacillus*, *Clostridium* and *Streptomyces*. However where genome information is available all members of these genera encode an *amtB*, *glnK* operon, that in the Actinobacteria is an *amtB*, *glnK*, *glnD* operon. Whether any of these genes influences nitrogen fixation in the diazotrophic members of these genera is unknown.

2.3. Cyanobacteria

Nitrogen fixation genes have been identified in a number of cyanobacteria but have been characterised in most detail in *Anabaena* PCC7120, *Anabaena variabilis* ATCC 29413 and *Synechococcus* strain RF-1. Nitrogen-fixing cyanobacteria can be divided into those species in which nitrogen fixation occurs in differentiated cells called heterocysts e.g. *Anabaena* PCC7120 or *Nostoc punctiforme* and non-heterocystous organisms such as *Gloeotheca*. When species such as *Anabaena* are deprived of a source of fixed nitrogen about every tenth vegetative cell in the cyanobacterial filament differentiates into a morphologically and physiologically distinct heterocyst. During heterocyst differentiation an 11kb DNA element is excised from within the *nifD* gene to form a contiguous *nifHDK* operon (Golden *et al.*, 1985). Whilst this process is triggered by nitrogen deprivation the signal transduction cascade that brings it about is not yet completely described.

Whilst species such as *N. punctiforme* contain a single major *nif* gene cluster, *Anabaena variabilis* (ATCC29413) encodes two *nif* clusters (*nif1* and *nif2*) and a *vnf* gene cluster encoding a vanadium nitrogenase (Thiel *et al.*, 2002). Whereas the *nif1*- and *vnf*-encoded nitrogenases function in the heterocyst, the *nif2*-encoded enzyme functions in all cells but only under anaerobic conditions (Thiel *et al.*, 1997). Consequently whereas expression of *nif1* and *vnf* is linked to the differentiation process, the *nif2* genes appear to be similar to the *nif* genes of non-heterocystous cyanobacteria and to be regulated directly by environmental factors such as availability of fixed nitrogen and oxygen tension.

The cyanobacteria have a distinct general nitrogen control system in which the central transcriptional regulator is NtcA, a member of the CRP (cAMP receptor protein) family (Herrero *et al.*, 2001). NtcA is widely distributed amongst cyanobacteria and has been identified in a number of nitrogen-fixing species including *Anabaena* PCC7120, *N. punctiforme*, *Cyanothece*, *Synechococcus* and *Trichodesmium*. The NtcA amino acid sequence is highly conserved and shows three strongly conserved regions. Region I covers around 70 residues and has many characteristics of cyclic-nucleotide-binding proteins and it has been proposed that this structural feature may represent a metabolite-binding site. The binding site of NtcA on DNA has been extensively studied and is characterised by a palindromic sequence GTA-N₈-TAC (Luque *et al.*, 1994; Jiang *et al.*, 2000). *In vitro* NtcA shows sequence-specific binding to this motif in the *glnA* promoter but fails to activate transcription from either the *glnA* or *ntcA* promoters even at saturating concentrations (Tanigawa *et al.*, 2002). However DNA binding by NtcA is significantly stimulated in the presence of 2-oxoglutarate which is then competent to activate transcription from both promoters (Tanigawa *et al.*, 2002; Vazquez-Bermudez *et al.*, 2002). These data strongly suggest that 2-oxoglutarate is the major regulator of NtcA-dependent transcription and that unlike the Proteobacteria 2-oxoglutarate does not act through the P_{II} protein. However recent data from *Synechocystis* indicate that P_{II} may be required for NtcA-dependent transcription activation under certain physiological conditions (Aldehni *et al.*, 2003).

NtcA mutants of *Anabaena* PCC7120, *Anabaena variabilis* ATCC 29413 and *N. punctiforme* are impaired in heterocyst development and are unable to fix nitrogen (Frias *et al.*, 1994; Wei *et al.*, 1994; Herrero *et al.*, 2001). As heterocyst

development occurs in response to combined nitrogen deprivation the involvement of NtcA is understandable and two groups of genes, *hetC* and *devBCA*, that are required for heterocyst development appear to be directly regulated by NtcA (Muro-Pastor *et al.*, 1999; Herrero *et al.*, 2001).

An *Anabaena* PCC7120 *ntcA* mutant does not express the *nifHDK* genes (Frias *et al.*, 1994) and the NtcA protein has been reported to bind weakly *in vitro* to the *nifH* upstream region (Ramasubramanian *et al.*, 1994). However sequences in the *nifH* promoter, whilst showing some similarity to the NtcA consensus, do not match precisely (Herrero *et al.*, 2001). This could mean that the *nif* genes are regulated by an as yet unidentified protein or that the lack of a consensus NtcA promoter reflects a requirement for a higher intracellular NtcA concentration.

2.4. Archaea

Within the Archaea, nitrogen fixation has been found only in methanogenic species but not all methanogens fix nitrogen (Leigh, 2000). Studies of the regulation of *nif* genes in these organisms have been hampered by the problems of genetic analysis but genetic systems have now been developed in *Methanococcus maripaludis* which is also relatively fast growing and provides a good model system.

