Role of GlnB and GlnK in ammonium control of both nitrogenase systems in the phototrophic bacterium *Rhodobacter capsulatus*

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In most bacteria, nitrogen metabolism is tightly regulated and P\textsubscript{II} proteins play a pivotal role in the regulatory processes. *Rhodobacter capsulatus* possesses two genes (*glnB* and *glnK*) encoding P\textsubscript{II}-like proteins. The *glnB* gene forms part of a *glnB–glnA* operon and the *glnK* gene is located immediately upstream of *amtB*, encoding a (methyl-) ammonium transporter. Expression of *glnK* is activated by NtrC under nitrogen-limiting conditions. The synthesis and activity of the molybdenum and iron nitrogenases of *R. capsulatus* are regulated by ammonium on at least three levels, including the transcriptional activation of *nifA1*, *nifA2* and *anfA* by NtrC, the regulation of NifA and AnfA activity by two different NtrC-independent mechanisms, and the post-translational control of the activity of both nitrogenases by reversible ADP-ribosylation of NifH and AnfH as well as by ADP-ribosylation independent switch-off. Mutational analysis revealed that both P\textsubscript{II}-like proteins are involved in the ammonium regulation of the two nitrogenase systems. A mutation in *glnB* results in the constitutive expression of *nifA* and *anfA*. In addition, the post-translational ammonium inhibition of NifA activity is completely abolished in a *glnB–glnK* double mutant. However, AnfA activity was still suppressed by ammonium in the *glnB–glnK* double mutant. Furthermore, the P\textsubscript{II}-like proteins are involved in ammonium control of nitrogenase activity via ADP-ribosylation and the switch-off response. Remarkably, in the *glnB–glnK* double mutant, all three levels of the ammonium regulation of the molybdenum (but not of the alternative) nitrogenase are completely circumvented, resulting in the synthesis of active molybdenum nitrogenase even in the presence of high concentrations of ammonium.

INTRODUCTION

The phototrophic non-sulfur purple bacterium *Rhodobacter capsulatus* is able to fix atmospheric dinitrogen by either a conventional molybdenum-containing (*nif*-encoded) nitrogenase or an alternative heterometal-free (*anf*-encoded) nitrogenase. Synthesis and activity of both nitrogenase systems are tightly controlled at different levels in response to ammonium availability (for a review, see Masepohl & Klipp, 1996; Masepohl et al., 2002). At one level, the ammonium-dependent transcriptional control of the *nifA1*, *nifA2* and *anfA* genes (which encode specific transcriptional activators of all the other *nif* and *anf* genes) is mediated by a regulatory cascade which is similar to the Ntr system of enteric bacteria. The *R. capsulatus* Ntr system consists of the signal transduction protein GlnB (see below) and the two-component regulatory system NtrB/NtrC (Kranz & Foster-Hartnett, 1990). Under nitrogen-limiting conditions, the sensor kinase NtrB phosphorylates, and thereby activates, the response regulator NtrC (Cullen et al., 1996; Kranz & Foster-Hartnett, 1990). In addition to the NtrC-dependent activation, *anfA* transcription is repressed by MopA and MopB in the presence of molybdenum (Kutsche et al., 1996). At a second regulatory level, the activity of NifA1 and NifA2 is inhibited in response to ammonium (Hübner et al., 1993; Paschen et al., 2001). The characterization of *R. capsulatus* NifA1 mutants which are able to activate *nif* gene transcription in the presence of ammonium revealed that the N-terminal domain of NifA is involved in this post-translational control mechanism (Paschen et al., 2001). Finally, at a third level of control, the activity of both nitrogenases is regulated in response to ammonium and darkness via reversible ADP-ribosylation of the dinitrogenase reductases NifH and AnfH mediated by DraT (dinitrogenase reductase ADP-ribosyltransferase) and DraG (dinitrogenase-reductase-activating glycohydrolase) and by a DraT/DraG-independent switch-off mechanism (Masepohl et al., 1993; Pierrard et al., 1993; Yakunin &
Hallenberg, 1998). Recently it has been shown that the (methyl-) ammonium transporter AmiB, but not its homologue AmiY, may act as an ammonium sensor which is involved in controlling nitrogenase activity via DraT/DraG and also via the DraT/DraG-independent mechanism (Yakunin & Hallenberg, 2002).

