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# Regulation of Nitrogen Fixation in the Phototrophic Purple Bacterium *Rhodobacter capsulatus*

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## Abstract

**In *R. capsulatus* synthesis and activity of the molybdenum and the alternative nitrogenase is controlled at three levels by the environmental factors ammonium, molybdenum, light, and oxygen. At the first level, transcription of the *nifA1*, *nifA2*, and *anfA* genes – which encode the transcriptional activators of all other *nif* and *anf* genes, respectively – is controlled by the Ntr system in dependence on ammonium availability. Mutations in *glnB* (coding for the signal transduction protein PII) result in significant expression of *nifA* and *anfA* in the presence of ammonium. In contrast to GlnB, the PII-paralogue GlnK is not involved in the Ntr signal transduction mechanism. In addition to ammonium control, transcription of *anfA* is inhibited by traces of molybdenum via the molybdate-dependent repressor proteins MopA and MopB. At the second level of regulation, activity of NifA1, NifA2, and AnfA is inhibited by ammonium in an NtrC-independent manner. This post-translational ammonium control of NifA activity is partially released in the absence of GlnK, and completely abolished in a *glnB*/*glnK* double mutant. In contrast, AnfA activity is still inhibited by ammonium in the *glnB*/*glnK* mutant background. At the third level of regulation, both GlnB and GlnK as well as the (methyl)-ammonium transporter AmtB are involved in ammonium control of the DraT/DraG system, which mediates reversible ADP-ribosylation of both nitrogenase reductases (NifH and AnfH) in response to changes in ammonium availability or light intensity. Most remarkably, in a *glnB*/*glnK* double mutant ammonium control of the molybdenum (but not of the alternative) nitrogenase is completely relieved, leading to synthesis of active nitrogenase in the presence of high concentrations of ammonium.**

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## Introduction

*Rhodobacter capsulatus* is a non-sulfur phototrophic purple bacterium which can grow with a variety of different nitrogen sources, such as ammonium, almost all amino acids, purines (e.g. xanthine, hypoxanthine), urea, polyamines (e.g. putrescine, spermidine) and with molecular nitrogen (Masepohl and Klipp, 1996; Moreno-Vivian *et al*., 1992; Leimkühler *et al*., 1998). *R. capsulatus* is able to fix atmospheric dinitrogen either by a molybdenum-containing nitrogenase (*nif*-encoded) or by an alternative iron-only nitrogenase (*anf*-encoded; Schneider *et al*., 1991; Schüddekopf *et al*., 1993). The organization of *nif* and *anf* genes has been reviewed recently (Masepohl and Klipp, 1996).

Like in many other bacteria, ammonium is the preferred nitrogen source in *R. capsulatus*, and consequently, the highly energy-demanding nitrogen fixation process is regulated by ammonium availability. In addition, $N_2$ fixation is controlled by the environmental factors molybdenum, light, and oxygen. Although the regulatory influence of these factors is taken into consideration, this review will mainly focus on the regulation of nitrogen fixation by ammonium.

The cellular nitrogen status controls expression of nitrogen fixation genes, as well as synthesis and activity of both nitrogenases at three levels (Figure 1). *R. capsulatus* measures the cellular nitrogen status by a nitrogen regulation (Ntr) system similar to that of enteric bacteria (see below). Under conditions of nitrogen depletion, NtrC activates transcription of the re-gulatory *nifA1*, *nifA2*, and *anfA* genes (level 1). In turn, in the absence of ammonium NifA and AnfA activate expression of all other *nif* and *anf* genes resulting in synthesis of nitrogenase. In addition to ammonium control of NtrC-mediated transcriptional regulation, ammonium leads to inhibition of NifA and AnfA activity (level 2) and to DraT/G-mediated reversible inactivation of both nitrogenases (level 3).

