Regulation of Biological Nitrogen Fixation[1,2]

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ABSTRACT Biological nitrogen fixation, a process found only in some prokaryotes, is catalyzed by the nitrogenase enzyme complex. Bacteria containing nitrogenase occupy an indispensable ecological niche, supplying fixed nitrogen to the global nitrogen cycle. Due to this inceptive role in the nitrogen cycle, diazotrophs are present in virtually all ecosystems, with representatives in environments as varied as aerobic soils (e.g., *Azotobacter* species), the ocean surface layer (*Trichodesmium*) and specialized nodules in legume roots (*Rhizobium*). In any ecosystem, diazotrophs must respond to varied environmental conditions to regulate the tremendously taxing nitrogen fixation process. All characterized diazotrophs regulate nitrogenase at the transcriptional level. A smaller set also possesses a fast-acting post-translational regulation system. Although there is little apparent variation in the sequences and structures of nitrogenases, there appear to be almost as many nitrogenase-regulating schemes as there are nitrogen-fixing species. Herein are described the paradigms of nitrogenase function, transcriptional control and post-translational regulation, as well as the variations on these schemes, described in various nitrogen-fixing bacteria. Regulation is described on a molecular basis, focusing on the functional and structural characteristics of the proteins responsible for control of nitrogen fixation. J. Nutr. 130: 1081–1084, 2000.

KEY WORDS: • nitrogen fixation • nitrogenase
• ADP-ribosylation
• dinitrogenase reductase ADP-ribosyltransferase
• dinitrogenase reductase-activating glycohydrolase

Nitrogen, as an essential dietary element, is derived primarily from animal and plant proteins. The source of biological nitrogen atoms in these proteins can be traced ultimately to nitrogen fixation. Although an increasing proportion of human nutritional nitrogen comes from ammonia fixed by industrial fertilizer production, roughly half of the 23 million metric tons of nitrogen consumed as human food sources (grains and livestock) comes from biological nitrogen fixation by bacteria (1). Although most bacteria assimilate nitrogen in the form of NH_4^+ for use in biosynthetic pathways, a more limited set of bacteria is able to convert atmospheric dinitrogen to NH_4^+. Biological nitrogen fixation is catalyzed by the nitrogenase complex. Nitrogenase catalyzes the conversion of N_2 to NH_4^+, as represented by: N_2ase

$$N_2 + 10H^+ + 8e^- + n\text{MgATP} \longrightarrow 2NH_4^+ + H_2 + n\text{MgADP} + nP_i \; (n \geq 16)$$

As can be seen by comparison of this reaction to the nitrogen assimilation reactions, nitrogen fixation is very expensive in biological energy equivalents, requiring large amounts of both reducing power and high energy phosphate (ATP). Obligate proton reduction occurs during nitrogenase catalysis, with a minimum of 1 mol of H_2 produced per mol of N_2 reduced (2). The proportion of electrons allocated to proton reduction increases under conditions of limiting electron flux, further increasing the consumption of MgATP (3).

In addition to protons, nitrogenase can reduce several other alternative substrates, which resemble N_2 on the basis of double or triple bonds in their structures. Acetylene has proven to be a particularly useful substrate in nitrogenase research because the reduction product, ethylene, is easily quantified by gas chromatography. Because acetylene and ethylene are both permeable to the bacterial envelope, nitrogenase activity may be measured in vivo as well as in vitro by the acetylene reduction method. Reduction of all substrates, except protons, can also be inhibited by CO, suggesting that proton reduction occurs by a slightly different pathway (3).

Properties of nitrogenase. A brief description of the properties of nitrogenase proteins is necessary for discussion of their regulation. The nitrogenase complex consists of two metalloproteins, highly conserved in sequence and structure throughout nitrogen-fixing bacteria (4). The protein containing the site of substrate reduction is nitrogenase molybdenum-iron (MoFe)[4] protein, also known as dinitrogenase or component I. The obligate electron donor to MoFe protein is nitrogenase iron protein (Fe protein), also known as dinitrogenase reductase or component II. The genes encoding MoFe protein and Fe protein, as well as accessory genes for electron transfer proteins, metal cluster synthesis and regulation comprise the *nif* regulon (4).