Evidence for a nitrogen regulon, i.e. a group of genes that are subject to nitrogen control by a common transcriptional regulator, comes from analysis of potential common sequences within promoters of genes that are expected or known to be nitrogen regulated. The upstream sequences of the *nifH* genes in *Methanobacterium thermoautotrophicum* and *Methanobacterium ivanovii* and of the *glnA* (glutamine synthetase) gene in *Methanococcus voltae* contain a common palindromic sequence GGAA – N₆ – TTCC (Sibold *et al.*, 1991; Souillard and Sibold, 1989; Possot *et al.*, 1989). Subsequent searches identified an identical sequence upstream of these same genes in *M. maripaludis*, of the *amtB*, *glnK* operons of *M. thermoautotrophicum* and *M. jannaschii*, and of the *M. jannaschii nadE* gene (Kessler and Leigh, 1999). The role of this regulatory sequence was demonstrated in the case of both the *nifH* and *glnA* genes of *M. maripaludis* where mutations that disrupt the palindrome lead to failure of ammonium to repress transcription (Cohen-Kupiec *et al.*, 1999; Cohen-Kupiec *et al.*, 1997). These data are consistent with a mechanism of negative control in which a repressor protein inhibits transcription of these N-regulated genes in the presence of ammonium. The proposed protein has yet to be identified although components of cell extracts have been shown to bind the *nifH* promoter (Cohen-Kupiec *et al.*, 1997).

The mechanism described above is apparently not universal among methanogenic diazotrophs. The proposed operator sequence is not present in the *nif* promoter of *M. thermoautotrophicum* strain Δ H but is found in strain Marburg of the same species. The sequence is also not present in the *nifH* promoters of *Methanosarcina barkeri* and *Methanosarcina mazei* so in these species a different regulation mechanism may be present (Chien *et al.*, 1998; Ehlers *et al.*, 2002). In *M. barkeri*, extracts from nitrogen-limited cells bind to the *nifH* promoter and a

substance present in ammonium-grown cells inhibits DNA binding by a transcription-associated protein or proteins suggesting that *nif* expression is positively controlled (Chien *et al.*, 1998).

3. NIF-SPECIFIC NITROGEN CONTROL

Transcriptional regulation of nitrogen fixation genes in response to availability of fixed nitrogen can minimally occur at just one level. In such cases the *nif* structural genes, e.g. *nifHDK*, are part of the general nitrogen regulon and are under the direct control of the global transcriptional regulator, which may be an activator or a repressor. This is apparently the situation in at least some of the Archaea such as *M. maripaludis* where the *nif* genes are subject to the same regulation as genes such as *amtB* or *nadE*. In the Gram-positive bacteria and the cyanobacteria, as discussed above, the regulatory circuits are not well characterised although it would appear that the cyanobacterial *nif* genes are not directly controlled. However in the very well documented Gram-negative systems a second level of regulation is almost always present. In these systems the *nif* structural genes are controlled by a *nif*-specific regulator and it is expression of this protein that is subject to global nitrogen control.

3.1. The NifA protein

The *nif*-specific regulator NifA, first identified in *K. pneumoniae*, is found in all diazotrophic members of the α , β and γ Proteobacteria. In the γ Proteobacteria *K. pneumoniae* and *A. vinelandii* and in the β Proteobacterium *Azoarcus*, *nifA* is part of a two gene operon and is located downstream of the *nifL* gene (Martin and Reinhold-Hurek, 2002; Merrick *et al.*, 1982; Bali *et al.*, 1992; Egner *et al.*, 2002). In all other cases it constitutes a single cistron. The presence of NifA in the δ Proteobacteria has not been investigated but in *Desulfovibrio gigas* the *nifH* gene does not have a characteristic NifA binding site upstream (Kent *et al.*, 1989).

The *nifA* or *nifLA* promoter provides the interface between the global and the *nif*-specific regulatory circuits and it is this promoter that is activated by the NtrC protein under nitrogen-limiting conditions. The exception to this is *A. vinelandii nifA*, which is not NtrC-dependent (Blanco *et al.*, 1993).

The NifA protein, like NtrC, is a σ^N -dependent transcriptional activator and consequently all *nifA*-dependent promoters are characterised by the recognition site for σ^N RNA polymerase, namely a -24,-12 type promoter with a consensus sequence TGGCAC – N₅ – TTGCA in which the GG at -24,-25 is invariant and the GC at -12,-13 is very highly conserved (Merrick, 1993; Barrios *et al.*, 1999). These promoters also contain binding sites for NifA (otherwise known as upstream activator sequences, UAS) that are typically located 80 to 100 bp upstream of the -24,-12 sequence and that conform to a consensus TGT – N₁₀ – ACA (Buck *et al.*, 1986). As σ^N RNA polymerase absolutely requires an activator protein in order to initiate transcription the *nif* genes are only expressed in the presence of an active form of NifA. The protein binds to the UAS and contacts the holoenzyme by a

DNA looping mechanism (Buck *et al.*, 1987) in which the looping event is often facilitated by the binding of Integration Host Factor (IHF) to the region between the UAS and the promoter (Hoover *et al.*, 1990). Formation of the open promoter complex and subsequent transcription initiation requires ATP hydrolysis which is catalysed by NifA (Austin *et al.*, 1990; Lee *et al.*, 1993).

The NifA proteins have three distinct domains: an N-terminal domain of between 170 and 250 residues that is a member of the GAF domain family (Ho *et al.*, 2000); a central domain of around 240 residues that is characteristic of all σ^{54} -dependent activators or Enhancer Binding Proteins (EBP) and that belongs to the AAA+ domain family (Neuwald *et al.*, 1999); and a C-terminal DNA-binding domain of around 50 residues. Although the NifA protein shares many features with NtrC it is not a classical response regulator protein. The N-terminal domain does not have the typical features of those proteins including the conserved aspartate residue (typically Asp54) that is the site of phosphorylation in proteins such as NtrC and indeed there is no evidence that NifA is phosphorylated under any conditions. Nevertheless this domain is of particular interest as it constitutes the regulatory domain of the protein and plays a major role in determining whether NifA is active.

