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Distinct Roles of P_{II}-Like Signal Transmitter Proteins and *amtB* in Regulation of *nif* Gene Expression, Nitrogenase Activity, and Posttranslational Modification of NifH in *Azoarcus* sp. Strain BH72

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P_{II}-like signal transmitter proteins, found in *Bacteria*, *Archaea*, and plants, are known to mediate control of carbon and nitrogen assimilation. They indirectly regulate the activity of key metabolic enzymes and transcription factors by protein-protein interactions with signal transduction proteins. Many *Proteobacteria* harbor two paralogous P_{II}-like proteins, GlnB and GlnK, whereas a novel third P_{II} paralogue (GlnY) was recently identified in *Azoarcus* sp. strain BH72, a diazotrophic endophyte of grasses. In the present study, evidence was obtained that the P_{II}-like proteins have distinct roles in mediating nitrogen and oxygen control of *nif* gene transcription and nitrogenase activity. Full repression of *nif* gene transcription in the presence of a combined nitrogen source or high oxygen concentrations was observed in wild-type and *glnB* and *glnK* knockout mutants, revealing that GlnB and GlnK can complement each other in mediating the repression. In contrast, in a *glnBK* double mutant strain in the presence of only GlnY, *nif* gene transcription was still detectable, albeit at a lower level, on nitrate or 20% oxygen. As another level of control, nitrogenase activity was regulated by at least three types of mechanisms in strain BH72: covalent modification of dinitrogenase reductase (NifH), probably by ADP-ribosylation, and two other, unknown means. Functional inactivation upon ammonium addition (switch-off) required the putative high-affinity ammonium transporter AmtB and GlnK, but not GlnB or GlnY. Functional inactivation in response to anaerobiosis did not depend on AmtB, GlnK, or GlnB. In contrast, covalent modification of NifH required both GlnB and GlnK and AmtB as response to ammonium addition, whereas it required either GlnB or GlnK and not AmtB when cells were shifted to anaerobiosis. In a *glnBK* double mutant expressing only GlnY, NifH modification was completely abolished, further revealing functional differences between the three P_{II} paralogues.

Bacteria encountering rapid changes in environmental conditions require complex regulatory networks to integrate the signals and to adapt the cellular machinery. The availability of nitrogen sources is an important parameter, resulting in rapid changes in enzyme activities and transcriptional activity. P_{II}-like proteins are central signal transmitter proteins in this regulatory network and occur in many *Proteobacteria* as two paralogous gene copies, *glnB* and *glnK* (44). A third copy (*glnY*) has recently been identified in the beta subgroup proteobacterium *Azoarcus* sp. strain BH72 (39). Depending on the cellular nitrogen status of the cell, a bifunctional uridylyl-transferase/hydrolase covalently modifies or demodifies the P_{II} protein. Under conditions of nitrogen deficiency, the P_{II}-like proteins in enteric bacteria occur mainly in the uridylylated form (for a review, see reference 2).

Depending on its state of modification, the P_{II} protein acts as a molecular switch by protein-protein interactions. One target in enteric bacteria is the adenylyltransferase, which regulates by covalent modification the activity of the key enzyme of ammonium assimilation, glutamine synthetase (30). Additionally, unmodified P_{II} (GlnB) inhibits autophsphorylation and activates the phosphatase activity of the target NtrB (29), which is part of a two-component regulatory system, resulting in a decrease in phosphorylated transcriptional regulator NtrC, thus preventing the transcription of Ntr-dependent operons (for a review, see reference 44).

The diversity of cellular responses to nitrogen raises the question whether additional proteins might act as receptors for P_{II}-like proteins. Most bacteria fixing N_2 react to a supply of ammonium by repression of transcription of nitrogenase structural genes, *nifHDK* (10, 41), and more rapidly by inactivation of nitrogenase activity (47, 53). NifA is the specific transcriptional activator of σ^{54}-dependent *nif* promoters, whose activity in enteric bacteria such as *Klebsiella pneumoniae* is regulated in response to combined nitrogen and oxygen by NifL (10).

The P_{II}-like protein GlnK is involved in the signal transduction cascade by relieving the NifL-dependent inactivation of NifA when combined nitrogen is limiting (20, 27). In several nitrogen-fixing bacteria, nitrogenase activity is also regulated at the posttranslational level. The so-called nitrogenase switch-off by ammonium depends on two different mechanisms. In some diazotrophs, such as *Rhodospirillum rubrum* (47), *Rhodobacter capsulatus* (31), and *Azospirillum brasilense* (17), the iron protein of nitrogenase (NifH) is subject to posttranslational modification, a reversible mono-ADP-ribosylation at a specific arginine residue. The *draT* gene product, an ADP-ribosyltransferase, covalently modifies nitrogenase, while DraG removes the ADP-ribosyl residue and thus reactivates nitrogenase (37).