In Escherichia coli and many other bacteria, GlnB and GlnD (uridylyltransferase/uridylyl removing enzyme) are key elements of the regulatory networks controlling nitrogen assimilation (Merrick & Edwards, 1995; Ninfa & Atkinson, 2000; Ar Condéguy et al., 2001). Both GlnD and GlnB are involved in sensing the nitrogen status of the cells by direct interaction with glutamine and 2-oxoglutarate, respectively. In E. coli, GlnB controls the activity of glutamine synthetase and the sensor kinase NtrB (Ninfa & Atkinson, 2000), whereas the GlnB proteins of Azospirillum brasilense, Herbaspirillum seropedicae and Rhodospirillum rubrum also play essential roles in nitrogen fixation, since glnB mutations lead to synthesis of inactive NifA in the latter three organisms (Benelli et al., 1997; de Zamaroczy et al., 1993; Liang et al., 1992; Souza et al., 1999; Zhang et al., 2000). In addition, many bacteria contain a second glnB-like gene designated glnK (for a review, see Thomas et al., 2000; Ar Condéguy et al., 2001). In most cases, glnK appears to be co-transcribed with amtB, which is located downstream of glnK, and encodes a high-affinity (methyl-) ammonium transporter, and expression of glnK–amtB is activated by NtrC. In contrast, Azotococcus sp. BH72 and Rs. rubrum contain three PiI-encoding genes, namely a glnB gene and two different glnK-like genes forming bicistronic operons with amtB-like genes (Martin et al., 2000; Zhang et al., 2001b). The additional copies of glnK are designated glnY (in Azotococcus) and glnJ (in Rs. rubrum).

In E. coli, GlnB and GlnK are structurally and functionally similar (Atkinson & Ninfa, 1998, 1999; van Heeswijk et al., 1996). However, recent studies demonstrated that in Klebsiella pneumoniae, only GlnK is involved in transmission of the nitrogen status to the NifL/NifA regulatory system, suggesting functional differences between GlnB and GlnK in this organism (Ar Condéguy et al., 1999; He et al., 1998; Holtel & Merrick, 1989; Jack et al., 1999). In addition, PiI-like proteins also have distinct roles in mediating nitrogen control of nitrogenase activity in some diazotrophic organisms (Klasson et al., 2001; Martin & Reinhold-Hurek, 2002; Zhang et al., 2001b). In Rs. rubrum as well as in Azotococcus sp. BH72, GlnB and one of the two GlnK-like proteins (GlnJ and GlnK, respectively) are involved in regulation of DraT/DraG activity, whereas the second GlnK-like protein apparently has no function in this regulatory mechanism (Martin & Reinhold-Hurek, 2002; Zhang et al., 2001b).

In this report, we characterized the glnK–amtB operon in R. capsulatus and analysed the roles of GlnB and GlnK in the regulation of nitrogen fixation by ammonium. We demonstrated that both GlnB and GlnK are involved in the control of the synthesis and activity of both the molybdenum and the iron-only nitrogenase. Most remarkably, only in a glnB–glnK double mutant strain are all levels of ammonium control of molybdenum nitrogenase completely circumvented, whereas at least the activity of the transcriptional activator of the alternative nitrogenase, AnfA, was still affected by ammonium in this mutant.

Preliminary results concerning the role of R. capsulatus GlnB and GlnK in the regulation of nitrogen fixation were presented at the 12th and 13th International Congress on Nitrogen Fixation (Drepper et al., 2000; Groß et al., 2002; Hallenberg et al., 2002).

**METHODS**

**Strains, media and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The growth conditions, media and antibiotic concentrations used to cultivate E. coli and R. capsulatus strains were as described previously (Klipp et al., 1988; Masepohl et al., 1988). R. capsulatus cultures were grown either in RCV minimal medium in order to derepress molybdenum nitrogenase, or in molybdenum-free AK-NL minimal medium for the expression of the alternative nitrogenase. To remove traces of molybdenum, the media were treated with activated carbon as described by Schneider et al. (1991). Conjugal plasmid transfer from E. coli S17-1 into R. capsulatus via filter mating was carried out as described previously (Klipp et al., 1988).

**Isolation of the R. capsulatus glnK gene and construction of a glnK–lacZ fusion plasmid.** The R. capsulatus glnK gene was isolated by a PCR-based strategy by amplification of a glnK internal 264 bp DNA fragment using degenerate primers based on conserved regions of GlnB proteins of different bacteria (5'-GCSATCATCAA-RCCSTTYCARGT-3' and 5'-CACRAAGATCTSCRTGCRAT-3'), where B is C, G or T, R is A or G, and S is C or G. Subsequently, a hybrid plasmid (pSG5II) carrying a 2127 bp SalI fragment was isolated by screening a size-fractionated gene bank by Southern hybridization using the amplified glnK fragment as a probe. As a basis for analysing glnK expression, a 1669 bp SalI–XhoI fragment (glnK') from pSG5II was cloned into the broad-host-range vector pML35-β-lacZ, resulting in hybrid plasmid pSG9II (glnK–lacZ).