The central domain is responsible for interaction with σ^N RNA polymerase and for ATP hydrolysis. The central domain of EBP proteins has been modelled and shown to contain seven highly conserved motifs of which the first (C1) is typical of the Walker A motif found in a wide range of proteins that bind and hydrolyse ATP (Osuna *et al.*, 1997). The roles of the other six motifs is unknown but studies of positive control mutants in the NifA protein of *Bradyrhizobium japonicum* identified region C3 and a highly conserved sequence (GAFTGA) as a candidate for interaction with σ^N RNA polymerase (Gonzalez *et al.*, 1998).

Between the central domain and the C-terminal domain is a variable region that characteristically divides the NifA proteins into two sub-families (Fig. 2). One group includes the NifA proteins from the γ Proteobacteria i.e. *Klebsiella*, *Enterobacter* and *Azotobacter*. These proteins contain a single conserved cysteine residue very near the end of the central domain. The second group includes the NifA proteins in the α and β Proteobacteria i.e. all the symbiotic rhizobia and members of the genera *Azospirillum*, *Rhodobacter*, *Rhodospirillum* and *Herbaspirillum*. These proteins contain two conserved cysteine residues at the end of the central domain (the second of which is equivalent to that in the γ Proteobacteria). They also have an additional two conserved cysteine residues, in a CXXXXC motif, in a region between the central domain and the DNA-binding domain. The NifA proteins that carry the CXXXXC motif are also distinguished by the fact that the activity of these proteins is oxygen sensitive, whereas those without the motif are oxygen resistant (Fischer *et al.*, 1988; Kullik *et al.*, 1989; Souza *et al.*, 1999).

The oxygen sensitivity of NifA has been studied in most detail in *Bradyrhizobium japonicum* where it was found that mutations in the cysteines of the CXXXXC motif resulted in an inactive protein. Mutation of the residues that separate the cysteines have no effect on activity whereas a change in the spacing

significantly decreases activity (Fischer *et al.*, 1989). All these data suggest that the motif may be involved in binding a metal ion but to date confirmation of this by purification of a NifA protein from this group has not been achieved. Oxygen-tolerant variants of NifA were successfully isolated in *Rhizobium meliloti* and all the alleles sequenced contained the same mutation causing a change from methionine to isoleucine in residue 217, near to the putative ATP binding site (Krey *et al.*, 1992). This led to a model proposing that at high oxygen concentrations the loss of NifA activity is due to a conformational change in the ATP binding site thereby abolishing ATP binding or hydrolysis.

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AV  --IRLLMSHRWPGNVRELENCLESAIMSEDGTTTRDVVSLTGVDNESPPLAAP--
KP  --IRLLMEYSWPGNVRELENCLESAVLSSEGLIDRDVILFNHRDNPALKASS--
EA  --VRLLMYTSWPGNVRELENCLESAVMTDEGLIDRDVILFNHHPALSVKPG--

BJ  --IDVLMSCKFPNGVRELENCIERTATLSAGTSTIVRSDFACSQGQLSTTLWKS--
RE  --IEVMSQCYFPGNVRELENCVRRATLARSSIVSSDFACKNSQCLSSLLWKT--
RL  --IDILSKCAFPGNVRELENCVQRTATLASSNTITSSDFACQDQCSSALLRKA--
RM  --LDHLSKCKFPNGVRELENCVRRATLARSKTITSSDFACQTDQCFSSRLWKG--
AC  --IDVLRRCYFPGNVRELENCIRRATLAHDAVITPHDFACDSGQCLSAMLWKG--

HS  --MKVMMNCYWPNGVRELENCVERTATMMRGDLITTEVHFSQQNKCLTKVLHEP--
AB  --LEVLRNCTWPGNVRELENCIERAATQSRDGIIRTESLCSLNLCSNVLFQY--
RC  --FDQICRCQFPGNERELENCVNRAAALSDGAIVLAEEACRQGAELSAELFRL--
RR  --LTAMGCNFPNGVRELENCVCRAATLAQDEVIQELGLSCHNDKCLSASLWQR--

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Figure 2. Location of conserved cysteine residues in the C-terminal region of NifA proteins. AV – *A. vinelandii*, KP – *K. pneumoniae*, EA – *E. agglomerans*, BJ – *B. japonicum*, RE – *R. etli*, RL – *R. leguminosarum*, RM – *R. meliloti*, AC – *A. caulinodans*, HS – *H. seropedicae*, AB – *A. brasilense*, RC – *R. capsulatus*, RR – *R. rubrum*. Vertical line indicates boundary between central and C-terminal domains.

3.2. The VnfA and AnfA proteins

Those diazotrophs that can synthesise alternative nitrogenases i.e. vanadium (*vnf*) or iron (*anf*) nitrogenases, regulate expression of the structural genes for these enzymes with specific NifA homologues designated VnfA and AnfA (Joerger *et al.*, 1989; Masepohl and Klipp, 1996). These proteins differ primarily from NifA in their N-terminal domains which are particularly characterised by the presence of two cysteine residues in a CXXXXC motif. Studies of amino acid substitutions in this region of *A. vinelandii* AnfA demonstrated that both cysteines (Cys21 and Cys 26) are required for AnfA activity (Premakumar *et al.*, 1994; Jepson and Austin, 2002). The data suggest the involvement of a metal cluster and purification of the isolated N-terminal domain of AvAnfA shows it to have a red/brown colour and spectral characteristics consistent with a 2Fe-2S cluster (Jepson and Austin, 2002).

