Regulation of Nitrogen Fixation in Photosynthetic Purple Nonsulfur Bacteria

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Abstract Biological nitrogen fixation (BNF) is the nitrogenase-catalyzed process in which dinitrogen (N_2) is reduced to ammonia (NH_3), the preferred nitrogen source in bacteria. All N_2-fixing or diazotrophic bacteria have molybdenum-nitrogenases. In addition, some diazotrophs possess one or two alternative Mo-free nitrogenases, namely a vanadium and/or an iron-only nitrogenase, which are less efficient than Mo-nitrogenase in terms of ATP-consumption per N_2 reduced. BNF is widespread in photosynthetic purple nonsulfur bacteria, which are capable of using light energy to generate ATP for nitrogenase activity. This review focusses on BNF regulation in the purple nonsulfur bacteria *Rhodobacter capsulatus*, *Rhodopseudomonas palustris*, and *Rhodospirillum rubrum*. *Rp. palustris* is one of few diazotrophs having both alternative nitrogenases, whereas *Rb. capsulatus* and *Rs. rubrum* have Fe-nitrogenases but no V-nitrogenase. Purple nonsulfur bacteria regulate BNF in response to ammonium, molybdenum, iron, oxygen, and light. BNF regulation involves common regulatory proteins including the two-component nitrogen regulatory system NtrB-NtrC, the transcriptional activator NifA, the nitrogen-specific sigma factor RpoN, the DraT-DraG system for posttranslational nitrogenase regulation, and at least two PII signal transduction proteins. When ammonium is limiting, NtrB phosphorylates NtrC, which in turn activates expression of *nifA* and other BNF-related genes. NifA and its homologs VnfA and AnfA activate expression of Mo, V, and Fe-nitrogenase genes, respectively, in concert with RpoN. DraT mediates nitrogenase switch-off by ADP-ribosylation upon ammonium addition or light deprivation, the latter condition causing energy depletion. DraG reactivates nitrogenase upon ammonium consumption or reillumination. PII-like proteins integrate the cellular nitrogen, carbon, and energy levels, and control activity of NtrB, NifA, DraT, and DraG. Beside these similarities in BNF regulation, there are species-specific differences. NifA is active as synthesized in *Rb. capsulatus*, but requires activation by PII in *Rp. palustris* and *Rs. rubrum*. Reversible ADP-ribosylation is the only mechanism regulating nitrogenase in *Rs. rubrum*, whereas *Rb. capsulatus* and *Rp. palustris* have additional ADP-ribosylation-independent mechanisms. Last but not least, molybdate directly

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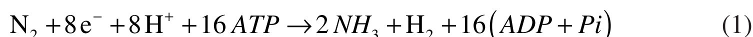
represses *anfA* transcription and hence, Fe-nitrogenase expression in *Rb. capsulatus*, whereas expression of the alternative nitrogenases in *Rp. palustris* and *Rs. rubrum* respond to Mo-nitrogenase activity rather than to molybdate directly.

Keywords Nitrogen fixation • Nitrogenase • Rhodobacter • Rhodopseudomonas • Rhodospirillum • Regulation

Introduction to Biological Nitrogen Fixation

Growth of all eukaryotes and most prokaryotes requires a fixed nitrogen source like ammonium, nitrate, or amino acids. Quite a few prokaryotes, however, can reduce the chemically inert molecular dinitrogen (N_2) to ammonia (NH_3) by a process called biological nitrogen fixation (BNF). No eukaryote is capable of directly fixing N_2 but several eukaryotes like legumes and termites make indirectly use of N_2 by forming symbiotic associations with nitrogen-fixing bacteria or archaea (Hongoh 2010; Oldroyd 2013). BNF depends on complex metalloenzymes called nitrogenases, which catalyze the overall reaction shown in Eq. (1), and require a theoretical minimum of 16 ATP per N_2 reduced (Igarashi and Seefeldt 2003). In addition to N_2 reduction, nitrogenases produce hydrogen gas (H_2) in an obligate side reaction and, in the absence of N_2, nitrogenases exclusively reduce protons to H_2. Nitrogenase-catalyzed production of H_2 as a biofuel has extensively been studied in photosynthetic bacteria (Adessi et al. 2016; Heiniger et al. 2012; Huang et al. 2010; McKinlay and Harwood 2010; Rey et al. 2007) and is discussed in more detail elsewhere in this book series.



$$\mathrm{N_2} + 8\,\mathrm{e}^- + 8\,\mathrm{H}^+ + 16\,ATP \rightarrow 2\,NH_3 + \mathrm{H_2} + 16\left(ADP + Pi\right) \tag{1}$$

All N_2-fixing (diazotrophic) bacteria and archaea possess a molybdenum-dependent nitrogenase containing the catalytic iron-molybdenum cofactor, FeMoco (Zhang and Gladyshev 2008). In addition to Mo-nitrogenase, some diazotrophs synthesize alternative Mo-free nitrogenases containing the iron-vanadium cofactor, FeVco, or the iron-only cofactor, FeFeco (Dos Santos et al. 2012; McGlynn et al. 2013). The three nitrogenases are encoded by distinct gene sets, namely *nifHDK* (Mo-nitrogenase), *vnfHDGK* (V-nitrogenase), and *anfHDGK* (Fe-nitrogenase). Beside the structural nitrogenase genes, diazotrophs have numerous genes involved in cofactor biosynthesis, electron supply, and regulation (see below).

In addition to N_2 and protons, nitrogenases reduce the artificial substrate acetylene (C_2H_2). Mo-nitrogenases reduce acetylene to ethylene (C_2H_4), whereas alternative nitrogenases reduce acetylene to ethylene and in part, to ethane (C_2H_6). In the laboratory, gas chromatography-based acetylene reduction assays have been established to quantify nitrogenase activity and to detect activity of alternative nitrogenases (Dilworth et al. 1988).

Mo, V, and Fe-nitrogenases consist of two components each, the catalytic dinitrogenases and the dinitrogenase reductases, the latter serving as the ultimate electron donors to their respective dinitrogenases (Curatti and Rubio 2014; Hu and Ribbe 2016). The three dinitrogenase reductases are collectively called Fe-proteins (homodimers of NifH, VnfH, and AnfH), all of which coordinate one [4Fe-4S] cluster involved in electron transfer. The Mo, V, and Fe-dinitrogenases are called MoFe-protein (heterotetramer of NifDK containing two FeMoco), VFe-protein (heterohexamer of VnfDGK containing two FeVco), and FeFe-protein (heterohexamer of AnfDGK containing two FeFeco), respectively. In addition to the catalytic cofactors, the dinitrogenases contain two P-clusters (see below) involved in electron transfer from the Fe-proteins to the catalytic cofactors.