In *Azoarcus* sp. strain BH72, a gene encoding a DraT ho-

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mologue (30% amino acid identity to DraT of *A. brasilense*) has been found upstream of *nifH* (Junker and Reinhold-Hurek, unpublished). The DraT/G system has recently been implicated as a target for P_{II}-protein-mediated regulation of nitrogenase activity in *R. rubrum* (62). Additionally, a physiological switch-off mechanism exists in some bacteria which does not involve a covalent modification of nitrogenase (46). The mechanism is still unknown; however, it has been suggested that the electron flow to nitrogenase may be involved (46). This is consistent with our study on the role of the ferredoxin FdxN, an electron donor to nitrogenase, which was shown to be essential for fast nitrogenase inhibition upon ammonium addition (14).

In many *Bacteria* and *Archaea*, the P_{II} paralogue *glnK* occurs in an operon with an *amtB* gene. *amtB* genes encode integral membrane proteins which were identified as high-affinity ammonium transporters in *Saccharamyces cerevisiae* (38) and *Arabidopsis thaliana* (45). An involvement in ammonium transport is also assumed for bacteria (9, 43, 54). It has been proposed that the conservation of physical linkage of *glnK* and *amtB* reflects a functional relationship and a physical interaction of these proteins (57).

An additional level of complexity is added to this regulatory system by the occurrence of two structurally and functionally similar P_{II} paralogues. In *Escherichia coli*, in contrast to *glnB*, *glnK* is expressed only under conditions of nitrogen limitation (3, 58). In the diazotroph *Klebsiella pneumoniae*, only GlnK and not GlnB regulates the activity of NifA or NifL (20, 27). However, findings in enteric bacteria cannot be generalized, and differential functions may vary depending on the bacterial species. In the alpha-proteobacterial diazotrophs *Azospirillum brasilense* and *Azorhizobium caulinodans*, the paralogue essential for free-living nitrogen fixation is GlnB (9), or both paralogues can complement each other (43), respectively.

The diazotroph *Azoarcus* sp. strain BH72 is an endophyte of grasses which can also infect rice (24, 51). It is a strictly respiratory bacterium which fixes nitrogen under microaerobic conditions, reaching steady states in a chemostat at 0.5 to 25 μM dissolved O_2 (23). Reporter gene studies have shown a regulation at the transcriptional level in response to O_2 and ammonium; *nifH*::*gus* expression was not detectable at or above 4% O_2 in the headspace or above 0.5 mM ammonium (12). Strain BH72 harbors three paralogous P_{II}-like proteins, all of which can be uridylylated and are thus likely to have a function in nitrogen sensing (39). Like *glnK*, the novel third paralogue, *glnY*, is physically linked with an amount gene (*amtY*). Unlike in *E. coli*, both paralogues GlnB and GlnK are abundant in *Azoarcus* spp. under conditions of nitrogen excess.

Interestingly, neither GlnK nor GlnB is essential for nitrogen fixation when GlnY is still present (39). GlnY can only be detected in a *glnK glnB* double mutant strain; however, expression levels are low on ammonium as the nitrogen source (39). This novel paralogue was found to be unusual, as it only occurred in the uridylylated state in vivo as shown by mass spectrometric analysis (39). In the present study P_{II}-like proteins were shown to have distinct roles for the physiological switch-off and posttranslational covalent modification of dinitrogenase reductase (NifH) upon addition of ammonium or anaerobiosis, underlining that they are paralogues and not homologues. Moreover, the AmtB protein was essential for ammonium-induced switch-off in *Azoarcus* sp. strain BH72, probably serving as an ammonium sensor transmitting the signal to membrane-associated GlnK.

(Preliminary accounts on the involvement of P_{II}-like proteins in regulation of nitrogenase activity or their membrane association were presented on the 12th International Congress on Nitrogen Fixation, Brazil, 1999 [50], or at the 4th European Nitrogen Fixation Conference, Spain, 2000.)

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. Growth of *Azoarcus* sp. strain BH72 on complex media for electroporation and mutant selection was performed as described earlier (11, 39). *E. coli* was grown in LB medium following standard protocols (4). To obtain N_2-fixing cells of *Azoarcus* sp., bacteria were precultured on SM + N medium (48), washed twice in N-free SM medium (48), adjusted to an optical density at 578 nm (OD_{578}) of 0.05 in the same medium, and incubated under microaerobic conditions in rubber stopper-sealed 1-liter Erlenmeyer flasks sparged with N_2 and adjusted to 1.5% O_2 and 1% acetylene in the headspace, with rotary shaking at 100 rpm.