**Construction of glnK and ntrC interposon mutants.** To construct a defined glnK interposon mutant, a 2.6 kb XhoI cassette carrying the gentamicin resistance gene from pWKR440 was inserted into the XhoI site located within the glnK coding region. The resulting hybrid plasmid, designated pSG5IIb1, was subsequently used to create R. capsulatus glnB single mutant strain SG26. In order to generate a glnB–glnK double mutant (TD166), the 264 bp PCR fragment encompassing an internal glnK fragment was cloned into a mobilizable derivative of pUC18, resulting in hybrid plasmid pSG2.1, which subsequently was integrated into the genome of R. capsulatus mutant strain PHU332 (glnB) by single cross-over recombination. Plasmid integration of pSG2.1 resulted in an R. capsulatus mutant strain containing a 3' truncated glnK gene, which was under control of the wild-type promoter. Expression of the truncated glnK gene resulted in a mutant GlnK protein in which the 28 C-terminal amino acid residues (encoding the C-loop and part of the B-loop) are substituted by 22 vector-encoded amino acid residues.

To construct a defined ntrC interposon mutant, the 2.6 kb SalI gentamicin resistance cassette from plasmid pWKR202 was inserted into the XhoI site of the mobilizable suicide hybrid plasmid pPBK1, resulting in hybrid plasmid pTD8I (ntrC::[Gm]).
Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td>DH5x</td>
<td>Host for plasmid amplification</td>
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<td><strong>R. capsulatus</strong></td>
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<td>B10S</td>
<td>Spontaneous Sm&lt;sup&gt;+&lt;/sup&gt; mutant of <em>R. capsulatus</em> B10</td>
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<td>Wang <em>et al.</em> (1993)</td>
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<td>glnK::[Km]</td>
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<td>PHU348</td>
<td>glnB::[Gm]</td>
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<td>ntrC::[Gm] (transconjugant of B10S with pTD8I)</td>
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<td><strong>Plasmids</strong></td>
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<td>pML5B&lt;sup&gt;+&lt;/sup&gt;/B&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Broad-host-range lacZ transcriptional fusion vectors, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Labes <em>et al.</em> (1990)</td>
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<td>pPHU231</td>
<td>Broad-host-range vector, Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Reyes <em>et al.</em> (1996)</td>
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<td>pPHU236</td>
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<td>P. Hübner, Basel</td>
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<td>pUC18 derivative carrying glnK</td>
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<td>pTD6-5I</td>
<td>pPHU231 derivative carrying the constitutively expressed <em>αnfA</em> gene</td>
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<td>pTD8I</td>
<td>pPBK1 derivative carrying <em>ntrC</em>::[Gm]</td>
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<td>pWK202</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;−&lt;/sup&gt; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pWKR440</td>
<td>Tc&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;−&lt;/sup&gt;</td>
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Construction of hybrid plasmid pTD6-5I constitutively expressing *αnfA* (*αnfA*<sup>+</sup>). A 1756 bp *Kasl−EcoRI* fragment encompassing *R. capsulatus* *αnfA* was cloned into the mobilizable broad-host-range vector pPHU231. Subsequently, a 1489 bp *HindIII−Sall* cassette carrying the kanamycin resistance gene (*aphII*) from transposon *Tn5* was inserted at the *KasI* site upstream of the *αnfA* gene. In the resulting hybrid plasmid pTD6-5I expression of *αnfA* was driven by the constitutively expressed *aphII* promoter (*αnfA*<sup>+</sup>).

**β-Galactosidase assays.** *R. capsulatus* strains carrying *lacZ* fusions were cultured photoheterotrophically as batch cultures in RCV (for strains carrying the glnK::lacZ fusion) or AK-NL (for strains carrying the *αnfA–lacZ* fusion) minimal medium with either 15 mM ammonium or 10 mM serine as sole nitrogen source until the late-exponential growth phase. β-Galactosidase activities were determined by the SDS/chloroform method as described previously (Miller, 1972; Hübner *et al.* 1991).

**Western analysis.** Cell-free protein extracts of *R. capsulatus* were isolated as described before (Masepohl *et al.*, 1988). Proteins were separated on SDS-polyacylamide gels with an acrylamide concentration gradient of 10–15%, and subsequently blotted onto PVDF membranes (Roth). Detection of NifA1, NifH and AnfH proteins was performed using the ECL kit (Amersham Pharmacia Biotech).

**Nitrogenase ADP-ribosylation and in vivo activity assays.** *R. capsulatus* strains were grown phototrophically in 3 ml cultures (in 15 ml Hungate tubes) in either RCV or AK-NL minimal medium supplemented with 5 mM serine under an argon atmosphere. Nitrogenase activity was measured in whole cells by the acetylene reduction method using a Hewlett Packard gas chromatograph model 5890 series II with a Chrompack alunina GC column for the acetylene/ethylene/ethane separation. The formation of ethane was routinely monitored as an indication of the activity of the alternative nitrogenase (Wang *et al.*, 1993). Nitrogenase-mediated
H\textsubscript{3} evolution was determined as described previously (Klein et al., 1991). For the results shown in Fig. 2, \textit{in vivo} nitrogenase activity was analysed by the acetylene reduction method as described previously (Yakunin et al., 1999). Culture samples (25–50 \textmu l) were removed from the vials at the times indicated in Fig. 2 as described previously (Yakunin & Hallenbeck, 2002) and equal amounts of total protein (1 \mu g per well) were loaded onto polyacrylamide gels. Immunoblotting with chemiluminescence detection was used to monitor the modification state of Fe–protein essentially as described previously (Yakunin & Hallenbeck, 1998).