Expression of both *vnf* and *anf* structural genes is under nitrogen control. In both *A. vinelandii* and *R. capsulatus*, *anfA* expression is regulated by ammonium but whereas in *R. capsulatus* this control is mediated by NtrC this is not the case in *A. vinelandii* (Premakumar *et al.*, 1998; Kutsche *et al.*, 1996; Toukdarian and

Kennedy, 1986; Kennedy *et al.*, 1991). Expression of the *A. vinelandii* *vnfA* gene is not subject to nitrogen control so in this case all nitrogen regulation occurs at the level of VnfA activity (Premakumar *et al.*, 1998).

3.3. Regulation of NifA activity

In NifA-dependent systems the regulation of *nif* gene expression is achieved through regulation of *nifA* expression and by regulation of NifA activity. Whilst *nifA* expression is almost invariably Ntr-regulated the control of NifA activity occurs in a variety of ways that vary from organism to organism. The data available to date indicate that in those organisms that have an oxygen-resistant NifA protein, namely *Klebsiella*, *Azotobacter* and *Enterobacter*, nitrogen control of NifA activity is regulated by its partner protein NifL in concert with a P_{II} protein. By comparison, in those organisms where NifA is oxygen sensitive, nitrogen control of NifA activity is mediated directly by a P_{II} protein.

3.3.1. *Klebsiella pneumoniae*

In *K. pneumoniae*, NifA activity is regulated in response to the cellular nitrogen status by its partner protein NifL (Merrick *et al.*, 1982). Expression of the *nifL* and *nifA* genes is controlled by NtrC and is translationally coupled so that NifL and NifA are expressed stoichiometrically, consistent with the proposal that they form a protein complex (Henderson *et al.*, 1989; Govantes *et al.*, 1996). Inactivation of *nifL* or overexpression of *nifA* leads to constitutive activation of the *nif* genes in the presence of fixed nitrogen or oxygen (Arnott *et al.*, 1989).

The NifL protein is predicted to comprise two domains. The N-terminal domain of around 290 residues contains a PAS motif, a widely distributed motif found in many sensor proteins and particularly associated with sensors of oxygen, redox and light (Zhulin *et al.*, 1997; Taylor and Zhulin, 1999). *K. pneumoniae* NifL is a flavoprotein with FAD bound to the N-terminal domain and most probably associated with the PAS motif (Schmitz, 1998). The C-terminal domain of around 200 residues shows significant homology to the histidine protein kinase transmitter domain but does not contain the highly conserved histidine residue that characterises these proteins (Drummond and Wootton, 1987). Studies of mutations in *nifL* suggested that the oxygen and fixed nitrogen sensing properties of the protein were separable (Sidoti *et al.*, 1993) and led to the search for a factor that could modulate the nitrogen response of NifL.

Studies of the classical components of the Ntr system, namely GlnB and GlnD excluded both these proteins (Holtel and Merrick, 1989; Edwards and Merrick, 1995) and it was the recognition that the γ Proteobacteria, such as *E. coli* and *K. pneumoniae*, encode a second P_{II} protein designated GlnK (van Heeswijk *et al.*, 1996) that finally suggested another candidate. Mutations in *glnK* were then indeed found to impair nitrogen control by *Klebsiella* NifL leading to a model in which GlnK interacted with NifL under nitrogen-limiting conditions thereby allowing NifA to be active (He *et al.*, 1998; He *et al.*, 1997; Jack *et al.*, 1999). The GlnK and GlnB

proteins are 68% identical and hence as both can be present in the cell at the same time the question arises as to how they are distinguished with respect to their interaction with NifL (Arcondéguy *et al.*, 1999). Studies in *E. coli* have suggested that the T-loop of the P_{II} proteins plays a major role in interactions with P_{II} target proteins (Jiang *et al.*, 1997; Pioszak *et al.*, 2000; Martinez-Argudo and Contreras, 2002). The T-loops of both *E. coli* and *K. pneumoniae* GlnB and GlnK differ at just three residues and changing a single residue (Asp54) in GlnB to the Asn54 found in GlnK dramatically increases the ability of GlnB to behave like GlnK with regard to regulation of NifL activity. Two changes in the T-loop (giving a Asp54Asn, Thr43Ala GlnB protein) essentially confers full GlnK activity on GlnB (Arcondéguy *et al.*, 2000).

Regulation of NifL activity by GlnK is surprisingly not dependent upon uridylylation of GlnK, raising the question of how NifL could respond to a rapid change in the intracellular nitrogen status (He *et al.*, 1998; Edwards and Merrick, 1995). However recent studies suggest that changes in the cellular localisation of both GlnK and NifL in response to nitrogen status may play a major role in this regulatory system. In *K. pneumoniae*, as in nearly all bacteria, the *glnK* gene is linked to *amtB* which encodes a high affinity ammonium transporter. The *glnK,amtB* operon is regulated by NtrC and is only expressed under nitrogen limitation (Jack *et al.*, 1999). In *E. coli*, GlnK associates specifically with AmtB in the membrane and this association is markedly increased in response to a rapid increase in N status e.g. after an ammonium shock (Coutts *et al.*, 2002). This rapid association of GlnK to AmtB serves to regulate AmtB activity but will also quickly deplete the intracellular pool of GlnK. If, as expected, a similar mechanism operates in *K. pneumoniae* then an increase in N status could serve to provide the rapid depletion of the GlnK pool with consequent liberation of NifL which would then inhibit NifA activity. This model is further enhanced by the recognition that some 55% of NifL protein is membrane-associated in nitrogen-limited conditions whereas less than 10% of NifL is found in the membrane fraction of cells growing in nitrogen sufficiency (Klopprogge *et al.*, 2002).