For nitrogenase derepression analyses, precultivation was performed in the presence of either 10 mM NH_4Cl or KNO_3 as the sole nitrogen source, and cells were transferred to microaerobic conditions (0.8% O_2) as described above at an initial OD_{578} of 0.05 in 30 ml of medium and grown for 10 h. For nitrogenase switch off (repression) analyses, N_2-fixing cells were incubated as described above on N-free medium followed by addition of 0.2 and 2 mM NH_4Cl or KNO_3. Alternatively, cells were transferred to an anaerobic Erlenmeyer flask containing 1% acetylene using a 50-ml syringe.

Membrane isolation. Cells were grown under conditions of N_2 fixation or on VM-ethanol (complex) medium (52) as described above and harvested at 4°C by centrifugation for 10 min at 5,000 × *g*. The cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.0) and sonicated five times (30-min pulses each, 40-W output) using a Branson Sonifier 250. Cellular debris was removed by centrifugation (20 min, 20,000 × *g*), and the remaining supernatant was used for membrane sedimentation by ultracentrifugation (2 h, 200,000 × *g*). The remaining supernatant was referred to as the soluble cytoplasmic fraction, whereas the isolated membrane fractions were washed twice with 50 mM sodium phosphate buffer (pH 7.0) followed by two salt washes using the same buffer supplemented with 600 mM NaCl to remove nonspecifically or loosely bound proteins. The insoluble pellet after ultracentrifugation was referred to as the membrane fraction.

Determination of nitrogenase and β-glucuronidase activity. Nitrogenase activity of batch cultures was determined by using the acetylene reduction method (13). Activity of β-glucuronidase was measured quantitatively using the method described earlier (28) as modified (12) and expressed in Miller units, defined as $E_{420} \times 1{,}000/t$ (minutes) × OD_{600}.

DNA and RNA analyses. Isolation of chromosomal DNA was carried out as described previously (22). Other DNA techniques followed standard protocols (4). Genomic clones were characterized by restriction mapping and Southern blot analyses with digoxigenin-labeled gene probes (39). DNA was sequenced from both strands as described previously (26). Homology searches were carried out using the Blast program (1). Prediction of membrane protein topology of AmtB and AmtY was performed using the program TopPred2. The *amtB* and *amtY* sequences of *Azoarcus* sp. strain BH72 have been assigned GenBank accession numbers AF430400 and AF430401.

Construction of plasmids for marker exchange mutagenesis and a *nifH*::*gusA* reporter gene fusion. Knockout mutants BHGLKK (*glnK*), BHGLBS (*glnB*), and BHGLKKBS (*glnBK*) had been obtained by insertion of resistance cartridges (39). The *amtB* gene was inactivated by cloning the kanamycin resistance cartridge (1.3 kb) of pUC4K into the *Srf* I restriction site (635 bp downstream of *amtB* start codon) of pDZD17.1, resulting in plasmid pDM3. Plasmid pDZD17.1 was generated by insertion of a 3.6-kb *Hin*dIII/*Spe*I fragment of pDZD17 (39) into pBluescriptSK. The orientation of the kanamycin cassette resulting in a nonpolar mutation was checked by restriction digestion (*Eco*RI/*Hin*dIII).

Marker exchange mutant strain BHABK (*amtB*) was obtained by transformation of *Azoarcus* sp. strain BH72 by electroporation with the suicide plasmid pDM3. Southern blot analysis using a *glnK* gene probe (39) verified the correct chromosomal integration of the resistance cartridge since the hybridizing 5.1-kb *Eco*RI fragment harboring the *glnK-amtB* operon shifted to 6.3 kb in size.

TABLE 1. Bacterial strains, cloning vectors, and recombinant plasmids used

Strain, vector, or plasmid	Relevant genotype or properties	Source or reference
E. coli DH5α	F′ *recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44* (λ^- *thi-1 relA1* ϕ80d *lacZ*Δ*M15* Δ(*lacZYA-argF′*)*U169*	19
Azoarcus sp.		
BH72	Wild type	49
BHGLKK	Km^r, BH72 *glnK*::Km^r	39
BHGLBS	Sm/Sp^r, BH72 *glnB*::Sm/Sp^r	39
BHGLKKBS	Km^r, Sm/Sp^r, BH72 *glnK*::Km^r, *glnB*::Sm/Sp^r	39
BHABK	Km^r, BH72 *amtB*::Km^r	This study
Cloning vectors		
pBluescript SK	Ap^r, ColE1 origin	Stratagene
pBK-CMV	Km^r, Neo^r, ColE1 origin, fl(−) origin, simian virus 40 origin	Stratagene
pUC4K	Km^r, Neo^r, ColE1 origin	Pharmacia
pLAFR3	Tet^r, low-copy cosmid vector	56
Recombinant plasmids		
pDZD17	Km^r, *glnk-amtB* locus on a 5.8-kb chromosomal *Sau*IIIAI fragment of strain BH72 in pBK-CMV	39
pDZD17.1	Ap^r, 3.6-kb *Hin*dIII/*Spe*I fragment of pDZD17 cloned in pBluescript SK	This study
pDM3	Ap^r, Km^r, 1.3-kb Km^r cassette of pUC4K cloned in *Srf*I restriction site of pDZD17.1 (600 bp downstream of *amtB* start codon)	This study
pEGN3.1	Ap^r, *nifH*::*uidA* transcriptional fusion on pUC19	12
pNHGus	Tet^r, 3.3-kb *Hin*dIII/*Eco*RI fragment harboring *nifH*::*uidA* fusion of pEGN3.1 cloned in pLAFR3	This study
pKKOM	Tet^r, 1.5-kb *Srf*I/*Kpn*I fragment of pDZD17 cloned in pLAFR3	This study