**RESULTS AND DISCUSSION**

**Regulation of \textit{glnK} expression**

Like many proteobacteria, \textit{R. capsulatus} contains two \textit{P\textsubscript{II}}-encoding genes, \textit{glnB} and \textit{glnK} (Drepper et al., 2000; Masepohl et al., 2002). The \textit{R. capsulatus glnK} gene region was isolated by a PCR-based strategy (Methods). The \textit{glnk} gene is associated with \textit{amtB}, encoding a high-affinity (methyl-) ammonium transporter (Yakunin & Hallenbeck, 2002), while the previously described \textit{glnB} gene is cotranscribed with \textit{glnA}, encoding glutamine synthetase (Kranz et al., 1990). The deduced \textit{R. capsulatus GlnK} protein consists of 112 amino acid residues and is 59% identical to GlnB of \textit{R. capsulatus}. An even higher degree of identity (88%) exists for the GlnK proteins of \textit{R. capsulatus} and \textit{Rhodobacter sphaeroides} (Qian & Tabita, 1998). The presence of two DNA sequences 137 and 154 bp upstream of the \textit{R. capsulatus glnK} translational start codon exhibiting strong similarity to NtrC-binding sites (GC-N\textsubscript{7}-T-N\textsubscript{3}-GC; Foster-Hartnett & Kranz, 1994) suggested that expression of \textit{glnK}–\textit{amtB} is regulated by NtrC in dependence on ammonium availability. To verify this assumption, regulation of \textit{glnK} expression was examined in \textit{R. capsulatus} wild-type (B105) and an \textit{ntrC} mutant strain (TD50) containing the reporter plasmid pSG9I carrying a \textit{glnK}–\textit{lacZ} fusion. Expression of \textit{glnK} was eightfold higher in wild-type cells grown under nitrogen limitation (2125 Miller units) compared to ammonium-grown cells (256 Miller units). This transcriptional activation was found to depend on NtrC, since disruption of the \textit{ntrC} gene led to a drastic decrease of expression of the \textit{glnK}–\textit{lacZ} fusion in the absence of ammonium (246 Miller units). Similarly, NtrC-mediated activation of \textit{glnK} has been described for other bacteria, including \textit{E. coli}, \textit{K. pneumoniae} and \textit{Azospirillum brasilense} (Atkinson & Ninfa, 1998; de Zamaroczy, 1998; He et al., 1998; Jack et al., 1999; van Heeswijk et al., 1996). In addition to NtrC-mediated \textit{glnK} activation under N-limiting conditions, low but significant \textit{glnK} expression occurred in an \textit{ntrC} mutant background in both the presence and absence of ammonium, whereas no \textit{\beta}-galactosidase activity was detectable in the strains containing the vector plasmid pML5 carrying the promoter-less \textit{lacZ} gene (data not shown). These findings suggest that transcription of \textit{glnK} might start at a second NtrC-independent promoter leading to a constitutive low level expression of the \textit{glnK}–\textit{amtB} operon. This would be similar to the situation found for the \textit{glnK}-like \textit{glnZ} gene in \textit{Azospirillum brasilense} (de Zamaroczy, 1998), where transcription of \textit{glnZ} starts from two different promoters (P1 and P2). Expression analysis of the \textit{glnZ} gene revealed that only transcription from the major P1 promoter (but not from the weaker P2 promoter) is regulated by NtrC in dependence on the cellular nitrogen status.

**Ammonium control of synthesis and activity of molybdenum nitrogenase is completely abolished in a \textit{glnB}–\textit{glnK} double mutant**

To study the function of \textit{R. capsulatus} GlnB and GlnK in the regulation of nitrogen fixation, single \textit{glnB} and \textit{glnK} mutant strains (PHU332 and SG26) as well as a \textit{glnB}–\textit{glnK} double mutant (TD166) were constructed (Methods). In contrast to the situation in \textit{glnK} single mutant strain SG26 (based on interposon mutagenesis; \textit{glnK}::Gm), the \textit{glnK} gene in the double mutant strain TD166 was disrupted by plasmid integration mutagenesis using pSG2.1 (Methods). At this point it should be noted that the phenotypes of single \textit{glnK} interposon and plasmid integration mutants were essentially the same (B. Masepohl, B. Lucas & T. Drepper, unpublished results). The influence of \textit{glnB} and \textit{glnK} single and double mutations on the \textit{nif}-encoded nitrogenase system was analysed at three different levels of regulation, namely the ammonium-dependent regulation of \textit{nifA} expression (level 1), the post-translational ammonium control of NifA activity (level 2) and the post-translational ammonium control of nitrogenase activity (level 3).