3.3.2. *Azotobacter vinelandii*

The NifLA system of *A. vinelandii*, whilst sharing many properties with that of *K. pneumoniae*, has a number of distinct features. Firstly the *nifLA* operon is not regulated by the Ntr system but is constitutively expressed (Blanco *et al.*, 1993) consequently all *nif* regulation in *Azotobacter* is achieved by controlling the activity of NifA. The NifL and NifA proteins of *Azotobacter* have proved to be much more amenable to purification and analysis *in vitro* than those of *Klebsiella* and hence much more is known of the detailed molecular nature of the proteins and their mode of action (Austin *et al.*, 1994; Dixon, 1998).

A. vinelandii NifL is highly homologous to *K. pneumoniae* NifL. The protein is also a flavoprotein containing FAD in the N-terminal domain (Hill *et al.*, 1996). The C-terminal domain shows a higher level of homology to the histidine protein kinases and binds both ATP and ADP (Söderbäck *et al.*, 1998). This domain is competent

to inhibit NifA activity in response to ADP *in vitro* and in response to the level of fixed nitrogen *in vivo* (Söderbäck *et al.*, 1998). Furthermore the domain includes a histidine within the motif that typically contains the phosphorylated histidine (Blanco *et al.*, 1993). However mutational analysis showed that many amino acid substitutions at this position do not impair NifL activity and there is no evidence that *Azotobacter* NifL is ever subject to phosphorylation (Woodley and Drummond, 1994).

A mutation in the *A. vinelandii glnD* gene (formerly called *nfrX*) prevented *nif* gene expression but could be suppressed by a secondary mutation in *nifL* (Contreras *et al.*, 1991). This suggested that uridylylation of a regulatory component may be necessary to prevent inhibition of NifA activity by NifL. *A. vinelandii* is atypical amongst the γ Proteobacteria in having only a single P_{II} protein which is encoded in a *glnK,amtB* operon (Meletzus *et al.*, 1998). Studies both *in vivo* and *in vitro* show that the inhibitory activity of the NifL protein is stimulated by interaction with non-uridylylated P_{II} (either the native *Azotobacter* GlnK or *E. coli* GlnB) (Reyes-Ramirez *et al.*, 2001; Little *et al.*, 2000; Little *et al.*, 2002; Rudnick *et al.*, 2002). Consequently the system is quite distinct from that in *Klebsiella* because in *Azotobacter* GlnK interacts with NifL under conditions of nitrogen excess and stimulates it to inhibit transcriptional activation by NifA. The inhibitory activity of NifL is then relieved under nitrogen-limiting conditions because the elevated levels of 2-oxoglutarate modulate the interaction of NifL with NifA (Little *et al.*, 2000). The interaction of GlnK is with the C-terminal histidine protein kinase-like domain of NifL and is abolished by a single amino acid substitution (Glu44Cys) in the T-loop of GlnK or by uridylylation of GlnK (Little *et al.*, 2002).

3.3.3. *Azoarcus*

The nitrogen control system in *Azoarcus* is not yet fully described. However *Azoarcus* has been shown to synthesise three P_{II}-like proteins, GlnB, GlnK and GlnY of which the last two are encoded in operons (*glnK,amtB*; *glnY,amtY*) along with AmtB-like proteins. Transcription of *nif* genes is repressed by ammonium or nitrate in wild-type and *glnB* or *glnK* mutants but in a *glnB,glnK* double mutant some transcription was detectable in ammonium and almost full expression was found in nitrate-grown cells (Martin and Reinhold-Hurek, 2002). These data suggest that either GlnB or GlnK can mediate *nif* repression (possibly through NifLA) in *Azoarcus*.

3.3.4. *Azospirillum brasilense*

A. brasilense NifA belongs to the oxygen-sensitive group of NifA proteins and does not have a partner NifL protein. The first indication that a P_{II}-like protein might be involved in the nitrogen regulation of NifA activity came from the observation that in *A. brasilense* a *glnB* mutant was Nif⁻ (Liang *et al.*, 1992) whilst a *glnK* mutant had no effect on nitrogen fixation (de Zamaroczy, 1998). As the *glnB* mutation did

not affect *nifA* expression it appeared that GlnB is required to maintain the active form of NifA. Deletions within the N-terminal domain of NifA restore *nif* gene expression, suggesting that GlnB is required to activate NifA by preventing the inhibitory effect of its N-terminal domain (Arsene *et al.*, 1996). Mutation of residue Tyr18 to Phe in the N-terminal domain of NifA results in an active NifA that does not require GlnB. However, whether GlnB interacts directly with NifA or modulates the activity of another protein that in turn regulates NifA activity remains unsolved (Arsene *et al.*, 1999). A *glnB* Tyr51Phe mutant and a *glnD* mutant both exhibit a Nif⁻ phenotype (Arsene *et al.*, 1999; van Dommelen *et al.*, 2002) which is consistent with the fact that during nitrogen fixation, *A. brasilense* GlnB is uridylylated (de Zamaroczy, 1998) and suggests that it is GlnB-UMP that is required for NifA activation.