MoFe protein is a 230-kDa $\alpha_2\beta_2$ tetramer of the *nifD* and *nifK* gene products. Each MoFe protein tetramer contains two pairs of metalloclusters unique to MoFe protein, i.e., two molydenum-iron-sulfur-homocitrate clusters (FeMo-co) and two $[Fe_8S_7]$ clusters (P-cluster). FeMo-co consists of two partial cubanes ($[Fe_4S_3]$ and $[MoFe_3S_3]$) bridged by three sulfides, with homocitrate coordinated to the Mo atom. FeMo-co is completely encompassed by the three domains of the α subunit and is the presumed site of substrate reduction (5). Note that

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[4] Abbreviations used: DRAG, dinitrogenase reductase-activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyltransferase; FeMo-co, iron-molybdenum cofactor; Fe protein, nitrogenase iron protein; MoFe protein, nitrogenase molybdenum-iron protein.

the FeMo-co is structurally distinct from the molybdopterin cofactors found in human oxidase enzymes and nitrate reductase in plants. FeMo-co gives rise to the characteristic electron paramagnetic resonance spectrum of MoFe protein. The P-clusters consist of two distorted iron-sulfur partial cubanes with redox-dependent structure (6). The P-clusters are located at the interface of the α and β subunits and are thought to be intermediates in the electron transport pathway between Fe protein and FeMo-co (5).

Fe protein is a 64-kDa α_2 dimer of the *nifH* gene product. A single, regular $[Fe_4S_4]$ cubane is symmetrically coordinated between the subunits by Cys97 and Cys132 from each subunit. This $[Fe_4S_4]$ cluster is the redox-active center directly involved with electron transfer to MoFe protein. The $[Fe_4S_4]$ cluster of Fe protein cycles between the reduced (1+) state and the oxidized (2+) state during electron transfer to MoFe protein. An all-ferrous (0) state of Fe protein has also been described (7), but the physiologic relevance of this species is unclear. Each Fe protein dimer can bind two nucleotide molecules, at sites distal from the $[Fe_4S_4]$ active site (3). Binding of MgATP at these sites causes a conformational change in Fe protein. The two subunits rotate toward each other, extruding the $[Fe_4S_4]$ cluster toward the protein surface (and surmised interaction with the P-clusters of MoFe protein) by 4 Å (8). This conformational change is thought to be a key step in the catalytic cycle of nitrogenase.

Using data obtained from the nitrogenase systems of *Clostridium pasteurianum*, *Klebsiella pneumoniae* and *Azotobacter vinelandii*, a general sequence of events in the catalytic cycle of nitrogenase can be described. MgATP binding to reduced Fe protein shifts the redox potential of the $[Fe_4S_4]^{2+/1+}$ couple from about −300 mV to nearly −450 mV vs. standard hydrogen electrode. A concomitant MgATP-induced conformational change apparently promotes interaction of Fe protein with MoFe protein (3). Upon complex formation, an additional conformational change of Fe protein shifts the redox potential of the Fe_4S_4 cluster by an additional −200 mV (9), making the transfer of a single electron from the Fe protein to MoFe protein energetically favorable. The hydrolysis of MgATP (bound to Fe protein) to MgADP and P_i is coupled to this electron transfer. After electron transfer and MgATP hydrolysis, the nitrogenase complex dissociates in the rate-limiting step of the cycle. Fe protein is then reduced by a low potential electron donor (a ferredoxin or flavodoxin in vivo), and MgADP is exchanged for MgATP. The catalytic cycle is repeated until a sufficient number of electrons have been transferred to completely reduce the FeMo-cobound substrate (3). Although Fe protein is the obligate electron donor for MoFe protein in all characterized nitrogenase systems, the in vivo electron donor for Fe protein is less stringently conserved. The NifJ → NifF → Fe protein electron donation pathway, described in *K. pneumoniae* (10), is replaced with a number of electron donors in other species, particularly ferredoxins of varying FeS cluster composition.