**Ammonium-dependent regulation of \textit{nifA} expression**

First, we examined accumulation of the transcription activator NifA1 in \textit{R. capsulatus} wild-type and in the mutant strains PHU332, SG26 and TD166 (Fig. 1a). For this purpose, cells were cultured under nitrogenase-depressing (−N) or -repressing (+N) conditions prior to protein extraction and Western analysis using a NifA1-specific antiserum raised against a synthetic oligopeptide corresponding to the N-terminal 17 amino acid residues (Paschen et al., 2001). In the wild-type strain, NifA1 accumulated only in N-limited cells, which is consistent with NtrC-mediated transcriptional control of the \textit{nifA} gene (Fig. 1a, lanes 1 and 2). As expected, inactivation of \textit{glnB} led to accumulation of a low level of NifA1 protein in ammonium-grown cells (Fig. 1a, lanes 3 and 4). In contrast, the \textit{glnK} mutant SG26 (Fig. 1a, lanes 5 and 6) showed a pattern of NifA1 accumulation that was similar to the wild-type. These data are corroborated by analysis of a \textit{nifA}–\textit{lacZ} fusion in the respective mutant backgrounds (Hübner et al., 1993; A. Paschen, S. Groß & W. Klipp, unpublished results). In the \textit{glnB}–\textit{glnK} double mutant TD166, synthesis and/or accumulation of NifA1 was greatly enhanced under both N-limiting and N-sufficient conditions (Fig. 1a, lanes 7 and 8). Thus it seems likely that \textit{R. capsulatus} GlnB is the predominant signal transducing protein for the first regulatory level (Ntr-mediated control of \textit{nifA} transcription), while GlnK is
Lanes: 1 and 2, B10S (wild-type); 3 and 4, PHU332 (glnB); 5 and 6, SG26 (glnK); 7 and 8, TD166 (glnB–glnK).

Fig. 1. Immunodetection of NifA1 and NifH in glnB and glnK mutant strains. R. capsulatus wild-type and mutant strains were grown in RCV minimal medium under nitrogenase-derepressing (10 mM serine as N-source; odd-numbered lanes) or repressing (20 mM ammonium as N-source; even-numbered lanes) conditions, before protein extracts were analysed by Western blots using antibodies against NifA1 (a) or NifH (b). The arrowhead marks the presence of a faint NifA1 band in mutant strain PHU332 (glnB) under ammonium-sufficient conditions (lane 4). Lanes: 1 and 2, B10S (wild-type); 3 and 4, PHU332 (glnB); 5 and 6, SG26 (glnK); 7 and 8, TD166 (glnB–glnK).

unable to fully substitute for GlnB in regulating activity of the sensor kinase NtrB.

Post-translational ammonium control of NifA activity

Previous studies demonstrated that constitutive (NtrC-independent) expression of nifA1 leads to a high-level accumulation of the NifA1 protein in both the absence and the present of ammonium, but NifA1-mediated nifH transcription was still inhibited by ammonium (Paschen et al., 2001). To further analyse the roles of GlnB and GlnK in this post-translational ammonium control of NifA activity, we examined the same protein extracts which were prepared for the NifA1 detection (Fig. 1a) by Western blot analysis using a NifH-specific antiserum (Fig. 1b). Most remarkably, extremely high amounts of NifH accumulated in the glnB–glnK double mutant TD166 in both the absence and presence of ammonium (Fig. 1b, lanes 7 and 8), suggesting that ammonium control of synthesis and activity of NifA is completely abolished in the absence of both PII-like proteins. In contrast to the situation described for TD166, in the glnB mutant strain PHU332 (where only GlnK is present), no accumulation of NifH was found under ammonium-sufficient conditions (Fig. 1b, lane 4), although significant amounts of NifA1 were present in this mutant strain under +N conditions (Fig. 1a, lane 4). These data clearly demonstrate that GlnK is sufficient for ammonium inhibition of NifA activity. Since mutant strain SG26 (glnK–glnB+) did not accumulate NifA1 and consequently did not synthesize NifH under +N conditions (Fig. 1a, b, lane 6), it remains speculative whether GlnB (like GlnK) might also be sufficient for ammonium control of NifA activity.