3.3.5. *Herbaspirillum seropedicae*

As in *A. brasilense*, in *H. seropedicae* a *glnB* mutant is Nif⁻, whilst *nifA* expression, which is NtrC-dependent, would be expected to be constitutive in this background (Benelli *et al.*, 1997). Studies of the *H. seropedicae* NifA protein *in vivo* show that the full length protein expressed in *A. brasilense* is active only under low oxygen and in the absence of ammonium, but NifA is not active when expressed in *E. coli* or *K. pneumoniae* (Souza *et al.*, 1999). By contrast, an amino terminally truncated NifA is still active in the presence of ammonium in *A. brasilense*, *E. coli* or *K. pneumoniae*, indicating that the N-terminal domain is involved in nitrogen control. Furthermore when expressed *in trans*, this domain can inhibit the activity of the truncated NifA (Souza *et al.*, 1999; Monteiro *et al.*, 1999a; Monteiro *et al.*, 1999b). If GlnB were to interact with the N-terminal domain then the inactivity of *H. seropedicae* NifA in *E. coli* could be due to the absence of the cognate P_{II}.

3.3.6. *Rhodobacter capsulatus*

R. capsulatus is unique in having two *nifA* genes that encode virtually identical proteins that can substitute for each other and that differ only in their 19 N-terminal residues (Masepohl *et al.*, 1988; Paschen *et al.*, 2001). Expression of both genes is regulated by NtrC (Foster-Hartnett and Kranz, 1992) but strains expressing either *nifA*₁ or *nifA*₂ from a constitutive promoter in an *ntrC* mutant still show inhibition of *nifH* transcription in ammonium, again suggesting post-translational control of NifA activity (Hübner *et al.*, 1993). Mutations in the N-terminal domain of NifA1 result in ammonium-tolerant NifA proteins suggesting that, as in other NifA proteins, this domain is involved in regulation of activity (Paschen *et al.*, 2001).

R. capsulatus encodes two P_{II} proteins, GlnB and GlnK. Mutations in *glnB* do not affect NifA activity whereas in a *glnK* mutant NifA partially escapes ammonium inhibition and in a *glnB,glnK* double mutant ammonium control is completely abolished (Masepohl *et al.*, 2002). Yeast two-hybrid studies indicate that both GlnB and GlnK can interact with NifA suggesting that these proteins directly

mediate nitrogen control of NifA activity though GlnB apparently only partially substitutes for GlnK (Masepohl *et al.*, 2002).

In contrast to *nif* regulation, *anfH* is still inhibited by ammonium in a *glnB,glnK* double mutant and this inhibition is also independent of NtrC. Hence the ammonium control of AnfA activity must occur by a completely different mechanism (Masepohl *et al.*, 2002).

3.3.7. *Rhodospirillum rubrum*

R. rubrum can synthesise three P_{II}-like proteins, GlnB, GlnJ and GlnK, of which the last two are encoded in operons that also encode ammonium transport-like proteins, *glnJ,amtB₁* and *glnK,amtB₂* (Zhang *et al.*, 2001; Zhang *et al.*, 2000; Johansson and Nordlund, 1999; Johansson and Nordlund, 1997). A Δ *glnB* mutant has no nitrogenase activity whereas a *glnB* Tyr51Phe mutant shows around 10% of wild-type nitrogenase activity. Expression of *R. rubrum nifA* from a multicopy plasmid does not restore nitrogenase activity in a Δ *glnB* mutant whereas a *glnB* Tyr51Phe mutant is complemented (Zhang *et al.*, 2000). The effects of the *glnB* mutations are not on *nifA* expression and therefore GlnB appears to be essential for NifA activity. There is no specific evidence for a GlnB-NifA interaction but the data do suggest that GlnB-UMP promotes activation of NifA (Zhang *et al.*, 2001).

3.4. Regulation of VnfA and AnfA activity

The activities of *A. vinelandii* VnfA and AnfA and of *R. capsulatus* AnfA are all subject to nitrogen control. In *R. capsulatus* this control appears to occur by a novel mechanism that is independent of NtrC, GlnB and GlnK (Masepohl *et al.*, 2002). In *A. vinelandii* the activities of both VnfA and AnfA are nitrogen regulated but ammonium repression of both proteins is relieved when the N-terminal domains are deleted (Drummond *et al.*, 1995). The role of *A. vinelandii* GlnK in this regulation has not been investigated.

4. NITROGEN CONTROL OF NITROGENASE ACTIVITY

4.1. ADP ribosylation

The final level at which nitrogen fixation can be subject to control in response to availability of fixed nitrogen is by regulation of the activity of the nitrogenase enzyme itself. In 1950, studies of nitrogen fixation in *Rhodospirillum rubrum* first demonstrated that the addition of ammonium to nitrogen-fixing cultures led to a rapid decrease in nitrogenase activity (Gest *et al.*, 1950). This property was not exhibited by other diazotrophs that were available to study at the time and subsequent studies showed the inhibitory effect to be reversible and the length of the inhibitory period to be correlated with the concentration of ammonium added (Schick, 1971). Extensive biochemical studies finally led to the recognition that

inactivation of nitrogenase was due to ADP ribosylation of residue Arg101 on one of the two subunits of NifH, the Fe protein of nitrogenase (also called dinitrogenase reductase) (Pope *et al.*, 1985). The process requires the concerted action of two enzymes; dinitrogenase reductase ADP-ribosyl transferase (DRAT) which transfers the ADP-ribose moiety of NAD to Arg101 of NifH (Lowery *et al.*, 1986), and dinitrogenase reductase activating glycohydrolase (DRAG) which reactivates NifH by removing ADP-ribose (Saari *et al.*, 1986). The structural genes for both DRAG (*draG*) and DRAT (*draT*) have been cloned from *R. rubrum* (Fitzmaurice *et al.*, 1989).