Transcriptional regulation of nitrogenase. Nitrogen fixation is regulated at the transcriptional level in response to environmental oxygen and ammonium levels. Because the nitrogenase components are oxygen labile, it is advantageous for bacteria to repress transcription when oxygen levels are high. It is also advantageous to repress the expression of the metabolically expensive nitrogenase system when the cellular level of fixed nitrogen is sufficiently high. The degree to which each stimulus affects transcription is characteristic of the particular diazotroph. Expression of nitrogenase in symbiotic diazotrophs is fairly insensitive to ammonium because export of ammonium to their symbiont suppresses ammonium levels. The expression of *nif* genes in free-living diazotrophs is more sensitive to cellular ammonium levels (11).

As with the nitrogen assimilation and the nitrogenase mechanisms, the paradigm of transcriptional regulation is derived from studies on *K. pneumoniae*. In this model, control of *nif* gene expression focuses on NifA (the *nifA* gene product), a σ^{54} (*rpoN* gene product)-dependent transcriptional activator, responsible for control of all major *nif* gene cluster transcription. Transcription of *nifA* is under the control of the *ntrBC* gene products, which comprise a global two-component transcriptional activator system, responsible for cellular nitrogen regulation (11). In the paradigm system, *K. pneumoniae*, the *nifA* gene is cotranscribed with *nifL*, which encodes a redox- and nitrogen-responsive regulatory flavoprotein (NifL). NifL acts as a negative regulator of NifA, effectively adding another level of regulation in response to oxygen and fixed nitrogen. Oxidized NifL is also sensitive to the presence of nucleotides in vitro, with increased inhibition especially in response to ADP (12). The means by which NifL inhibits NifA remain to be determined.

Deviations from the *K. pneumoniae* paradigm exist in nearly all nitrogen fixation organisms of research interest (**Fig. 1**). In *A. vinelandii* (11) and *Rhodospirillum rubrum* (Y. Zhang, unpublished results), expression of *nifA* is not under the control of the *ntrBC* gene products, and it remains unclear whether *nifA* expression is under nitrogen control. In *Rhizobium meliloti*, redox-dependent control of *nifA* expression occurs in response to *fixL* and *fixJ*, which encode a two-component regulatory system responsive to oxygen (11). This system apparently replaces the *ntrBC* control found in *K. pneumoniae*. *R. meliloti* also lacks NifL, but NifA still appears to be inhibited by oxygen stimulus (13). Similarly, there is no evidence for NifL in *Rhodobacter capsulatus*. *R. capsulatus* contains *nif*-related genes analogous to *ntrBC*, but the expression of an *rpoN*-like gene is found to be sensitive to oxygen and fixed nitrogen status (11). Also, *R. capsulatus* contains two copies of *nifA*, which respond differently to ammonium (14). Clearly, nitrogenase transcriptional control mechanisms must be elucidated separately for any given diazotroph.

Post-translational regulation of nitrogenase. Because of the metabolically demanding nature of nitrogen fixation, an additional layer of nitrogenase regulation is present in a few free-living diazotrophs. To prevent unproductive nitrogen fixation during energy-limiting or nitrogen-sufficient conditions, the nitrogenase complex is rapidly, reversibly inactivated by ADP-ribosylation of Fe protein. The ADP-ribosylation system has been identified in *R. rubrum* and *R. capsulatus* (purple, nonsulfur photosynthetic bacteria), *Azospirillum brasilense* and *Azospirillum lipoferum* (microaerophilic, associative bacteria), and *Chromatium vinosum* (a purple sulfur bacterium) (15). The ADP-ribosylation system of *R. rubrum*, the organism in which post-translational nitrogenase regulation was first identified, remains the model organism under investigation. The basic model for ADP-ribosylation in *R. rubrum* is shown in **Figure 2**, showing the roles of the NAD^+-dependent enzyme, dinitrogenase reductase ADP-ribosyltransferase (DRAT), and its partner, dinitrogenase reductase-activating glycohydrolase (DRAG).