Biosynthesis of the Mo-nitrogenase cofactors ([4Fe-4S] cluster, P-cluster, and FeMoco) is complex and requires several *nif* gene products including NifU, NifS, NifB, NifV, NifE, NifN, NifH, NifD, and NifK as shown for *Klebsiella pneumoniae* and *Azotobacter vinelandii* (Curatti and Rubio 2014; Hu and Ribbe 2016; and the references therein). Briefly, NifU and NifS function as the scaffold protein and sulfur donor, respectively, for biosynthesis of [4Fe-4S] clusters, which are either inserted into apo-NifH or serve as building blocks for P-cluster and FeMoco formation. The P-cluster is formed in situ on the apo-NifDK protein, whereas the FeMoco is synthesized ex situ prior to insertion into the apo-NifDK protein. P-cluster biosynthesis starts with the transfer of two [4Fe-4S] clusters to the apo-NifDK protein followed by NifH-mediated reductive coupling to form the [8Fe-7S] or P-cluster. FeMoco biosynthesis starts with the coupling of two [4Fe-4S] clusters on NifB involving S-adenosylmethionine-dependent carbon (C) insertion to form an [8Fe-9S-C] cluster. This cluster is further processed on the NifEN scaffold by insertion of Mo and homocitrate (the product of homocitrate synthase, NifV) resulting in the [Mo-7Fe-9S-C-homocitrate] cluster or FeMoco, which is finally inserted into the apo-NifDK protein.

NifU, NifS, NifB, and NifV are required for activity of Mo, V, and Fe-nitrogenases in *A. vinelandii* indicating that the biosynthetic pathways of FeMoco, FeVco, and FeFeco overlap to a certain extent (Drummond et al. 1996; Kennedy and Dean 1992). Formation of FeVco involves the Vnf-specific NifEN homolog, VnfEN, instead of NifEN (Hu and Ribbe 2016; and the references therein). Possibly, the last steps of FeFeco biosynthesis occur in situ on the AnfDGK protein, since no NifEN homolog is required for Fe-nitrogenase activity (Schüddekopf et al. 1993).

Diazotrophs regulate BNF in response to several environmental factors including ammonium, molybdenum, iron, oxygen, and in case of photosynthetic bacteria, light. Since BNF is a highly energy-demanding process, diazotrophs typically induce nitrogenase expression only when ammonium, the product of BNF, is limiting. Mo-nitrogenase is more efficient than the alternative nitrogenases in terms of consumption of ATP and reductant per N_2 reduced (Hu et al. 2012; Schneider et al. 1997) and hence, expression of alternative nitrogenases is typically repressed as long as Mo-nitrogenase is active. Most bacteria possess *modABC* genes encoding

high-affinity ABC transporters, which support uptake of molybdate, the only bio-available form of molybdenum, under Mo-limiting conditions (Zhang and Gladyshev 2008; Zhang and Gladyshev 2010). All three nitrogenases are irreversibly damaged by oxygen (Blanchard and Hales1996; Chisnell et al. 1988; Gollan et al. 1993) and diazotrophs have evolved different strategies to cope with this problem.

Diazotrophic and non-diazotrophic bacteria utilize similar proteins to sense the cellular nitrogen status and to control nitrogen assimilation. Among these proteins are the bifunctional uridylyltransferase/uridylyl-removing enzyme GlnD, the PII signal transduction proteins GlnB and GlnK, the two-component regulatory system NtrB-NtrC, and the ammonium transporter AmtB, which are best characterized in the non-diazotrophic enterobacterium *Escherichia coli* (van Heeswijk et al. 2013; and the references therein).

Briefly, GlnD senses the cellular nitrogen status through the glutamine level (Jiang et al. 1998a). Under low glutamine levels (N-limiting conditions), GlnD modifies GlnB and GlnK by uridylylation of conserved tyrosine residues within their T-loops. Under high glutamine levels (N-replete conditions), GlnD catalyzes the reverse reaction by hydrolyzing GlnB-UMP and GlnK-UMP. Trimeric PII proteins can be fully uridylylated (PII-UMP_3), partially uridylylated (PII-UMP_2 or PII-UMP_1), or completely unmodified (PII). PII proteins directly sense the cellular carbon and energy status by binding 2-oxoglutarate (2OG) and ATP/ADP, respectively (Radchenko et al. 2013). 2OG joins nitrogen and carbon metabolism as it serves as the carbon skeleton for ammonium assimilation by the GS-GOGAT (glutamine synthetase–glutamate synthase) pathway. Taken together, PII proteins integrate the cellular nitrogen (glutamine), carbon (2OG), and energy (ATP/ADP) levels, and transduce these signals to target proteins by physical interaction.

Under N-limiting conditions, the response regulator NtrC is phosphorylated by its cognate sensor kinase NtrB (Jiang et al. 1998b). In turn, NtrC-P activates transcription of *glnA* encoding glutamine synthetase, the *glnK-amtB* operon, and genes required for generation of ammonia from "poor" nitrogen sources like amino acids. Under N-replete conditions, unmodified GlnB forms a complex with NtrB to stimulate dephosphorylation and hence, inactivation of NtrC. In parallel, unmodified GlnK forms a complex with AmtB thereby inhibiting ammonium uptake under N-replete conditions.

This review deals with the regulation of nitrogen fixation in photosynthetic purple nonsulfur bacteria, which are capable of using light energy to generate the ATP required for nitrogenase activity. Purple nonsulfur bacteria are known for their extreme metabolic versatility enabling growth under photoautotrophic, photoheterotrophic, chemoautotrophic, and chemoheterotrophic conditions (Madigan et al. 1984). BNF is widespread in purple nonsulfur bacteria and has been extensively studied in *Rhodobacter capsulatus*, *Rhodopseudomonas palustris*, and *Rhodospirillum rubrum*, whose complete genome sequences have been determined (Larimer et al. 2004; Madigan et al. 1984; Munk et al. 2011; Strnad et al. 2010). In addition to Mo-nitrogenase, *Rb. capsulatus* and *Rs. rubrum* synthesize Fe-nitrogenases (Davis et al. 1996; Lehman and Roberts 1991; Schneider et al. 1991; Schneider et al. 1997),

whereas *Rp. palustris* is one of the few diazotrophs synthesizing Mo, V, and Fe-nitrogenases (Oda et al., 2005).

Organization of Nitrogen Fixation Genes in Purple Nonsulfur Bacteria

All diazotrophs including *Rb. capsulatus*, *Rp. palustris*, and *Rs. rubrum* contain a common set of nitrogen fixation genes, namely the structural genes of Mo-nitrogenase (*nifH*, *nifD*, and *nifK*) and genes involved in [4Fe-4S] cluster, P-cluster, and FeMoco biosynthesis (*nifU*, *nifS*, *nifB*, *nifV*, *nifE*, and *nifN*) (Curatti and Rubio 2014; Hu and Ribbe 2016; Larimer et al. 2004;MacKellar et al. 2016; Masepohl and Klipp 1996; Munk et al. 2011; Oda et al. 2005; Strnad et al. 2010; Wang et al. 2013). These common *nif* genes cluster with species-specific *nif* genes involved in regulation, electron transport to nitrogenase, and genes of unknown function (Fig. 1). Expression of common and species-specific *nif* genes requires the central transcriptional activator NifA, which enhances transcription by RNA polymerase containing the nitrogen-specific sigma factor RpoN (also called NtrA or σ^{54}) as is the case in other proteobacterial diazotrophs (see below). NifA proteins consist of an N-terminal GAF domain involved in the response to the cellular nitrogen status, a central AAA domain involved in the interaction with RNA polymerase and ATP hydrolysis, and a C-terminal HTH (helix-turn-helix) domain involved in binding to promoter DNA (Fischer 1994). Noteworthy, *Rb. capsulatus* synthesizes two structurally and functionally highly similar NifA proteins: NifA1 and NifA2 (Masepohl et al. 1988; Paschen et al. 2001).