## Review

### The Nitrogen Regulation (Ntr) System of *R. capsulatus*

In enteric bacteria a general nitrogen regulation system that senses the cellular nitrogen status is responsible for controlling the expression of many genes concerned with nitrogen metabolism (for a review, see Ninfa and Atkinson, 2000). This Ntr system comprises five gene products: a bifunctional uridylyltransferase/uridylyl-removing enzyme encoded by *glnD*, a trimeric signal transduction protein (PII) encoded by *glnB*, a PII-paralogue encoded by *glnK*, and a two-component regulatory system encoded by *ntrB-ntrC*. In response

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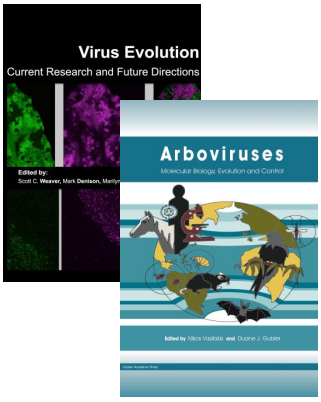


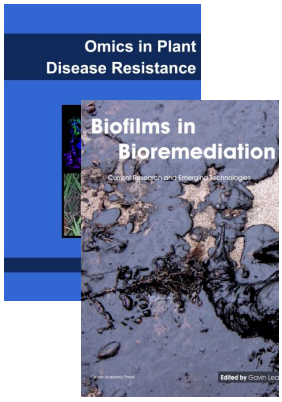


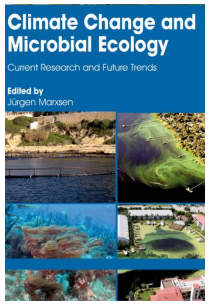


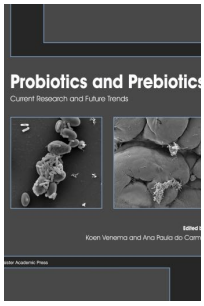


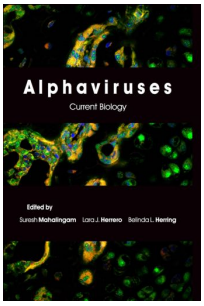



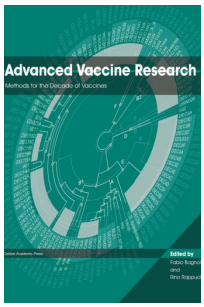


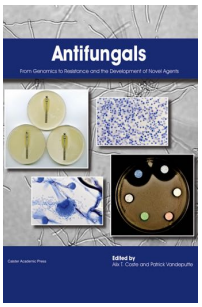


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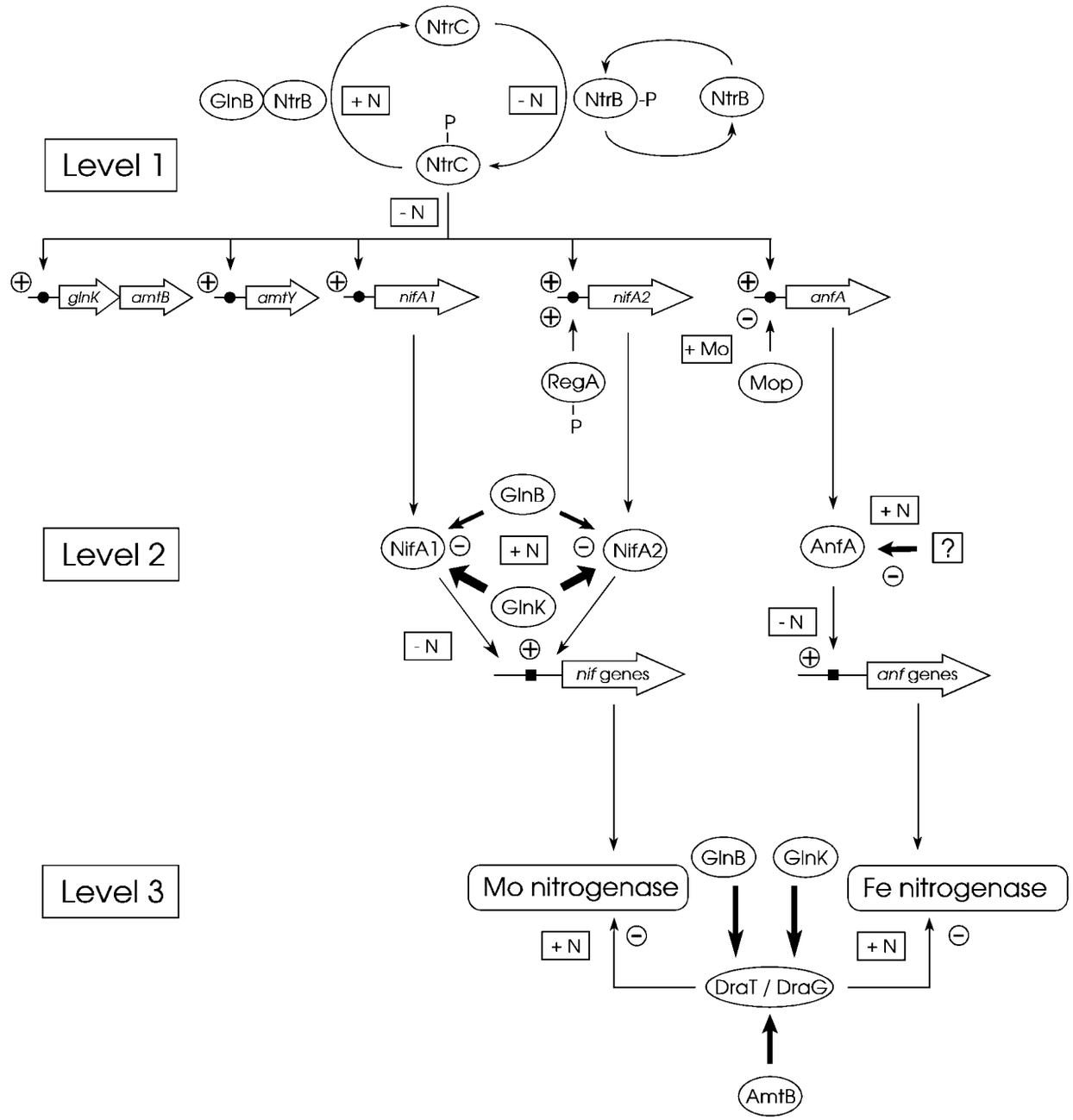


Figure 1. Model of the nitrogen fixation regulon in *R. capsulatus*. The model decribes the regulatory cascade controlling expression of nitrogen fixation genes and activity of the molybdenum nitrogenase (Mo nitrogenase, *nif*-encoded) and the alternative nitrogenase (Fe nitrogenase, *anf*-encoded) in response to ammonium availability. Nitrogenase-repressing conditions (presence of ammonium) or -derepressing conditions are symbolized by [+N] or [– N]. Presence of molybdenum is indicated by [+ Mo]. For