ADP-ribosylation of Fe protein occurs at a specific arginine residue (Arg101 in *R. rubrum*) by the formation of an α-N-glycosidic bond between the guanidino nitrogen atom of arginine and the terminal ribose of ADP-ribose (15). Structurally, this ADP-ribose is identical to the modifying groups attached by bacterial ADP-ribosylating toxins, the causative agents of cholera and diphtheria. In nitrogenase, the presence of the ADP-ribose group apparently prevents association of Fe protein with MoFe protein, rather than blocking electron transfer

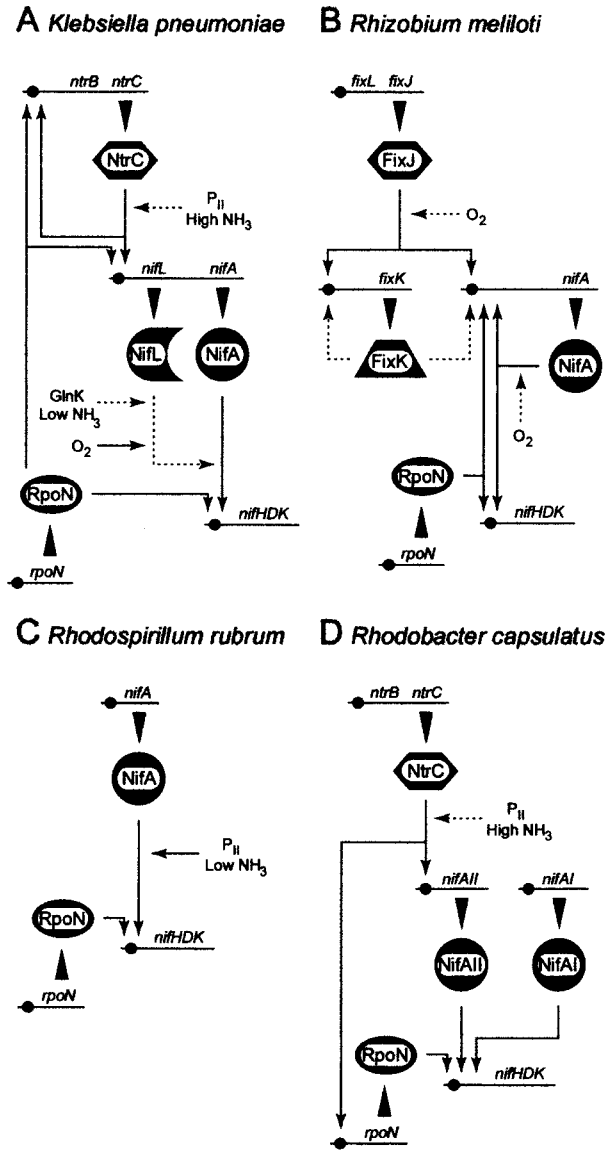

FIGURE 1 Comparative models of transcriptional regulation of *nif* genes in (*A*) *Klebsiella pneumoniae*, (*B*) *Rhizobium meliloti*, (*C*) *Rhodospirillum rubrum* and (*D*) *Rhodobacter capsulatus*. PII (the *glnB* gene product) and GlnK are global nitrogen regulation proteins of similar structure. RpoN is the σ^{54} subunit of the RNA polymerase complex. Functionally similar regulatory proteins are symbolized identically in each panel. Solid and dashed lines indicate positive and negative regulation, respectively. Solid circles indicate promoter sites. *R. meliloti* model adapted from (27).

between complexed Fe protein and MoFe protein (15). ADP-ribosylated Fe protein differs from unmodified Fe protein in only a few characteristics. The two subunits of the inactive Fe protein dimer are not equivalent because ADP-ribosylation occurs on only one subunit. Modified Fe protein retains the native $[Fe_4S_4]$ cluster, which can be chemically oxidized and reduced. It also retains the oxygen lability of the active Fe protein. Although ADP-ribosylated Fe protein cannot hydrolyze MgATP, it still has the ability to bind MgATP and to undergo the conformational change that gives access of the $[Fe_4S_4]$ cluster to chelators (15). It can also act in a role in synthesis and insertion of FeMo-co into MoFe protein (16).