Electron transport to nitrogenase in *Rb. capsulatus* involves the *rnfABCDGEH* genes (Jeong and Jouanneau 2000; Jouanneau et al. 1998; Kumagai et al. 1997; Schmehl et al. 1993), which are lacking in *Rs. rubrum* and *Rp. palustris*. Instead, the latter two strains contain the *fixABCD* genes, whose products form the major electron transport pathway in *Rs. rubrum* (Edgren and Nordlund 2004) and possibly also in *Rp. palustris* (Huang et al. 2010).

Many diazotrophs including *Rb. capsulatus* and *Rp. palustris* have *iscN*-*nifUSVW* operons, whereas *Rs. rubrum* lacks an *nifS* gene at the corresponding position between *nifU* and *nifV*. However, *Rs. rubrum* contains three *nifS*-like genes elsewhere in the chromosome, one of which possibly serves as a sulfur donor for biosynthesis of iron-sulfur clusters under N_2-fixing conditions.

The structural genes of Fe-nitrogenase *anfHDGK* and the Fe-nitrogenase-associated genes *anfOR* form conserved operons in *Rh. capsulatus*, *Rp. palustris*, and *Rs. rubrum* (Fig. 2) (Larimer et al. 2004; Munk et al. 2011; Oda et al. 2005; Schüddekopf et al. 1993; Strnad et al. 2010). Expression of these *anf* operons is activated by AnfA, an NifA-like regulator (Kutsche et al. 1996; Schüddekopf et al. 1993). Activation of the V-nitrogenase-related genes *vnfH*, *vnfDGK*, and *vnfENX* in *Rp. palustris* depends on VnfA, another NifA-like activator. Like NifA, AnfA and VnfA act in concert with the sigma factor RpoN.

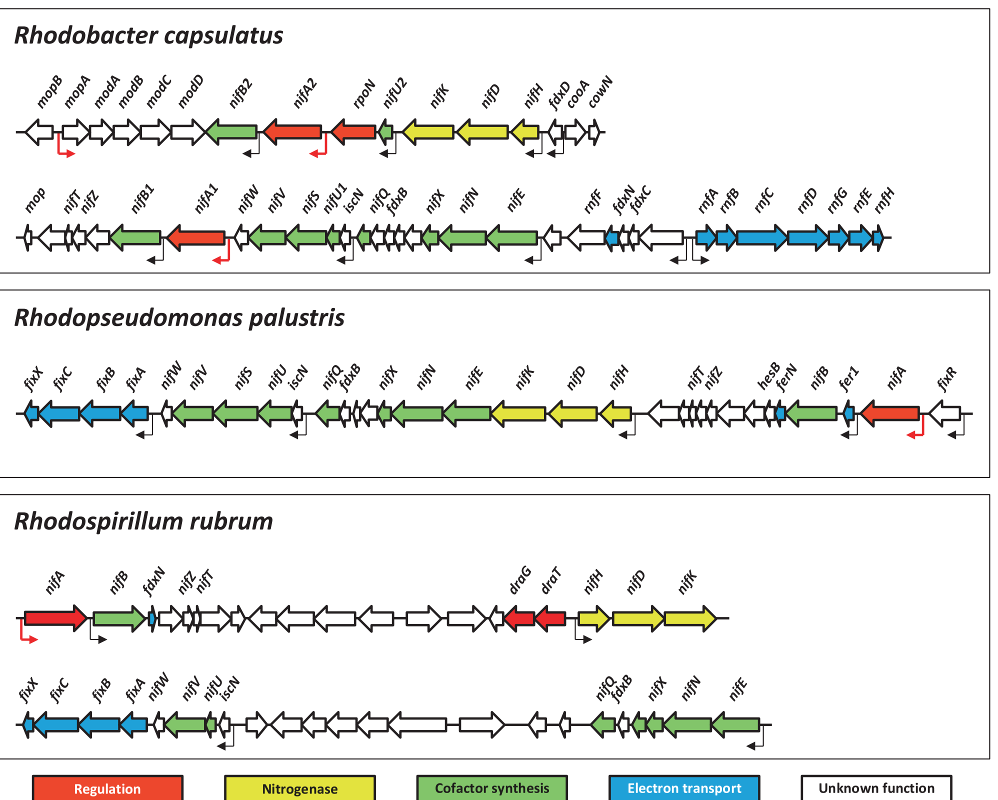


Fig. 1 Organization of Mo-nitrogenase-related genes. Genetic maps are based on the genome sequences of *Rh. capsulatus* SB 1003 (Strnad et al. 2010), *Rp. palustris* CGA009 (Larimer et al. 2004), and *Rs. rubrum* S1 (Munk et al. 2011). *Bent arrows* in *red* or *black mark* possible NtrC and RpoN recognition sequences, respectively

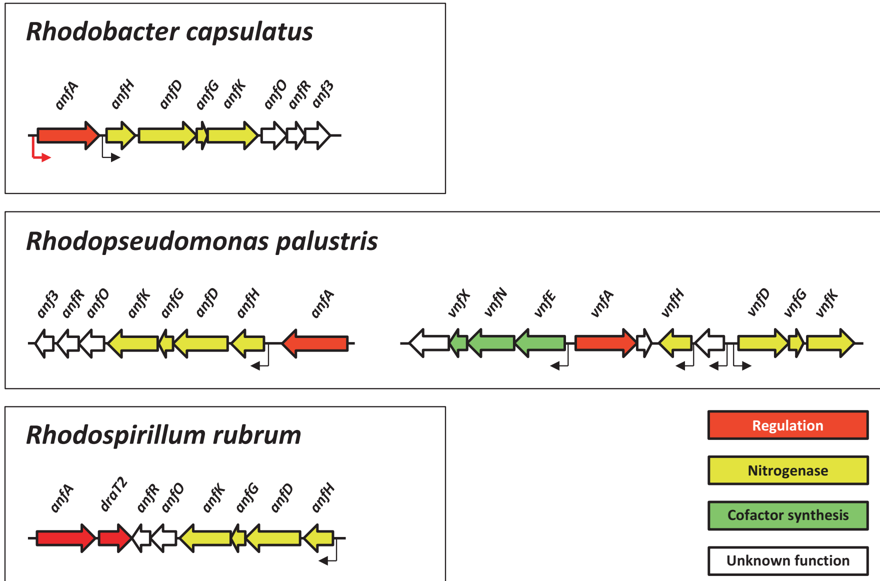


Fig. 2 Organization of Fe and V-nitrogenase-related genes. Genetic maps are based on the genome sequences of *Rh. capsulatus* SB 1003 (Strnad et al. 2010), *Rp. palustris* CGA009 (Larimer et al. 2004), and *Rs. rubrum* S1 (Munk et al. 2011). *Bent arrows* in *red* or *black mark* possible NtrC and RpoN recognition sequences, respectively

Cascade Activation of Nitrogen Fixation in *Rhodobacter capsulatus*

Rb. capsulatus is capable of growing with many different nitrogen sources including ammonium, urea, most amino acids, and N_2 (Hillmer and Gest 1977; Masepohl et al. 2001). Expression of urease and N_2 fixation genes strictly depends on NtrC (Hübner et al. 1991; Kranz and Haselkorn 1985; Kutsche et al. 1996; Masepohl et al. 2001). As described above for *E. coli*, *Rb. capsulatus* NtrC is phosphorylated, and thus activated, by NtrB under ammonium-limiting conditions (Cullen et al. 1996). In contrast to NtrC from *E. coli* and other bacteria, which require the nitrogen-specific sigma factor RpoN to activate transcription of their target genes, *Rb. capsulatus* NtrC activates gene expression in concert with the housekeeping sigma factor RpoD (Bowman and Kranz 1998; Foster-Hartnett et al. 1994).