description of the regulatory effects of other environmental factors, see text. Filled circles symbolize $\sigma^{70}$-dependent promoters activated by NtrC~P, and filled squares mark $\sigma^{54}$-dependent promoters activated by NifA or AnfA. Yeast two-hybrid studies suggest direct GlnB-NtrB, GlnB-NifA1, GlnB-NifA2, GlnB-DraT, GlnK-NifA1, and GlnK-NifA2 interactions.

to the cellular glutamine/2-ketoglutarate ratio, PII is regulated by reversible GlnD-mediated uridylylation at tyrosine residue 51. When cells are N-limited, PII is uridylylated, and thus is unable to interact with the sensor kinase NtrB. Under these conditions NtrB promotes the phosphorylation of the response regulator NtrC, and NtrC~P can function as a transcriptional activator of its target genes.

*R. capsulatus* contains genes homologous to *glnD*, *glnB*, *ntrB*, and *ntrC*, and therefore, regulatory mechanisms similar to those of the Ntr system of enteric bacteria have been proposed (Hübner *et al*., 1991; Kranz and Foster-Hartnett, 1990; Masepohl and Klipp, 1996). This assumption was verified by *in vitro* reconstitution of the NtrB/NtrC system (Cullen *et al*., 1996). Like many other bacteria, *R. capsulatus* contains a second *glnB*-like gene (*glnK*), which is closely linked to the *amtB* gene coding for a putative (methyl)-ammonium transporter (Drepper *et al*., 2000; Klipp *et al*., 2000). Both GlnB and GlnK contain the conserved tyrosine residue at position 51, suggesting that the activity of the two signal transduction proteins is regulated by GlnD-mediated uridylylation.