The role of GlnB and GlnK in the regulation of NifA activity has previously been examined in several diazotrophic bacteria, including Azospirillum brasilense, H. seropedicae, Rs. rubrum, K. pneumoniae, Azotobacter vinelandii and Azorhizobium caulinodans. There seem to be three different mechanisms of NifA regulation by PII-like proteins. (i) In Azospirillum brasilense, H. seropedicae and Rs. rubrum, GlnB is essential for NifA activity (Benelli et al., 1997; de Zamaroczy et al., 1993; Liang et al., 1992; Zhang et al., 2000), most likely involving a GlnB-mediated activation of preformed NifA under N-limiting conditions. (ii) In K. pneumoniae and Azotobacter vinelandii, GlnK functions as a signal transduction protein modulating the activity of NifL, which inactivates NifA under nitrogen-sufficient conditions (Arcondéguy et al., 1999; He et al., 1998; Holtel & Merrick, 1989; Jack et al., 1999; Little et al., 2000, 2002; Reyes-Ramirez et al., 2001; Rudnick et al., 2002). (iii) In contrast, neither GlnB nor GlnK is required for NifA activity under ammonium depletion in Azorhizobium caulinodans (Michel-Reydellet & Kaminski, 1999) and R. capsulatus (this study), but they seem to play a role in inactivation of NifA in the presence of ammonium.

Post-translational ammonium control of nitrogenase activity

Since a glnB–glnK double mutation led to constitutive expression of molybdenum nitrogenase, we asked whether nitrogenase was active in the presence of ammonium. This question was addressed by examination of the in vivo nitrogenase activity of R. capsulatus strain TD166 via the acetylene reduction assay and by measurement of H2 production (Table 2). Indeed, mutant strain TD166 exhibited high levels of nitrogenase activity in the presence of fixed nitrogen, demonstrating that a glnB–glnK double mutation leads to circumvention of all ammonium-dependent regulatory mechanisms inhibiting synthesis and activity of molybdenum nitrogenase. Furthermore, nitrogenase activity in TD166 was clearly enhanced under nitrogen-limiting conditions compared to the values obtained for the wild-type under the corresponding conditions. It is worth mentioning that in the glnB–glnK mutant the acetylene reduction activity of nitrogenase was four times higher than in the wild-type, whereas H2 production was only slightly (1.5-fold) enhanced. This observation might be explained by increased H2 consumption via uptake hydrogeneration, since expression of the R. capsulatus hupSL genes (encoding the small and large subunit of the uptake hydrogenase) is induced by H2 via the HupT/HupR two-component system.
system (Colbeau & Vignais, 1992; Toussaint et al., 1997; Dischert et al., 1999).

As described above, the glnB–glnK double mutant strain TD166 accumulated extremely large amounts of NifH protein and exhibited high levels of nitrogenase activity in the presence of ammonium. However, a protein band corresponding to the ADP-ribosylated form of NifH was clearly visible in extracts of all tested strains (even under \(2N\) conditions; Fig. 1b). Since harvesting of cells for protein extraction involved a centrifugation step (leading to darkness-induced ADP-ribosylation of NifH in the wild-type), we asked whether the ADP-ribosylated form of NifH was already present in the ammonium-grown cultures or appeared upon exposure of the cells to darkness.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Acetylene reduction*</th>
<th>(\text{H}_2) production†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(-N)</td>
<td>(+N)</td>
</tr>
<tr>
<td>B10S</td>
<td>Wild-type</td>
<td>921 ± 199</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>TD166</td>
<td>glnB glnK</td>
<td>3680 ± 902</td>
<td>1766 ± 681</td>
</tr>
</tbody>
</table>

*Activities are given in nmol ethylene produced h\(^{-1}\) (mg protein\(^{-1}\)). The results represent the means and standard deviations of six parallel assays.
†The values of nitrogenase-dependent \(\text{H}_2\) production assay are given in nmol \(\text{H}_2\) produced h\(^{-1}\) (mg protein\(^{-1}\)).

In order to discriminate between these two possibilities, we used highly nitrogen-limited (HNL) cultures, obtained by growing cultures to early stationary phase in RCV medium with \(N_2\) as sole nitrogen source. These growth conditions have previously been shown to be optimal for analysis of the ammonium-dependent switch-off response (Yakunin & Hallenbeck, 1998). To avoid darkness-induced ADP-ribosylation of NifH during cell harvesting, protein extracts were prepared using the rapid boiling method as described previously (Yakunin & Hallenbeck, 2002). Under these experimental conditions, switch-off of nitrogenase activity with a concomitant ADP-ribosylation of Fe-protein was readily observed in wild-type cells upon the addition of 200 \(\mu\)M ammonium to the culture (Fig. 2a). By contrast, the glnB–glnK double mutant strain TD166 was incapable

![Fig. 2](image-url)