Upon phosphorylation, *Rb. capsulatus* NtrC activates transcription of *nifA1*, *nifA2*, *mopA-modABC*, and *anfA* (Fig. 3). Activation involves binding of NtrC to sequences similar to the *Rb. capsulatus* NtrC binding site consensus CGCC–N_9–GGC–N_{4-14}–CGCC–N_9–GGC (Foster-Hartnett and Kranz 1994; Kutsche et al. 1996). NifA1 and NifA2 differ only in their very N-terminal amino acid residues, and consequently, can functionally substitute for each other in transcriptional activation of Mo-nitrogenase genes (Masepohl et al. 1988; Paschen et al. 2001). Expression of Fe-nitrogenase genes is activated by AnfA (Kutsche et al., 1996). Transcriptional activation by NifA1, NifA2, and AnfA depends on RpoN as is the

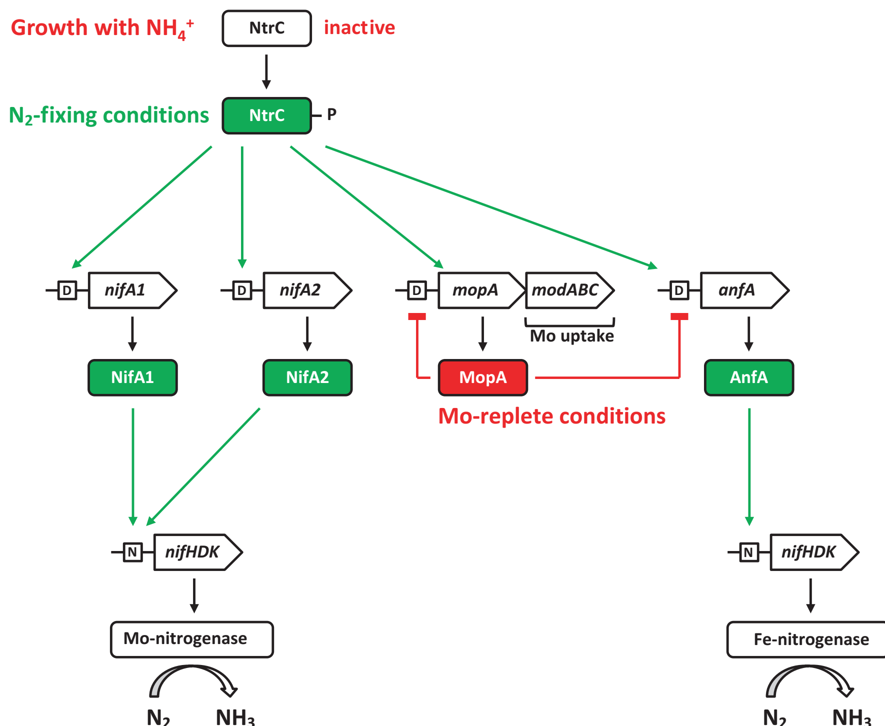


Fig. 3 Cascade regulation of nitrogen fixation in *Rh. capsulatus*. During growth with ammonium, NtrC is inactive, but is activated by phosphorylation upon ammonium consumption. NtrC-P activates RpoD-dependent promoters (boxed D), whereas NifA1, NifA2, and AnfA activate RpoN-dependent promoters (boxed N). MopA represses transcription of the *mopA-modABC* and *anfA* genes under Mo-replete conditions. For clarity, the second Mo-responsive regulator, MopB, is not shown

case in other proteobacterial diazotrophs (Hübner et al. 1991; Schüddekopf et al. 1993). The *Rb. capsulatus rpoN* gene forms part of the *nifU2-rpoN* operon, whose expression is activated by NifA1, NifA2, and presumably also by AnfA (Cullen et al. 1994; Preker et al. 1992). A weak primary NtrC-independent promoter located in the *nifU2-rpoN* intergenic region drives initial expression of *rpoN*, while a secondary promoter upstream of *nifU2* is required to increase *rpoN* expression under N_2-fixing conditions (Cullen et al. 1994).

All *Rb. capsulatus nif* promoters including the *nifU2* promoter as well as the *anfH* promoter contain sequences highly similar to the RpoN binding site consensus CTGC–N_8–TTGC typically located at position −24/−12 relative to the transcription start site (Fig. 1) (Morett and Buck 1989; Schmehl et al. 1993). The *nif* promoters are preceded by sequences similar to the NifA binding site consensus TGT–N_{10}–ACA (Morett and Buck 1988). As expected for an AnfA-dependent promoter, the *anfH* promoter lacks an NifA binding site; however, the AnfA binding site has not yet been identified.

Ammonium Inhibition of Nitrogen Fixation in *Rhodobacter capsulatus*

The levels of *Rb. capsulatus* NtrC remain constant under N-limiting and N-replete conditions, but NtrC activity clearly responds to the cellular nitrogen status (Cullen et al. 1998). Ammonium keeps NtrC in its dephosphorylated inactive state, thus preventing expression of the *nifA1*, *nifA2*, and *anfA* genes, and consequently, all the other nitrogen fixation genes (Foster-Hartnett and Kranz 1992; Preker et al. 1992).

Ammonium addition to an N_2-fixing *Rb. capsulatus* culture causes three effects, namely (1) inactivation of NtrC-P by dephosphorylation, (2) inhibition of NifA1, NifA2, and AnfA activity, and (3) "switch-off" of Mo and Fe-nitrogenases (Drepper et al. 2003; Hallenbeck 1992; Hallenbeck et al. 1982; Jouanneau et al. 1983; Masepohl et al. 1993; Paschen et al. 2001; Pierrard et al. 1993a, b; Schüddekopf et al. 1993).

Ammonium-induced inactivation of NtrC prevents further expression of *nifA1*, *nifA2*, and *anfA*. A strain lacking GlnB expresses *nifA1* (and probably also *nifA2* and *anfA*) even in the presence of ammonium (Drepper et al. 2003). NtrB specifically interacts with GlnB but not with GlnK (Pawlowski et al. 2003) suggesting that inactivation of *Rb. capsulatus* NtrC is catalyzed by an NtrB-GlnB complex exhibiting phosphatase activity as described above for *E. coli*.

Ammonium inhibition of NifA1, NifA2, and AnfA activity prevents further expression of all the other nitrogen fixation genes (Paschen et al. 2001; Schüddekopf et al. 1993). Either GlnB or GlnK is sufficient to inhibit NifA1 and NifA2, whereas a strain lacking both PII signal transduction proteins no longer inhibits activity of the NifA regulators (Drepper et al. 2003). Both NifA proteins interact with GlnB and GlnK (Pawlowski et al. 2003) suggesting that NifA inhibition is mediated by physical contact with the PII proteins. The strain lacking both PII proteins still expresses Mo-nitrogenase (Drepper et al. 2003) indicating that the *Rb. capsulatus* NifA proteins are active as synthesized and do not require activation by PII as is the case in *Rp. palustris* and *Rs. rubrum* (Heiniger et al. 2012; Rey et al. 2007; Zhang et al. 2000, 2004; Zhou et al. 2008; Zhu et al. 2006). In contrast to PII-mediated NifA inhibition in *Rb. capsulatus*, AnfA inhibition is not relieved in the strain lacking both PII proteins indicating that ammonium inhibition of NifA and AnfA involves different mechanisms (Drepper et al. 2003).