Under conditions of nitrogen starvation *R. capsulatus* NtrC~P activates transcription of a number of genes including *glnB-glnA*, *glnK-amtB*, *amtY*, *nifA1*, *nifA2*, *anfA*, *mopA-modABCD*, and the *ure* operon (Figure 1; Bowman and Kranz, 1998; S. Groß and W. Klipp, unpublished; Klipp *et al*., 2000; Masepohl *et al*., 2001). These genes code for PII, glutamine synthetase (GlnA), the PII-paralogue GlnK, two putative ammonium transporters (AmtB, AmtY), the transcriptional activators of the two nitrogenase systems (NifA1, NifA2, AnfA), a molybdenum repressor of the Anf system (MopA), a high affinity molybdate uptake system (ModABCD), and urease, respectively. However, mutations in *R. capsulatus ntrC* do not result in a ''classical'' Ntr phenotype as found in enterobacteria, since *R. capsulatus* NtrC is not required for the utilization of amino acids as an N source (Keuntje *et al*., 1995; Moreno-Vivian *et al*., 1992). In contrast to all other members of the NtrC family, *R. capsulatus* NtrC does not require $\sigma^{54}$ (NtrA, RpoN) but instead activates transcription of the above mentioned genes together with RNA polymerase (RNA-P) containing the $\sigma^{70}$-like house-keeping sigma factor (Bowman and Kranz, 1998; Foster-Hartnett *et al*., 1994).

*R. capsulatus* contains two functional copies of *nifA* (Figure 1. *nifA1* and *nifA2*), which can substitute for each other (Masepohl *et al*., 1988). Both NifA proteins are almost identical to each other, and differ only in their 19 N-terminal amino acid residues (Hübner *et al*., 1993; Paschen *et al*., 2001). In the absence of ammonium both NifA1 and NifA2 activate expression of all other *nif* genes in concert with RNA-P containing $\sigma^{54}$, leading to synthesis of the molybdenum nitrogenase.

NtrC-mediated expression of *R. capsulatus anfA* under conditions of nitrogen limitation is inhibited by traces of molybdate (Kutsche *et al*., 1996; Wang *et al*., 1993). This molybdate-dependent inhibition is released in mutants carrying either lesions in the high-affinity molybdenum uptake system (*modABCD*) or a double deletion of *mopA* and *mopB*, which encode ModE-like transcriptional repressor proteins binding to dyad symmetric promoter elements that overlap the transcription start (Pau *et al*., 1997). Although MopA and MopB can substitute for each other in repression of the *anfA* and the *mopA-modABCD* operon, only MopB (but not MopA) is essential for the molybdate-dependent expression of the *dorCDA* operon encoding dimethylsulfoxide reductase in *R. capsulatus* (Solomon *et al*., 2000). In the absence of ammonium AnfA activates expression of the *anfHDGK* operon and of several *nif* genes required for the alternative nitrogenase in concert with RNA-P containing $\sigma^{54}$, resulting in synthesis of the iron-only nitrogenase (Schüddekopf *et al*., 1993).

In enteric bacteria NtrB (in concert with GlnB or GlnK) promotes the dephosphorylation of NtrC under conditions of N-excess (Ninfa and Atkinson, 2000), and as a result, the activator properties of NtrC are diminished and expression of NtrC-dependent promoters is switched off. In *R. capsulatus*, a *glnB* mutant shows significant expression of NtrC-dependent promoters in the presence of ammonium indicating that GlnK is unable to fully substitute for GlnB in regulating NtrB activity. This result was corroborated by analyzing protein-protein interactions using the yeast two-hybrid system. A strong interaction between GlnB and NtrB could be shown, whereas GlnK was unable to interact with NtrB (A. Pawlowski, S. Groß, and W. Klipp, unpublished).