The genes encoding DRAT (*draT*) and DRAG (*draG*) are cotranscribed from a non-*nif* operon, which includes a third gene (*draB*) of unknown function. The configuration of the *draTGB* operon is conserved in *A. brasilense* (Y. Zhang, unpublished results) and *A. lipoferum* (17). *R. capsulatus*, however, lacks *draB* (18). DRAT acts as a 30-kDa monomer with high specificity toward oxidized, MgADP-bound Fe protein, possessing no measurable activity with other arginine residues or water as the ADP-ribose acceptor (15,19). The amino acid sequence of DRAT is not highly similar to those of the bacterial toxins, but the structural domains are expected to be similar and some key residues are conserved. Surprisingly, the Fe proteins from *K. pneumoniae* and *A. vinelandii* (which lack the *dra* operon) are better substrates for *R. rubrum* DRAT than the *R. rubrum* Fe protein itself. There are no measurable reverse or glycohydrolytic reactions catalyzed by DRAT. The removal of the ADP-ribose group is instead catalyzed by dinitrogenase reductase-activating glycohydrolase (DRAG), which restores fully active Fe protein with an intact Arg101 side chain. DRAG is a 32-kDa monomeric binuclear manganese enzyme that is capable of cleaving the α-N-glycosidic bond of a number of analogs of ADP-ribosylarginine (15). However, only the reduced, MgATP-bound form (not the MgADP-bound or nucleotide-free forms) of ADP-ribosylated Fe protein is a substrate for DRAG (15,19). Although the exact modes of interaction of DRAT and DRAG with Fe protein are unknown, it is believed that each binds the same surface of Fe protein as does MoFe protein, as evidenced by the inhibition of cellular nitrogenase activity by overexpressed DRAT (20).

Although the means by which DRAT and DRAG are each regulated are not well understood, it is known that the activity of each enzyme is regulated in vivo (21,22). Because the in vivo activation and inactivation rates are reflected by in vitro assay rates using purified components, it is believed that the

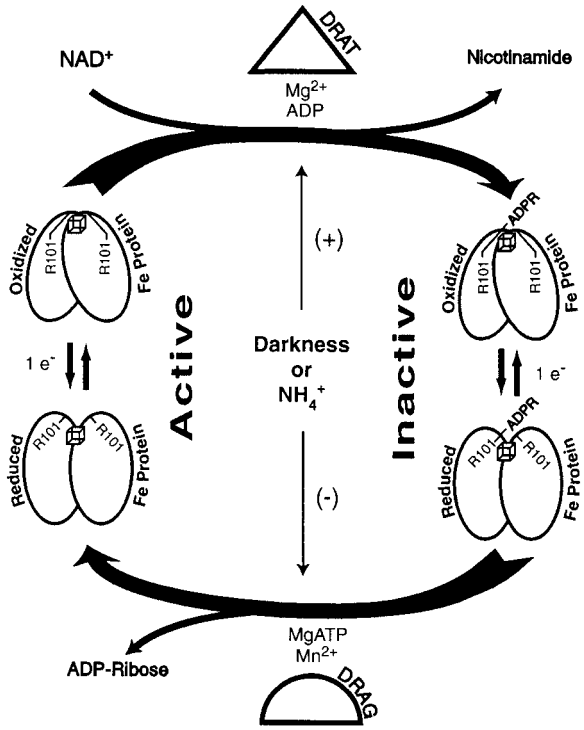

FIGURE 2 Model of in vivo nitrogenase Fe protein regulation in *Rhodospirillum rubrum* by reversible ADP-ribosylation. Small molecules required for dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase-activating glycohydrolase (DRAG) activities in vitro are shown beside the enzyme symbols. Adapted from (19).

regulatory signals involve either negative effectors or known assay components. As noted above, DRAT and DRAG have opposite specificities for MgADP- and MgATP-bound Fe protein. However, cellular fluctuations in ATP and ADP levels during inactivation/activation cycles are insufficient to account for the dramatic nitrogenase activity regulation (15). The recently demonstrated sensitivity of DRAT and DRAG toward the redox state of Fe protein suggests the possibility that DRAT and DRAG may be regulated by sensing the cellular energy and redox status directly from the state of Fe protein (19). The cellular NAD^+ concentration has also been suggested as a possible positive effector for DRAT (23). Apparently, unregulated variants of DRAG have altered divalent cation affinities (K. Kim, unpublished results), suggesting a potential means of regulation by weak, reversible binding of Fe^{2+} or Mn^{2+} ion in DRAG.