Ammonium addition to an N_2-grown culture rapidly represses activity of Mo and Fe-nitrogenases, an effect immediately reversed upon ammonium consumption (Hallenbeck 1992; Hallenbeck et al. 1982; Jouanneau et al. 1983; Masepohl et al. 1993; Pierrard et al. 1993a). In *Rb. capsulatus*, nitrogenase "switch-off" is caused by at least two mechanisms, one blocking activity of the Fe-proteins, NifH and AnfH, by ADP-ribosylation, and another possibly blocking the ATP or the electron supply to nitrogenase (Förster et al. 1999; Pierrard et al. 1993a, b). Evidence for the second mechanism comes from the observation that *Rb. capsulatus* strains expressing mutant NifH proteins, which are no longer ADP-ribosylated, as well as a *draTG* mutant strain still exhibit ammonium-induced nitrogenase switch-off (Förster et al. 1999;

Pierrard et al. 1993a; Yakunin and Hallenbeck 1998b). Ammonium-induced nitrogenase switch-off independent of ADP-ribosylation has been reported in many other diazotrophs, but the underlying mechanisms are unknown (Huergo et al. 2012; and the references therein). In *Rb. capsulatus*, a further nitrogenase switch-off mechanism called "magnitude response" reflects the amount of added ammonium (Yakunin and Hallenbeck 1998b; Yakunin et al. 1999).

ADP-ribosylation is catalyzed by DraT (dinitrogenase reductase ADP-ribosyl transferase), whereas DraG (dinitrogenase reductase-activating glycohydrolase) mediates the reverse reaction (Huergo et al. 2012; Nordlund and Högbom 2013; and the references therein). ADP-ribosylation at arginine residue 101 of one subunit of the *Rb. capsulatus* NifH homodimer is sufficient to prevent electron transfer to the MoFe protein and consequently, N_2 reduction by Mo-nitrogenase (Jouanneau et al. 1989). Proper regulation of nitrogenase modification and switch-off requires GlnB, GlnK, and AmtB, and disruption of the *amtB* gene abolishes ADP-ribosylation and switch-off (Drepper et al. 2003; Tremblay et al. 2007; Tremblay and Hallenbeck 2008; Yakunin and Hallenbeck 2002).The *amtB* strain exhibits wild-type growth properties with ammonium as sole nitrogen source indicating that AmtB is dispensable for ammonium uptake, but primarily serves as an ammonium sensor for ammonium-induced switch-off of nitrogenase (Tremblay and Hallenbeck 2009).

Figure 4 shows a model of DraTG-mediated ammonium regulation of Mo-nitrogenase and possibly also of Fe-nitrogenase in *Rb. capsulatus*. The mechanisms of DraT activation and DraG inactivation can be summarized as follows: (1) Under N_2-fixing conditions, both PII proteins are uridylylated, but upon ammonium addition, GlnK-UMP and GlnB-UMP are deuridylylated. (2) Next, DraG is inactivated by membrane sequestration as a ternary DraG-GlnK-AmtB complex and DraT is activated by complex formation with GlnB. In turn, the GlnB-DraT complex mediates ADP-ribosylation of the Fe-protein. (3) Upon ammonium consumption, DraG is released from the membrane and reactivates the Fe-protein by removing the ADP-ribose moiety.

Ammonium Regulation of Nitrogen Fixation in *Rhodopseudomonas palustris*

Expression and activity of Mo-nitrogenase in *Rp. palustris* is regulated at three levels, namely (1) control of *nifA* transcription, (2) control of NifA activity, and (3) switch-off control of Mo-nitrogenase as is the case for *Rb. capsulatus* (Heiniger et al. 2012; Rey et al. 2007). Ammonium regulation at all three levels involves PII proteins in both diazotrophs. In contrast to *Rb. capsulatus*, which has two PII genes, *glnB* and *glnK*, *Rp. palustris* has three PII genes forming part of the *glnB*-*glnA* and the *glnK1*-*amtB1*-*glnK2*-*amtB2* clusters (Connelly et al. 2006). GlnB, GlnK1, and GlnK2 undergo uridylylation under ammonium-starved (N_2-fixing) conditions, but are deuridylylated under ammonium-replete conditions. Under N_2-fixing conditions, NtrC activates transcription of the *nifA* gene (level 1). Only after binding to GlnB,

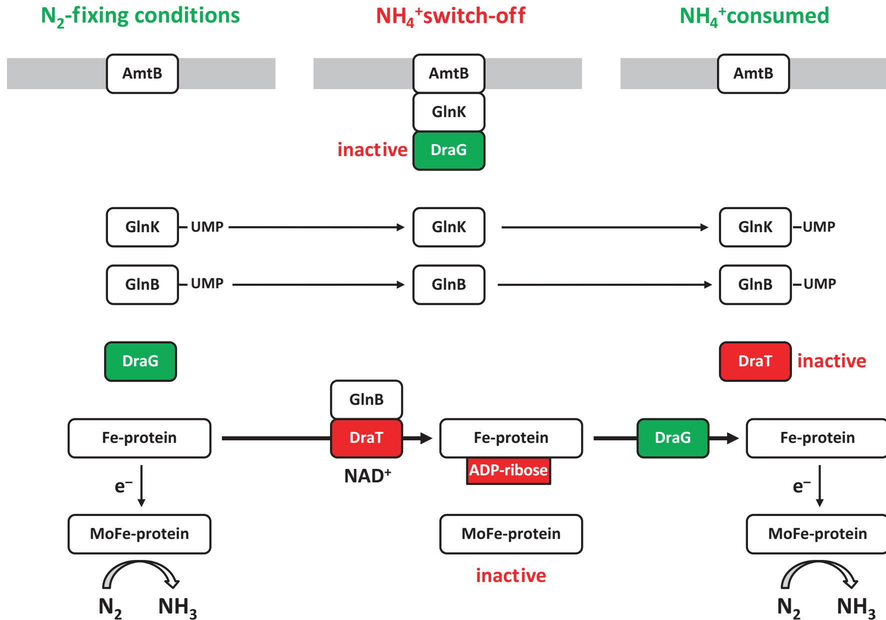


Fig. 4 Model of ammonium-responsive nitrogenase regulation in *Rb. capsulatus*. Upon ammonium addition to a nitrogen-fixing culture, GlnK-UMP and GlnB-UMP are deuridylylated. In turn, DraT is activated by GlnB, while DraG is inactivated by GlnK-mediated membrane sequestration. DraT-mediated ADP-ribosylation of the Fe-protein prevents electron (e^-) transfer to the MoFe-protein. Upon ammonium consumption, DraG is released from the membrane and reactivates the Fe-protein by removing the ADP-ribose moiety

Rp. palustris NifA is capable of activating Mo-nitrogenase gene expression (level 2). Upon ammonium addition to an N_2-grown culture, GlnK2 and DraT2 form a complex to inactivate Mo-nitrogenase by ADP-ribosylation. In addition, *Rp. palustris* DraT2 possibly regulates electron transfer to nitrogenase as discussed for *Rb. capsulatus* DraT (Förster et al. 1999; Heiniger et al. 2012; Pierrard et al. 1993a, b). Like Mo-nitrogenase, the V and Fe-nitrogenases in *Rp. palustris* are modified upon ammonium addition (Heiniger and Harwood 2015).