Fig. 2. Ammonium-induced nitrogenase switch-off and ADP-ribosylation of NifH in the wild-type strain B10S (a) and the glnB–glnK mutant strain TD166 (b). HNL cultures were inoculated and grown photosynthetically in modified liquid RCV medium. Samples were withdrawn at the indicated times for the measurement of \(\text{in vivo}\) nitrogenase activity and the ADP-ribosylation status of Fe-protein as described by Yakunin et al. (1999). Results are presented as the total amounts of ethylene produced per vial. Ammonium was added as an anoxic solution to a final concentration of 200 \(\mu\)M at the time indicated by the arrow.
of carrying out either nitrogenase modification or switch-off of in vivo nitrogenase activity (Fig. 2b). Essentially the same results were obtained with these strains when a higher concentration (1 mM) of ammonium was used (data not shown). These results clearly demonstrate that in the glnB–glnK double mutant, both ammonium-induced short-term responses (ADP-ribosylation of NifH as well as the DraT/DraG-independent switch-off) are completely abolished. Therefore, ADP-ribosylation of NifH in the glnB–glnK mutant, as shown in Fig. 1(b), is probably caused by darkness-induced switch-off, indicating that this response is independent of the GlnB/GlnK-signalling system.

Similar results concerning the role of PII-like proteins in controlling DraT/DraG activity have recently been described for several other diazotrophic bacteria, including Rs. rubrum, Azorarcus sp. strain BH72 and Azospirillum brasilense (Klassen et al., 2001; Martin & Reinhold-Hurek, 2002; Zhang et al., 2000, 2001a, b). In all cases, the PII signal transduction proteins play an important role in regulating not only synthesis of nitrogenase but also activity of the enzyme complex in response to the cellular nitrogen status.

Recent studies demonstrated that R. capsulatus strains disrupted for the amtB gene (which is located immediately downstream of glnK) were defective in regulating in vivo molybdenum nitrogenase activity in response to ammonium but not to darkness (Yakunin & Hallenbeck, 2002). Since glnK mutations used in this study are thought to be polar onto amtB expression, we cannot exclude that loss of ammonium control of nitrogenase in the glnB–glnK double mutant is partially due to the absence ofAmtB. However, at least GlnB also appears to be involved in the regulation of DraT/DraG activity, since ammonium-dependent nitrogenase control was also absent in a single glnB mutation background (T. Drepper, B. Masepohl, A. F. Yakunin & P. C. Hallenbeck, unpublished results). To elucidate the specific roles of GlnB, GlnK and AmtB in control of nitrogenase activity in more detail, further studies based on defined mutant strains are required. For this purpose, a glnK mutant strain carrying a marker-less in-frame deletion, which should be non-polar onto amtB expression, has been constructed (B. Masepohl and coworkers, unpublished results). Analysis of this deletion strain in comparison with appropriate glnB and amtB mutants is currently under investigation.

The molybdenum and alternative nitrogenases are regulated differently in response to ammonium

Since a glnB–glnK double mutation led to complete loss of ammonium control of molybdenum nitrogenase, we asked whether this also holds true for the alternative nitrogenase. For this purpose, appropriate glnB and glnK single and double mutants were constructed in an nifHDK deletion background (KS36) to avoid any interference of molybdenum nitrogenase during analysis of the alternative nitrogenase. The resulting mutant strains were grown phototrophically in molybdenum-free medium (AK-NL) in the absence or presence of ammonium prior to determination of in vivo nitrogenase activity via the acetylene reduction assay. Under nitrogen limitation, all tested mutant strains exhibited nitrogenase activities comparable to that of the parental strain KS36 (data not shown). Unlike the situation with molybdenum nitrogenase, no activity of the alternative nitrogenase was detectable in the glnB–glnK double mutant under +N conditions (data not shown). Therefore, in contrast to ammonium control of the nif-encoded nitrogenase, absence of both GlnB and GlnK was not sufficient for synthesis of an active alternative nitrogenase in the presence of ammonium.

To determine at which regulatory level this PII-independent ammonium control of the alternative nitrogenase occurs, we first analysed expression of the anfA gene under nitrogen-sufficient and nitrogen-limiting conditions. For this purpose, the reporter plasmid pKS131A carrying an anfA–lacZ fusion (Kutsche et al., 1996) was introduced into R. capsulatus strains KS94A (anfA), TD52 (ntrC anfA), TD53 (glnB anfA) and TD120 (glnK anfA). As shown before, transcription of anfA was strongly induced under −N compared to +N conditions, and expression of anfA was strictly dependent on NtrC (Table 3; Kutsche et al., 1996). A mutation in glnB resulted in constitutive expression of anfA regardless of the absence or presence of ammonium in the growth medium. In contrast, the glnK mutant TD120 showed a pattern of anfA expression similar to the wild-type. These findings are in agreement with the current regulatory model where most of the NtrC molecules are permanently in the phosphorylated, active form when GlnB is absent, since GlnK appears to be unable to substitute for GlnB in regulating activity of NtrB.