**Regulation of NifA and AnfA Activity**

A *glnB* mutation results in significant expression of *nifA1* and *nifA2* in the presence of ammonium, but NifA-mediated *nifH* transcription is still inhibited under these conditions (Table 1; Drepper *et al*., 2000). This inhibition is independent of NtrC as de-monstrated by analysis of *R. capsulatus ntrC* mutant strains overexpressing *nifA1* or *nifA2* from a constitutive promoter (Drepper *et al*., 2000; Hübner *et al*., 1993; Kern *et al*., 1998). Two lines of evidence suggest that ammonium regulation of *nifH* gene expression occurs at the post-translational level controlling NifA activity (Figure 1; level 2). (i) *R. capsulatus* strains overexpressing *nifA1* accumulate high levels of NifA1 both in the absence or presence of ammonium as shown by Western analysis using NifA1-specific antibodies (Drepper *et al*., 2000). As shown by time course experiments, NifA1 is highly stable in the presence of ammonium suggesting that inactivation of NifA in response to ammonium is not due to proteolytic degradation of NifA. (ii) Single mutations in the N-terminal domain of NifA1 resulted in ammonium-tolerant NifA mutant proteins mediating *nifH* gene expression under N-excess (Paschen *et al*., 2001).

In contrast to lesions in *glnB*, a *glnK* mutation leads to low level expression of *nifH* in the presence of ammonium (in *R. capsulatus* strains constitutively expressing *nifA*), indicating that NifA activity partially escapes ammonium inhibition in this mutant background (Table 1). Furthermore, in a *glnB*/*glnK* double mutant ammonium control of NifA activity is completely abolished. Therefore, GlnB can only partially substitute for GlnK in mediating ammonium inhibition of NifA activity. As shown by yeast two-hybrid studies, both GlnB and GlnK interact with NifA1 and NifA2 (S. Groß, and W. Klipp, unpublished), suggesting that ammonium inhibition of NifA activity is directly mediated by these signal transduction proteins.

A *glnB* mutation results in constitutive expression of *anfA* (Drepper *et al*., 2000). In contrast to the *nif*

Table 1. Effect of GlnB and GlnK on the expression and activity of both nitrogenase systems.

| | *glnB*$^-$ | *glnK*$^-$ | *glnB*$^-$/*glnK*$^-$ |
|---|---|---|---|
| *Level 1:* | | | |
| transcription | | | |
| – of *nifA1* and *nifA2* | constitutive | N-regulated | constitutive |
| – of *anfA* | constitutive | N-regulated | constitutive |
| *Level 2:* | | | |
| post-translational control[(a)] | | | |
| – of NifA activity | + | ± | – |
| – of AnfA activity | + | + | + |
| *Level 3:* | | | |
| ADP-ribosylation[(b)] | | | |
| – of NifH | ± | + | – |
| – of AnfH | ± | + | – |
| Activity[(c)] | | | |
| – of the Mo nitrogenase | – | – | + |
| – of the Fe nitrogenase | – | – | – |

[(a)] Analysis of post-translational control of NifA and AnfA activity requires NtrC-independent expression of *nifA* and *anfA* from constitutive promoters. Activity of the activator proteins is defined by the ability to mediate transcription of *nifH-lacZ* or *anfH-lacZ* fusions, or can be followed by Western analysis using nitrogenase reductase-specific antibodies. Post-translational control of the activity of the transcriptional activators is present (+), partially abolished (±), or completely abolished (–).
[(b)] DraT-mediated modification of nitrogenase reductase is not affected (+), reduced (±), or completely abolished (–) as measured by the appearance of the ADP-ribosylated form of NifH and AnfH.
[(c)] Nitrogenase activity was measured by the acetylene reduction assay in the presence of ammonium.

regulon, *anfH* expression is still inhibited by ammonium in a *glnB*/*glnK* double mutant. This inhibition is also independent of NtrC, and therefore, ammonium control of AnfA activity involves a GlnB/GlnK-independent regulatory mechanism (T. Drepper, and W. Klipp, unpublished).