Rp. palustris strains synthesizing mutant NifA* proteins with single amino acid substitutions or small deletions in the Q-linker constitutively express nitrogenase and produce H_2 even in the presence of ammonium (Heiniger et al. 2012; Rey et al. 2007). The Q-linker is located between the nitrogen-responsive GAF domain and the RNA polymerase-binding AAA domain (Fischer 1994). Three observations explain, how the *nifA** mutants bypass the elaborated regulatory cascade otherwise limiting N_2 fixation to ammonium-starved conditions in the wild-type. First, *Rp. palustris* synthesizes low amounts of NifA independent of NtrC activation. Second, mutant NifA* proteins do not require activation by GlnB and thus, appear to be more active than wild-type NifA proteins. Consequently, NifA* strains overexpress Mo-nitrogenase explaining at least in part resistance against DraT2-mediated nitrogenase switch-off. Third, DraT2

activity requires GlnK2, whose expression depends on NtrC, which is synthesized only at low levels in the presence of ammonium (Conlan et al. 2005). NtrC activates transcription of the *ntrC* gene in *Rp. palustris* (Conlan et al. 2005), whereas NtrC is constitutively synthesized *Rb. capsulatus* (Cullen et al. 1998).

Ammonium Regulation of Nitrogen Fixation in *Rhodospirillum rubrum*

Transcription of *nifA* completely or for the most part depends on NtrC in *Rb. capsulatus* and *Rp. palustris*, respectively (Foster-Hartnett and Kranz 1992; Heiniger et al. 2012; Hübner et al. 1993; Preker et al. 1992; Rey et al. 2007), whereas NtrC appears to be dispensable for *nifA* expression in *Rs. rubrum* (Zhang et al. 1995). However, the *Rs. rubrum nifA* gene is preceded by a possible NtrC binding site (Fig. 1) suggesting that NtrC contributes to *nifA* expression. Disruption of *ntrC* impairs nitrogenase switch-off in *Rs. rubrum*, likely because NtrC is required for maximal *glnBA* expression, and GlnB is essential for DraT activation (Cheng et al. 1999; Zhang et al. 1995).

Rs. rubrum NifA is synthesized in an inactive form, which requires activation by GlnB as is the case in *Rp. palustris* (Zhang et al. 2000, 2001, 2004). Neither of the other two PII proteins synthesized by *Rs. rubrum*, GlnK and GlnJ, can substitute for GlnB in NifA activation. GlnD is essential for NifA activation indicating that only GlnB-UMP but not its unmodified form, GlnB, is capable of activating NifA (Zhang et al. 2005). GlnB* variants mediating NifA activity in a strain lacking GlnD contain single amino acid substitutions in the T-loop apparently mimicking the uridylylated form of GlnB (Zhang et al. 2004; Zhu et al. 2006). NifA* variants no longer requiring activation by GlnB-UMP contain amino acid substitutions in the N-terminal GAF domain, which is involved in interaction between wild-type NifA and GlnB-UMP (Fischer 1994; Zhou et al. 2008). Nitrogenase activity is still switched-off by ammonium in *Rs. rubrum nifA** strains, whereas ammonium switch-off is mostly relieved in *Rp. palustris nifA** strains as described above (Heiniger et al. 2012; Rey et al. 2007; Zhou et al. 2008). *Rs. rubrum nifA** strains lacking DraT, however, exhibit high nitrogenase activity in the presence of ammonium.

DraT-mediated ADP-ribosylation appears to be the only mechanism controlling nitrogenase activity in *Rs. rubrum* (Zhang et al. 1996). In contrast, nitrogenase activity is controlled by two mechanisms, one DraT-dependent and another DraT-independent mechanism, in many other diazotrophs including *Azoarcus* sp. strain BH72, *Azospirillum brasilense*, *Herbaspirillum seropedicae*, and *Rb. capsulatus* (Förster et al. 1999; Fu and Burris 1989; Huergo et al. 2012; Oetjen and Reinhold-Hurek 2009; Pierrard et al. 1993a, b; Yakunin and Hallenbeck 1998b; Zhang et al. 1996).

Rs. rubrum has three PII genes forming part of the *glnB-glnA*, *glnJ-amtB1*, and *glnK-amtB2* operons (Munk et al. 2011). Upon ammonium addition to an N_2-grown culture, DraT is activated by interaction with unmodified GlnB, and DraG is inacti-

vated by membrane sequestration involving AmtB1, unmodified GlnJ, and possibly an unknown membrane protein (Nordlund and Högbom 2013; Teixeira et al. 2008; Wang et al. 2005; Wolfe et al. 2007; Zhang et al. 2006).

Darkness Regulation of Nitrogenase

Like ammonium addition, light deprivation causes nitrogenase switch-off in photosynthetic bacteria (Huergo et al. 2012; Nordlund and Högbom 2013; Pierrard et al. 1993b; Selao et al. 2011; Yakunin and Hallenbeck 2002; Zhang et al. 1995, 2001, 2006). In *Rs. rubrum*, ammonium and darkness-induced nitrogenase regulation by reversible ADP-ribosylation involve the same proteins, namely DraT, DraG, GlnB, GlnJ, and AmtB1, but the signaling mechanisms transducing the cellular nitrogen and energy levels differ (Teixeira et al. 2010; Zhang et al. 2001, 2006). While ammonium addition to an N_2-grown culture causes a big increase in the cellular glutamine concentration leading to GlnD-mediated deuridylylation of GlnB-UMP and GlnJ-UMP, light deprivation does not affect the glutamine pool or induce PII demodification on a big scale (Li et al. 1987; Teixeira et al. 2010). Full uridylylation of trimeric PII prevents "plug-in" interaction with AmtB, whereas partially uridylylated PII proteins form a complex with AmtB in *A. brasilense* (Rodrigues et al. 2011). Hence, DraG inactivation in *Rs. rubrum* may either be achieved by GlnJ-independent membrane sequestration or involve complex formation between partially deuridylylated GlnJ and AmtB1 (Huergo et al. 2012; Nordlund and Högbom 2013).

Iron Regulation of Electron Transport to Nitrogenase

Rb. capsulatus utilizes two parallel acting electron transport pathways to nitrogenase, the RnfABCDGEH-FdxN and the NifJ-NifF pathway, in which the ferredoxin FdxN and the flavodoxin NifF act as the ultimate electron donors to NifH and possibly also to AnfH (Gennaro et al. 1996; Hallenbeck and Gennaro 1998; Jeong and Jouanneau 2000; Jouanneau et al. 1998; Kumagai et al. 1997; Schmehl et al. 1993; Yakunin et al. 1993; Yakunin and Hallenbeck 1998a). The Rnf proteins form an energy-coupling NADH oxidoreductase complex that catalyzes the reduction of FdxN. The NifJ protein is a pyruvate-flavodoxin oxidoreductase mediating electron transfer from pyruvate to NifF. In contrast to the situation in *Rb. capsulatus*, the NifJ-NifF pathway constitutes the sole electron transport pathway to nitrogenase in *K. pneumoniae* (Hill and Kavanagh 1980; Shah et al. 1983).