Although the nitrogenase-inactivating conditions of nitrogen sufficiency (NH_4^+) and energy limitation (darkness) give rise to convergent signal transduction pathways, it remains unclear where the pathways converge. Inhibition of glutamine synthetase perturbs both responses, suggesting an intermediary role for glutamine (21). However, the cellular concentration of glutamine is relatively unaffected by the modification and demodification of Fe protein (24). Also, genetic perturbations of nitrogen control genes (*glnB, ntrBC*) yield results that do not support the model of closely related signal transduction pathways because the effects on ammonia response appear to be independent of the darkness response (25, Y. Zhang, unpublished results).

The response of DRAT and DRAG activities to exogenous inactivation effectors is not species specific. Plasmid-borne *draTG* genes from *A. brasilense* restored the wild-type phenotype to *dra* mutants of *R. rubrum* (26), so that Fe protein was inactivated in response to darkness, but not anaerobicity. Transformants of *K. pneumoniae* carrying a plasmid containing *draTGB* from *R. rubrum* have been shown to reversibly ADP-ribosylate Fe protein in response to exogenous ammonium (C. Halbleib, unpublished results). Thus, DRAT and DRAG appear to sense a global regulatory signal, present even in nitrogen-fixing bacteria that lack the *dra* operon.

The multiple layers and redundant mechanisms of nitrogenase regulation attest to the biological necessity of proper control of biological nitrogen fixation (27). Future research in the nitrogen fixation field will be required to clarify the regulatory mechanisms not only in the paradigm systems, but also in those organisms (e.g., legume-associated microbes) whose contributions to the global nitrogen cycle are most critical.

LITERATURE CITED

1. Socolow, R. H. (1999) Nitrogen management and the future of food: lessons from the management of energy and carbon. Proc. Natl. Acad. Sci. U.S.A. 96: 6001–6008.

2. Simpson, F. B. & Burris, R. H. (1984) A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science (Washington, DC) 224: 1095–1097.

3. Burgess, B. K. & Lowe, D. J. (1996) Mechanism of molybdenum nitrogenase. Chem. Rev. 96: 2983–3011.

4. Dean, D. R. & Jacobsen, M. R. (1992) Biochemical genetics of nitrogenase. In: Biological Nitrogen Fixation (Stacey, G., Burris, R. H. & Evans, H. J., eds.), pp. 763–834. Chapman and Hall, New York, NY.

5. Howard, J. B. & Rees, D. C. (1996) Structural basis of biological nitrogen fixation. Chem. Rev. 96: 2965–2982.

6. Peters, J. W., Stowell, M. H., Soltis, S. M., Finnegan, M. G., Johnson, M. K. & Rees, D. C. (1997) Redox-dependent structural changes in the nitrogenase P-cluster. Biochemistry 36: 1181–1187.

7. Angove, H. C., Yoo, S. J., Burgess, B. K. & Münck, E. (1997) Mossbauer and EPR evidence for an all-ferrous Fe_4S_4 cluster with S = 4 in the Fe protein of nitrogenase. J. Am. Chem. Soc. 119: 8730–8731.

8. Schindelin, H., Kisker, C., Schlessman, J. L., Howard, J. B. & Rees, D. C. (1997) Structure of ADP x AlF_4^--stabilized nitrogenase complex and its implications for signal transduction. Nature (Lond.) 387: 370–376.

9. Lanzilotta, W. N. & Seefeldt, L. C. (1997) Changes in the midpoint potentials of the nitrogenase metal centers as a result of iron protein-molybdenum-iron protein complex formation. Biochemistry 36: 12976–12983.

10. Shah, V. K., Stacey, G. & Brill, W. J. (1983) Electron transport to nitrogenase. Purification and characterization of pyruvate:flavodoxin oxidoreductase. The *nifJ* gene product. J. Biol. Chem. 258: 12064–12068.

11. Merrick, M. J. (1992) Regulation of nitrogen fixation genes in free-living and symbiotic bacteria. In: Biological Nitrogen Fixation (Stacey, G., Burris, R. H. & Evans, H. J., eds.), pp. 835–876, Chapman and Hall, New York, NY.

12. Hill, S., Austin, S., Eydmann, T., Jones, T. & Dixon, R. (1996) *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. Proc. Natl. Acad. Sci. U.S.A. 93: 2143–2148.