In addition, protein extracts isolated from the R. capsulatus strains KS36 (ΔnifHDK), TD202 (glnB ΔnifHDK), TD178 (glnK ΔnifHDK) and TD205 (glnB–glnK ΔnifHDK) were examined by Western analysis using a NifH antiserum, known to recognize AnfH (Fig. 3a). All cultures grown in the absence of ammonium contained comparable amounts of AnfH. Table 3 shows the results of β-galactosidase assays on extracts from these strains. Essential for regulation of nitrogenase synthesis is the expression of NifH. In the absence of ammonium, significant amounts of NifH were not detected. This inhibition of NifH synthesis is relieved in strains expressing constitutive levels of NtrC (ΔnifHDK). Therefore, in the presence of ammonium, β-galactosidase activities in these strains correlated with the expression levels of NtrC.

Table 3. Expression of anfA–lacZ (pKS131A) in different genetic backgrounds

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity†</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−N</td>
</tr>
<tr>
<td>KS94A</td>
<td>Parental strain</td>
<td>676 ± 13</td>
</tr>
<tr>
<td>TD52</td>
<td>ntrC</td>
<td>31 ± 20</td>
</tr>
<tr>
<td>TD53</td>
<td>glnB</td>
<td>531 ± 21</td>
</tr>
<tr>
<td>TD120</td>
<td>glnK</td>
<td>647 ± 69</td>
</tr>
</tbody>
</table>

*All strains carried an anfA mutation and a plasmid-borne anfA–lacZ fusion (pKS131A).
†Miller units (Miller, 1972) and standard deviations were calculated from three independent assays of each strain.
of the unmodified and modified forms of dinitrogenase reductase, designated AnfH and AnfHADP-R, respectively (in all cases an additional protein band with a lower molecular mass was detectable in these strains, which might correspond to a degradation product of AnfH). These data indicate that different mutations in $glnB$ and/or $glnK$ do not significantly affect expression of the alternative nitrogenase under nitrogen-limiting conditions. In contrast to the molybdenum nitrogenase system, the alternative nitrogenase was not expressed in the presence of ammonium in any of the tested mutant backgrounds including the $glnB$–$glnK$ double mutant (TD186). These data were further corroborated by examination of appropriate $R$. capsulatus strains carrying plasmid pTD6-5I ($anfA^c$).

Constitutive expression of $anfA^c$ driven by the $aphII$ promoter was verified by analysis of strains harbouring an $anfA^c$–$lacZ$ fusion (data not shown). Subsequently, activation of the $anfH$ promoter mediated by $anfA^c$ was examined by immunodetection of AnfH (Fig. 3b). In all tested strains, including the $glnB$–$glnK$ double mutant (TD186), significant amounts of AnfH protein were detectable only under ammonium-deficient conditions. These experiments strongly suggest that ammonium inhibition of AnfA activity involves an as-yet-identified regulatory mechanism. At present it remains speculative whether this new mechanism acts completely independently of GlnB and/or GlnK. However, ammonium control of NifA and AnfA activity is clearly different.

As might be expected, the $glnB$–$glnK$ double mutant strain TD205 was also impaired in the DraT-mediated ammonium-dependent post-translational modification of AnfH (data not shown), reflecting that the $P_II$-like proteins generally transmit the ammonium signal to the DraT/DraG system, which in turn controls the activity of both the molybdenum nitrogenase and the alternative nitrogenase.

**Fig. 3.** Immunodetection of AnfH in $glnB$ and $glnK$ mutant strains. The parental strain KS36 ($ΔnifHDK$) and selected mutant strains were grown in molybdenum-free AK-NL minimal medium under nitrogenase-derepressing (5 mM serine as N-source; odd-numbered lanes) or repressing (20 mM ammonium as N-source; even-numbered lanes) conditions, before protein extracts were analysed by Western blots using cross-reacting antiserum against NifH. Accumulation of AnfH was tested for strains carrying wild-type $anfA$ (a) or a constitutively expressed $anfA$ gene ($anfA^c$, pTD6-5I; b), respectively. Lanes: 1 and 2, KS36 ($ΔnifHDK$); 3 and 4, TD176 ($ΔnifHDK$/$glnB$); 5 and 6, TD178 ($ΔnifHDK$/$glnK$); 7 and 8, TD186 ($ΔnifHDK$/$glnB$/$glnK$).

**Fig. 4.** Model of signal transduction by $P_II$-like proteins in $R$. capsulatus. GlnB and GlnK are involved in different levels of regulation of synthesis and activity of both nitrogenase systems by ammonium. For details, see text.
in a glnB–glnK double mutant. In contrast to NifA, even in a glnB–glnK double mutant, AnfA activity is still inhibited by ammonium. At a third level, the PII-like proteins are involved in the control of the DraT/DraG system. This regulatory model has been corroborated by preliminary yeast two-hybrid studies (Pawlowski et al., 2003). Protein–protein interactions were demonstrated for GlnB–NtrB, GlnB–NifA1, GlnB–NifA2, GlnB–DraT, GlnK–DraT, GlnK–NifA1, GlnK–NifA2 and GlnK–DraT. In accordance with this model, we could not detect interaction of GlnK with NtrB.

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