Unlike the *rnf* and *fdxN* genes, the *Rb. capsulatus nifF* and *nifJ* genes are not contained in the major *nif* clusters (Fig. 1). However, the *nifF* gene belongs to the NifA regulon and accordingly, *nifF* is specifically expressed under N_2-fixing conditions as is the case for the *rnf* and *fdxN* genes (Gennaro et al. 1996; Schmehl et al. 1993). In contrast to *nifF*, the *nifJ* gene is expressed under ammonium-replete conditions and its

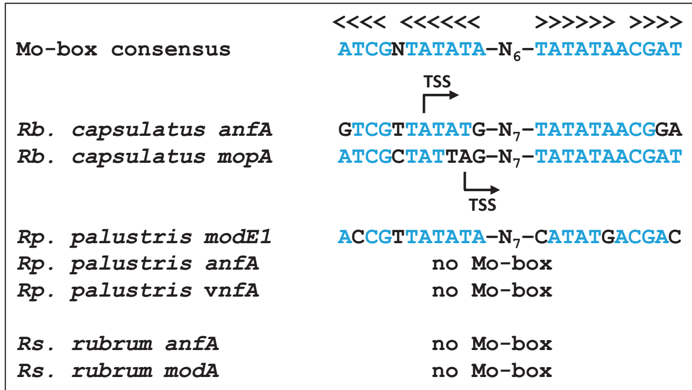
expression increases only slightly under N_2-fixing conditions indicating that NifJ function is not restricted to N_2 fixation (Yakunin and Hallenbeck 1998a). The NifJ-NifF pathway contributes significantly to electron transfer to nitrogenase under iron-replete conditions, but is essential under iron-limiting conditions (Gennaro et al. 1996; Yakunin et al. 1993; Yakunin and Hallenbeck 1998a). Accordingly, *nifF* expression and NifF accumulation is higher under iron-deficient than under iron-sufficient conditions, while *rnf* transcription and Rnf accumulation decreases upon iron limitation (Jouanneau et al. 1998). Apparently, *Rb. capsulatus* copes with iron limitation by replacing the iron-containing ferredoxin FdxN by the Fe-free flavodoxin NifF, but the iron-responsive mechanisms controlling *nifF* and *rnf* expression remain unknown to date.

Rs. rubrum lacks *rnfABCDGEH* genes but instead has *fixABCX* genes (Fig. 1), whose products form the major electron transport pathway to nitrogenase in this diazotroph (Edgren and Nordlund 2004). In addition, *Rs. rubrum* has an *nifJ*-like gene encoding a pyruvate-ferredoxin oxidoreductase (Edgren and Nordlund 2006). In both the FixABCX and the NifJ pathways ferredoxin N (encoded by the *fdxN* gene located downstream of *nifB*; Fig. 1) is the ultimate electron donor to nitrogenase (Edgren and Nordlund 2005, 2006). Like *Rs. rubrum*, *Rp. palustris* lacks *rnfABCDGEH* genes but has *fixABCX* genes (Fig. 1), which are essential for diazotrophic growth (Huang et al. 2010) suggesting that electron transport to nitrogenase in *Rp. palustris* involves a similar mechanism as in *Rs. rubrum*.

Regulation of Molybdate Uptake and Alternative Nitrogenases

Most bacteria synthesize high-affinity molybdate transporters (*modABC*-encoded) suggesting that they have to cope at least temporarily with Mo limitation (Zhang and Gladyshev 2008). Under Mo-replete conditions, *E. coli* represses *modABC* transcription by the molybdate-responsive one-component regulator ModE, thus limiting expression of the Mo uptake system to Mo-limiting conditions. ModE binds a palindromic sequence called Mo-box overlapping the *modA* transcription start site thereby preventing binding of RNA polymerase (Studholme and Pau 2003).

Rb. capsulatus has two *modE* homologs, *mopA* and *mopB*, belonging to divergently transcribed operons, *mopA*-*modABCD* and *mopB* (Fig. 1). Upon molybdate-binding MopA and MopB repress transcription of the *mopA*-*modABCD* and *anfA* genes by binding the Mo-boxes overlapping the transcription start sites of *mopA* and *anfA* (Fig. 5) (Kutsche et al. 1996; Müller et al. 2010; Wiethaus et al. 2006). Either MopA or MopB is sufficient to repress transcription from the *mopA* and *anfA* promoters. Beside its role as a repressor, MopA acts as a transcriptional activator of the *mop* gene encoding a molybdate-binding hexameric protein (Wiethaus et al. 2009). In contrast to the *mopA* and *anfA* Mo-boxes, which overlap the transcription start sites, the *mop* Mo-box is located at some distance upstream of the transcription start site as expected for an enhancer binding site. In line with the proposed role of the Mop protein in Mo storage, Mop accumulates to high levels with increasing Mo concentrations (Hoffmann et al. 2016).



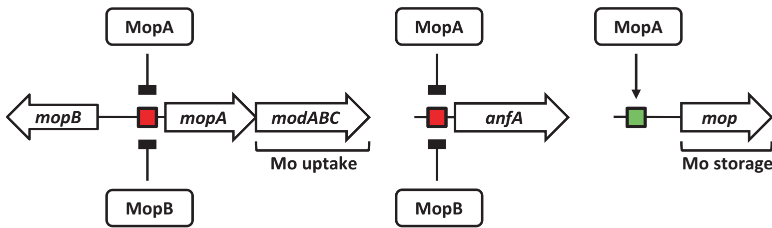


Fig. 5 Nitrogen fixation and molybdate transport-related Mo-boxes. Mo-boxes are highly conserved palindromic sequences (marked by *arrow heads*) serving as binding sites for ModE-type regulators (Studholme and Pau 2003). Conserved Mo-box nucleotides in the promoters of *Rb. capsulatus anfA* and *mopA*, and *Rp. palustris modE1* are highlighted in *blue*. *Rb. capsulatus* synthesizes two ModE-like regulators, MopA and MopB, which repress transcription of the *mopA-modABC* and *anfA* genes by binding Mo-boxes (*red squares*) overlapping the transcription start sites (TSS) of *mopA* and *anfA* (Kutsche et al. 1996). In addition, MopA activates *mop* transcription by binding the Mo-box (*green square*) preceding the *mop* TSS (Wiethaus et al. 2006)

While Mo represses *mopA*, the *mopB* gene is constitutively transcribed and accordingly, the MopA/MopB ratio varies in response to Mo availability (Hoffmann et al. 2016; Wiethaus et al. 2006, 2009). Under Mo-limiting conditions, MopA is more abundant than MopB, whereas only MopB is left under Mo-replete conditions. MopA and MopB form homodimers as well as heteromers (Wiethaus et al. 2009). Disruption of *mopB* enhances *mop* expression suggesting that MopA-MopB heteromer formation counteracts *mop* activation by MopA homodimers.

Since AnfA is essential for Fe-nitrogenase expression, *anfA* repression by MopA and MopB prevents Fe-nitrogenase expression at high Mo concentrations (Fig. 3). In contrast, Mo-nitrogenase levels increase with increasing Mo concentrations involving a yet unknown post-transcriptional control mechanism (Hoffmann et al. 2014a, 2016).