ADP ribosylation of nitrogenase has been reported in a variety of other diazotrophs and indeed the *draT* and *draG* genes have also been cloned from *R. capsulatus* (Masepohl *et al.*, 1993), *A. brasilense* (Zhang *et al.*, 1992) and *A. lipoferum* (Inoue *et al.*, 1996). In all cases they appear to constitute a *draT, draG* operon and in all but *R. capsulatus* they are located adjacent to the *nifHDK* operon. There is also evidence for the system in *Chromatium vinosum*, *Rhodopseudomonas palustris* and *Rhodopseudomonas viridis* and physiological responses consistent with ADP ribosylation have been reported in *Azoarcus*, *Azotobacter chroococcum*, *Azorhizobium sesbaniae* and *R. sphaeroides*. However *R. sphaeroides* does not contain *draTG* genes and does not apparently show ADP-ribosylation of nitrogenase (Yakunin *et al.*, 2001).

As DRAG and DRAT are encoded in an operon they are expressed together and their activities are post-translationally regulated *in vivo* as evidenced by studies in *R. rubrum*, *A. brasilense* and *R. capsulatus* of mutants lacking either of the enzymes (Liang *et al.*, 1991; Zhang *et al.*, 1993; Zhang *et al.*, 1992; Masepohl *et al.*, 1993). In nitrogen-fixing conditions DRAG is active and DRAT is completely inactive but following addition of a good nitrogen source e.g. ammonium, DRAG becomes inactive within a few minutes and DRAT becomes active leading to inactivation of NifH. Exhaustion of the nitrogen source leads to a reversal of this process. In *R. capsulatus* ADP ribosylation occurs not only on the molybdenum nitrogenase subunit NifH but also on the iron nitrogenase subunit AnfH (Masepohl *et al.*, 1993).

The introduction of the *R. rubrum* and *A. brasilense draTG* genes into *K. pneumoniae* proved to be a critical experiment as it demonstrated that DRAT and DRAG could regulate the activity of a heterologous nitrogenase in response to added nitrogen, indicating that the signal transduction system responsible for regulating the system was present in *K. pneumoniae* (Fu *et al.*, 1990). These data focussed attention on the Ntr system and in particular on the P_{II} proteins. Subsequent studies of P_{II} mutants in the heterologous *K. pneumoniae* system, in *R. rubrum* and in *R. capsulatus* have confirmed that the P_{II} proteins do indeed play a role in regulating ADP ribosylation.

R. capsulatus synthesizes two P_{II} proteins, GlnB and GlnK, and in a *glnB, glnK* double mutant nitrogenase is not subject to post-translational ammonium control indicating that one or both P_{II} proteins are necessary for DRAG activity (Masepohl *et al.*, 2002). *R. rubrum* is more complex in having three P_{II} proteins. A *glnK*, a *glnJ* or a *glnK, glnJ* mutant is unaffected in ADP ribosylation whereas a *glnB* or a *glnB, glnK* mutant shows some impairment in the response to ammonium and a

glnB, glnJ mutant shows almost no ammonium response (Zhang *et al.*, 2001). Hence it would appear that either GlnB or GlnJ can influence the activities of the DRAG/DRAT proteins. The precise mechanism of this control is not yet understood but given the mode of action of P_{II} in other systems it seems likely that the P_{II} proteins directly affect the DRAG and/or DRAT activities.

ADP ribosylation in *R. rubrum* also occurs in response to an energy shift as effected by moving cells into the dark but a *glnB, glnJ* double mutant is also impaired in this response indicating that these proteins also mediate a signal of energy limitation (Zhang *et al.*, 2001). This situation is distinct from that in *R. capsulatus* where a *glnB, glnK* double mutant responds normally to a light-dark shift (Masepohl *et al.*, 2002).

Whilst the P_{II} proteins have clearly been implicated in the signal transduction pathway controlling ADP ribosylation the precise signals are not yet identified. However in *R. capsulatus* mutations in ammonium transport genes also affect this process. *R. capsulatus* has two *amt* genes; *amtB* is linked to *glnK* in a *glnK, amtB* operon, and a second gene *amtY* is unlinked. An *amtB* mutant is completely defective in ADP ribosylation whilst an *amtY* mutant shows elevated levels of nitrogenase modification compared to wild-type in response to the same amount of ammonium (Masepohl *et al.*, 2002; Yakunin and Hallenbeck, 2000; Yakunin and Hallenbeck, 2002). The role of the Amt proteins in the signal transduction pathway is still unclear and, given the potential for Amt proteins to sequester P_{II} proteins in response to ammonium shock, these phenotypes could reflect indirect effects on the free intracellular pools of P_{II} protein (Coutts *et al.*, 2002).

NAD has been implicated as a potential signal molecule in some aspects of the control of ADP ribosylation in *R. rubrum* (Noren *et al.*, 1997; Noren and Nordlund, 1994; Soliman and Nordlund, 1992) and it is therefore of potential interest that P_{II} may also play a role in regulation of NAD synthetase. In both *H. seropedicae* and *Azoarcus* the structural gene for NAD synthetase (*nadE*) is genetically linked to a *glnB*-like gene giving rise to the possibility that this linkage reflects the potential for an interaction between the two proteins (Arcondéguy *et al.*, 2001; Martin *et al.*, 2000; Benelli *et al.*, 1997).