13. Krey, R., Pühler, A. & Klipp, W. (1992) A defined amino acid exchange close to the putative nucleotide binding site is responsible for an oxygen-tolerant variant of the *Rhizobium meliloti* NifA protein. Mol. Gen. Genet. 234: 433–441.

14. Klipp, W. & Paschen, A. (1998) Duplication of a transcriptional regulator as a mechanism for genetic control: NifAI and NifAII from *Rhodobacter capsulatus* differ in their susceptibility to ammonium control. In: Biological Nitrogen Fixation for the 21st Century (Elmerich, C., Kondorosi, A. & Newton, W. E., eds.), p. 131. Kluwer Academic Publishers, Dordrecht, The Netherlands.

15. Ludden, P. W. & Roberts, G. P. (1989) Regulation of nitrogenase activity by reversible ADP ribosylation. Curr. Top. Cell. Regul. 30: 23–56.

16. Shah, V. K., Hoover, T. R., Imperial, J., Paustian, T. D., Roberts, G. P. & Ludden, P. W. (1988) Role of *nif* gene products and homocitrate in the biosynthesis of iron-molybdenum cofactor. In: Nitrogen Fixation: Hundred Years After (Bothe, H., deBruijn, F. J. & Newton, W. E., eds.), pp. 115–120. Gustav Fischer, Stuttgart, Germany.

17. Inoue, A., Shigematsu, T., Hidaka, M., Masaki, H. & Uozumi, T. (1996) Cloning, sequencing and transcriptional regulation of the *draT* and *draG* genes of *Azospirillum lipoferum* FS. Gene 170: 101–106.

18. Masepohl, B., Krey, R. & Klipp, W. (1993) The *draTG* gene region of *Rhodobacter capsulatus* is required for post-translational regulation of both the molybdenum and the alternative nitrogenase. J. Gen. Microbiol. 139: 2667–2675.

19. Halbleib, C. M., Zhang, Y. & Ludden, P. W. (2000) Regulation of dinitrogenase reductase ADP-ribosyltransferase and dinitrogenase reductase-activating glycohydrolase by a redox-dependent conformational change of nitrogenase Fe protein. J. Biol. Chem. 275: 3493–3500.

20. Grunwald, S. K., Lies, D. P., Roberts, G. P. & Ludden, P. W. (1995) Posttranslational regulation of nitrogenase in *Rhodospirillum rubrum* strains overexpressing the regulatory enzymes dinitrogenase reductase ADP-ribosyltransferase and dinitrogenase reductase activating glycohydrolase. J. Bacteriol. 177: 628–635.

21. Kanemoto, R. H. & Ludden, P. W. (1984) Effect of ammonia, darkness, and phenazine methosulfate on whole-cell nitrogenase activity and Fe protein modification in *Rhodospirillum rubrum*. J. Bacteriol. 158: 713–720.

22. Liang, J. H., Nielsen, G. M., Lies, D. P., Burris, R. H., Roberts, G. P. & Ludden, P. W. (1991) Mutations in the *draT* and *draG* genes of *Rhodospirillum rubrum* result in loss of regulation of nitrogenase by reversible ADP-ribosylation. J. Bacteriol. 173: 6903–6909.

23. Norén, A., Soliman, A. & Nordlund, S. (1997) The role of NAD^+ as a signal during nitrogenase switch-off in *Rhodospirillum rubrum*. Biochem. J. 322: 829–832.

24. Kanemoto, R. H. & Ludden, P. W. (1987) Amino acid concentrations in *Rhodospirillum rubrum* during expression and switch-off of nitrogenase activity. J. Bacteriol. 169: 3035–3043.

25. Zhang, Y., Cummings, A. D., Burris, R. H., Ludden, P. W. & Roberts, G. P. (1995) Effect of an *ntrBC* mutation on the posttranslational regulation of nitrogenase activity in *Rhodospirillum rubrum*. J. Bacteriol. 177: 5322–5326.

26. Zhang, Y., Burris, R. H. & Roberts, G. P. (1992) Cloning, sequencing, mutagenesis, and functional characterization of *draT* and *draG* genes from *Azospirillum brasilense*. J. Bacteriol. 174: 3364–3369.

27. Fischer, H. M. (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. 58: 352–386.