In addition to ModABC, which imports molybdate at nanomolar concentrations in the environment, *Rb. capsulatus* synthesizes the oxyanion transporter PerO, which imports molybdate in micromolar ranges (Gisin et al. 2010). Besides molybdate,

PerO transports tungstate, vanadate, and sulfate. In contrast to the *modABC* genes, transcription of *perO* is not repressed by molybdate.

Like *Rb. capsulatus*, *Rp. palustris* has two *modE* genes, one of which, *modE1*, clusters with *modABC* genes, while the other is located at a distant position in the chromosome (Larimer et al. 2004). The *modE1* promoter contains a likely Mo-box (Fig. 5) indicating that ModE1 autoregulates its own expression in response to molybdate availability as is the case for *Rb. capsulatus* MopA. In contrast to the *Rb. capsulatus anfA* promoter, the *Rp. palustris anfA* and *vnfA* promoters do not encompass an obvious Mo-box suggesting that *anfA* and *vnfA* do not belong to the ModE1 regulon (see below). Unlike *Anabaena variabilis* ATCC 29413, which synthesizes a high-affinity vanadate transporter, VupABC, sustaining V-nitrogenase activity under vanadate-limiting conditions, *Rp. palustris* lacks *vupABC* homologs (Pratte and Thiel 2006).

Disruption of the Mo-nitrogenase genes induces expression of V and Fe-nitrogenases in *Rp. palustris* even at high molybdate concentrations otherwise sufficient to repress Fe-nitrogenase in *Rb. capsulatus* (Oda et al. 2005; Wang et al. 1993). Similar to the situation in *Rp. palustris*, *Rs. rubrum* strains lacking active Mo-nitrogenase express Fe-nitrogenase irrespective of Mo availability (Lehman and Roberts 1991). Hence, the mechanisms controlling expression of the alternative nitrogenases in *Rp. palustris* and *Rs. rubrum* differ from that in *Rb. capsulatus*.

Nitrogenase Protection Against Oxygen Damage

Mo, V, and Fe-nitrogenases are irreversibly damaged by oxygen (Blanchard and Hales 1996; Chisnell et al. 1988; Gollan et al. 1993), and thus, many diazotrophs synthesize nitrogenase only under anaerobic or microaerobic conditions. Other diazotrophs have evolved different strategies to protect nitrogenase at high ambient oxygen concentrations. Some filamentous cyanobacteria develop specialized N_2-fixing cells called heterocysts, which have thick cell walls limiting oxygen entry and lack the oxygen-evolving photosystem PSII. Most rhizobia express nitrogenase exclusively within special plant organs called nodules, in which oxygen partial pressure is sufficiently low. Other strategies involve cytochrome *bd* oxidase (*cydAB*-encoded) or the Shetna's protein II (*fesII*-encoded) mediating "respiratory" and "conformational" protection of nitrogenase, respectively, in *A. vinelandii*, *Gluconacetobacter diazotrophicus*, and *Rb. capsulatus* (Hoffmann et al., 2014a; Kelly et al. 1990; Moshiri et al. 1994; Schlesier et al. 2016; Ureta and Nordlund 2002). Conformational protection depends on a ternary complex formed by FeSII, the Fe-protein, and the MoFe-protein (Schlesier et al. 2016).

The *Rb. capsulatus* FeSII homolog, FdxD, supports diazotrophic growth via Mo-nitrogenase (but not via Fe-nitrogenase) under semiaerobic conditions (Hoffmann et al. 2014a). Expression of the *fdxD* gene, which is located immediately upstream of the *nifHDK* genes, is activated by NifA1 and NifA2 but not by AnfA. Hence, the *fdxD* gene belongs to the Mo-nitrogenase regulon, and its product specifically protects Mo-nitrogenase against oxygen damage.

NifA-dependent *fdxD* expression decreases with increasing oxygen concentrations (Hoffmann et al. 2014a). This regulation is possibly explained by oxygen sensitivity of NifA1 and NifA2, which belong to the class of oxygen-sensitive NifA regulators (Fischer 1994; Paschen et al. 2001). Members of this class contain an additional domain absent in oxygen-tolerant NifA proteins, the interdomain linker domain, which is located between the central AAA and the C-terminal HTH domain. The interdomain linker domain is implicated in metal (possibly Fe) binding and oxygen or redox sensing in *Bradyrhizobium japonicum* and *Herbaspirillum seropedicae* (Fischer et al. 1988, 1989; Oliveira et al. 2009).

Maximal *fdxD* expression requires both NifA1 and NifA2 (Hoffmann et al. 2014a), and maximal *nifA2* expression depends on the two-component regulatory system RegB-RegA (Elsen et al. 2000). In contradiction to the original assumption that oxygen directly inhibits RegB kinase activity (Mosley et al. 1994; Sganga and Bauer. 1992), the RegB-RegA system apparently responds to the cellular redox state (Elsen et al. 2000). Besides controlling nitrogen fixation (via *nifA2*), the RegB-RegA system regulates photosynthesis, carbon dioxide assimilation, and hydrogen oxidation, thus acting as a master regulator of important energy-generating and energy-consuming processes.

Nitrogenase Protection Against Carbon Monoxide Inhibition

Carbon monoxide (CO) inhibits all nitrogenase-catalyzed substrate reductions except for proton reduction by blocking intramolecular electron flow and hence, CO hampers N_2 fixation and diazotrophic growth (Hwang et al. 1973; Lee et al. 2009; Lockshin and Burris 1965; Rivera-Ortiz and Burris 1975; Shen et al. 1997; Yan et al. 2012). A small protein, CowN, sustains N_2-dependent growth of *Rb. capsulatus* and *Rs. rubrum* in the presence of CO (Hoffmann et al. 2014b; Kerby and Roberts 2011). CowN has been suggested to form a complex with nitrogenase like the Shetna protein but experimental evidence supporting this assumption is lacking (Kerby and Roberts 2011). In both *Rb. capsulatus* and *Rs. rubrum*, *cowN* expression is induced by CO, but *cowN* activation depends on different transcription activators in these species.

CO induction of *Rb. capsulatus cowN* expression is mediated by the CO-responsive regulator CooA (Hoffmann et al. 2014b), which belongs to the family of heme-containing transcription factors (Roberts et al. 2005). Expression of *cooA* is activated by NifA1 and NifA2, whereas AnfA represses *cooA* and consequently, *cowN*. Accordingly, CowN specifically sustains diazotrophic growth via Mo-nitrogenase but not Fe-nitrogenase-dependent growth in the presence of CO.

The *Rs. rubrum* CooA homolog activates expression of CO dehydrogenase genes, but is dispensable for *cowN* expression (Fox et al. 1996; Kerby and Roberts 2011; Shelver et al. 1995). Instead, *cowN* expression in *Rs. rubrum* requires another CO-responsive regulator, RcoM (Kerby et al. 2008; Kerby and Roberts 2011), which is lacking in *Rb. capsulatus*.

Genes similar to *cowN* are widespread in bacteria (Kerby and Roberts 2011). Apparently, all bacteria harboring a *cowN* homolog also possess *nifHDK* genes implying that CowN-mediated Mo-nitrogenase protection is a common mechanism. In contrast to strict ammonium repression of the *nifHDK* genes, however, *cowN* is only partially repressed by ammonium in *Rb. capsulatus* and *Rs. rubrum* suggesting that CowN function is not restricted to nitrogenase protection (Hoffmann et al. 2014b; Kerby and Roberts 2011).

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