### DraTG-Mediated Post-Translational Regulation of Nitrogenase Activity

The activity of nitrogenase is controlled by reversible ADP-ribosylation of NifH and AnfH mediated by DraT and DraG in response to ammonium or darkness (Figure 1; level 3; Masepohl *et al.*, 1993). Inactivation of dinitrogenase reductase by ADP-ribosylation is catalyzed by the *draT* gene product (dinitrogenase reductase ADP-ribosyltransferase), whereas restoration of nitrogenase activity is performed by the DraG protein (dinitrogenase reductase-activating glycohydrolase). In addition, nitrogenase activity is regulated by a second DraT/G-independent mechanism (Förster *et al.*, 1999; Pierrard *et al.*, 1993; Yakunin *et al.*, 1999). In a *glnB*/*glnK* double mutant both post-translational ammonium control mechanisms of nitrogenase activity are relieved (Table 1; Klipp *et al.*, 2000). In contrast, ADP-ribosylation of NifH and AnfH in response to darkness is not affected in a *glnB*/*glnK* double mutant.

As mentioned above, *R. capsulatus* contains two genes (*amtB*, *amtY*) coding for putative (methyl)-ammonium transporters. It has been suggested that AmtB could act as a signal transducer for ammonium availability (Thomas *et al.*, 2000). Both *R. capsulatus glnK-amtB* and *amtY* are part of the NtrC regulon (Figure 1; Klipp *et al.*, 2000; S. Groß, and W. Klipp, unpublished). An *amtB* mutant (but not an *amtY* mutant), in addition to being ineffective in methyl-ammonium uptake, is also completely defective in carrying out ADP-ribosylation of NifH in response to ammonium addition (Yakunin and Hallenbeck, 2000). As shown for the *glnB*/*glnK* double mutant, ADP-ribosylation in response to darkness is also unaltered in the *amtB* mutant. However, the *amtY* mutant shows higher levels of ADP-ribosylation than the wild type strain in response to the addition of the same amount of ammonium, suggesting that the lack of AmtY shunts additional ammonium through AmtB, and thus rendering this strain more sensitive to this effector. Interestingly, an *amtB*/*amtY* double mutant exhibited only low activity of the molybdenum nitrogenase (about 20% compared to the wild type) under nitrogen-limiting conditions. This low activity was reflected by a significantly reduced amount of nitrogenase in the mutant (S. Groß, T. Drepper, and W. Klipp, unpublished).

### Linkage of Nitrogen Fixation and Photosynthesis

The RegB/RegA signal transduction system is a global two-component regulatory system that activates photosynthesis, carbon assimilation, and nitrogen fixation genes, and represses hydrogenase structural genes and its own expression (Elsen *et al.*, 2000). RegA indirectly activates nitrogenase synthesis by binding to and activating the expression of *nifA2* but not of *nifA1*. Thus, although NtrC is absolutely required for *nifA2* expression, RegA acts as a coactivator of *nifA2*.

The *regA* gene is cotranscribed with *hvrA*, which encodes a histone-like protein homologous to *E. coli* H-NS. *R. capsulatus* HvrA is involved in low-light activation of the photosynthetic apparatus (Buggy *et al.*, 1994). A mutation in *hvrA* releases both ammonium and oxygen control of *nifH* expression in an *R. capsulatus* strain constitutively expressing *nifA1* (Kern *et al.*, 1998). This regulatory effect of the HvrA protein is mediated by binding of HvrA to the *nifH* promoter as demonstrated

by gel shift experiments (K. Raabe and W. Klipp, unpublished).

## Conclusions

This review describes mechanisms regulating synthesis and activity of the molybdenum-containing and the alternative nitrogenase in *R. capsulatus* in response to ammonium availability and other environmental factors. Regulation occurs at (at least) three levels including (i) transcriptional activation of the regulatory genes *nifA1*, *nifA2* and *anfA*, (ii) post-translational regulation of NifA and AnfA activity, and (iii) post-translational control of nitrogenase activity by reversible modification of NifH and AnfH. The complexity of these regulatory mechanisms is reflected by the number and diversity of regulatory proteins involved in control of nitrogen fixation. Among these are two two-component regulatory systems (NtrB/NtrC, RegB/RegA), two signal transduction proteins (GlnB, GlnK), three specific transcriptional activator proteins (NifA1, NifA2, AnfA), two molybdate-dependent repressor proteins (MopA, MopB), an ADP-ribosyl-transferase/glycohydrolase system (DraT, DraG), two (methyl)-ammonium transporter proteins (AmtB, AmtY), and a histone-like protein (HvrA).

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