4.2. Ammonium switch-off in methanogens

The phenomenon of ammonia switch-off is also found in the diazotrophic methanogens *Methanosarcina barkeri* and *Methanococcus maripaludis* (Lobo and Zinder, 1990; Kessler and Leigh, 1999). There is no evidence for ADP ribosylation in these organisms (Kessler *et al.*, 2001) and *draG* and *draT* homologues are not encoded in the completed genome sequences of the related diazotrophs *M. thermoautotrophicum* and *Methanosarcina mazei* Gö1. Hence it would seem likely that these organisms use an alternative method of post-translational modification.

The *nif* structural gene clusters of all the diazotrophic methanogens are characterised by the presence of two *glnB*-like genes, now designated *nifI*₁ and *nifI*₂, that are located between the nitrogenase structural genes *nifH* and *nifDK* (Sibold *et*

al., 1991; Souillard *et al.*, 1988; Kessler *et al.*, 2001; Kessler and Leigh, 1999; Kessler *et al.*, 1998; Chien and Zinder, 1996; Ehlers *et al.*, 2002). The P_{II} proteins encoded by these genes are from a quite distinct sub-group. The *nifH*-proximal gene (*nifI*₁) encodes a polypeptide of around 105 residues whereas the *nifH*-distal gene (*nifI*₂) encodes a polypeptide of 120-130 residues. Both polypeptides are highly homologous to proteins such as GlnB and GlnK and they are distinguished by the regions that correspond to the T-loop. Whereas the T-loop in GlnB or GlnK proteins is almost invariably 18 residues long, sequence alignments suggest that in NifI₁ the equivalent region is only 13 residues long and in NifI₂ it is 27 to 32 residues long. There is no conserved site for uridylylation or phosphorylation within the predicted T-loops suggesting that the proteins are either subject to a novel form of modification or, perhaps are not modified at all.

The NifI proteins are not restricted to the diazotrophic methanogens but are also found in the Firmibacteria (in *Clostridium acetobutylicum* and *Clostridium cellobioparum*) and in the δ Proteobacteria (in *Desulfovibrio gigas*). In all cases they are located downstream of *nifH* suggesting that they have a conserved function (Arcondéguy *et al.*, 2001).

The roles of the NifI proteins have been analysed in detail in *M. maripaludis* where the construction of in-frame deletions coupled with complementation analysis showed that both proteins are required for switch-off. The process of switch-off was reversible and did not affect *nif* gene transcription, *nifH* mRNA stability or NifH protein stability (Kessler *et al.*, 2001). The process mediated by the NifI proteins therefore appears to be quite novel and a number of possible mechanisms have been suggested. These include non-covalent association of nitrogenase with another protein factor (which could be NifI itself), reversible covalent modification of a Nif protein other than NifH or a reversible covalent modification that is not resolved on SDS PAGE (Kessler *et al.*, 2001).

The structure of the *nifH*, *nifI*₁, *nifI*₂, *nifD*, *nifK* operon implies a stoichiometric relationship between these five polypeptides and raises interesting questions about the likely structures of the NifI₁ and NifI₂ proteins. By comparison with all other P_{II} proteins they would be expected to form trimers but the 1:1 ratio of NifI₁ to NifI₂ could allow the formation of a hexameric protein. The occurrence in *Aquifex aeolicus* of a novel P_{II} gene, which appears to constitute a “duplicated” *glnB*-like gene where two similar coding sequences are fused in-frame, suggests that such a hexameric structure may be possible (Arcondéguy *et al.*, 2001).

4.3. Other mechanisms of ammonia switch-off

Mechanisms of ammonium-induced switch-off of nitrogenase that are independent of ADP ribosylation have been described in a number of cases. In the unicellular cyanobacterium *Gleotheca* a novel modification of NifH by palmitoylation has been reported (Gallon *et al.*, 2000). In *R. capsulatus* and *A. brasilense* a second mechanism for post-translational regulation of nitrogenase activity in addition to ADP ribosylation has also been reported but the mechanism underlying this control

is unknown (Yakunin and Hallenbeck, 1998; Pierrard *et al.*, 1993; Zhang *et al.*, 1996).

5. CONCLUSIONS

Our understanding of the regulation of the nitrogen fixation process in free-living diazotrophs has advanced considerably in the last decade with information coming from a wide range of model systems. These advances have led to a much more global view of the mechanisms that facilitate the very stringent control that is necessary to maximise the physiological benefits from diazotrophy. With regard to nitrogen control, members of the P_{II} protein family i.e. the GlnB, GlnK, NifI etc. proteins, clearly play a pivotal role in nearly all organisms and P_{II} is now being recognised as the critical signal transduction protein in a wide variety of aspects of bacterial nitrogen metabolism (Arcondéguy *et al.*, 2001). At the molecular level our understanding of the regulatory processes is advancing considerably in those organisms that have a NifA-dependent mode of control, though the ability to purify active NifA proteins (particularly of the oxygen-sensitive group) is still a major hurdle.

The heterocystous cyanobacteria are particularly complex owing to the integration of the regulation of nitrogenase expression and activity with that of heterocyst development. Nevertheless considerable information is now emerging and a broad outline of the major signal transduction pathways may be achieved fairly soon. The groups where there is still much to be learnt are the archaea and the Gram-positive diazotrophs. In the archaea the advent of genome sequences and good genetic systems in model organisms e.g. *Methanobacterium thermoautotrophicum* and *Methanosarcina mazei* shows considerable promise for the future but similar opportunities are not yet apparent in the Gram-positive diazotrophs.

In summary the challenge in the future is to begin to integrate our current knowledge into a whole-cell perspective of the genetic, biochemical and physiological processes that contribute to successful diazotrophy.

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