Plasmid pNHGus harboring a transcriptional *nifH*::*gusA* fusion was generated by cloning a 3.3-kb *Hin*dIII/*Eco*RI fragment of pEGN3.1 (12) into the low-copy vector pLAFR3 (56). pHNGus was transformed into *Azoarcus* sp. strain BH72 wild-type, BHGLBS, BHGLKK, and BHGLKKBS by the method of triparental mating using *E. coli*(pRK2013) as the helper strain (11).

SDS-PAGE and Western blotting. For the analysis of NifH modification, 1-ml aliquots of cell suspensions were removed from the culture, and proteins were precipitated immediately on ice with 100 μl of trichloroacetic acid (TCA) solution (1 g of TCA per ml) as described (61). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24, 35). For separation of the two forms of NifH protein, a 10% (wt/vol) acrylamide low cross-linker gel with a ratio of acrylamide to *N,N′*-methylenebis(bisacrylamide) of 172:1 was used (32).

Electroelution of proteins onto nitrocellulose membranes was performed as described previously (24) for 45 min at 8 V with a semidry electroblotter (Bio-Rad, Munich, Germany). The NifH protein of *Azoarcus* sp. strain BH72 was detected using antiserum against NifH of *Rhodospirillum rubrum*, kindly provided by R. Ludden (Madison, Wis.) as outlined previously (24). GlnB, GlnK, and GlnY were immunodetected using antisera raised against purified fusion proteins of *E. coli* maltose-binding protein (MalE) and GlnB, GlnK, and GlnY, respectively (39). Proteins were visualized using ECL Western blotting detection reagents (Amersham Pharmacia Biotech). Protein concentrations were determined by the Bio-Rad protein assay based on the method of Bradford (6).

Biochemical analyses. For analyses of NifH modification, protein extracts (sonicated cell extracts after centrifugation of cell debris; see membrane isolation) were incubated with RNase A (30 min at 30°C in 50 mM sodium phosphate buffer, pH 7.2), calf intestine alkaline phosphatase (30 min at 37°C in 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA), or snake venom phosphodiesterase I (30 min at 25°C in 100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 15 mM MgCl) using approximately 1 U of enzyme and 20 μg of protein extract per assay. Protein extracts were also treated with 1 M hydroxylamine (pH 7.0), 10 mM $HgCl_2$, 1 M HCl, or 1 M NaOH at 30°C for 6 h. Reaction mixtures were analyzed by SDS-PAGE, Western blotting, and immunodetection using NifH antiserum. Determination of ammonia concentrations in culture supernatants was performed using an enzymatic ammonium assay (Roche Molecular Biochemicals, no. 1112732) with a detection limit of 1 μM.

RESULTS

Effect of P_{II}-like proteins on nitrogen-regulated *nif* gene expression. *Azoarcus* sp. strain BH72 mutants in which the paralogues *glnB* or *glnK* had been inactivated showed equal growth rates on N_2 compared to the wild type, indicating that nitrogenase gene expression under derepressing conditions was not abolished in a *glnB* or *glnK* knockout mutant (39). In order to test the influence of the P_{II} paralogues on *nifH* gene transcription in response to combined nitrogen, nitrogenase gene expression was quantified using a *nifH*::*gusA* fusion on a stably replicated plasmid (pNHGus). Cells were cultivated aerobically on 10 mM of a combined nitrogen source (nitrate or ammonium), transferred to microaerobic conditions into medium containing 10 mM of the respective nitrogen source, and harvested for quantification of β-glucuronidase activity after 10 h of incubation. In wild-type cells, β-glucuronidase activity was strongly repressed by KNO_3 or NH_4Cl in comparison to N-free medium (Table 2). Mutants in which *glnB* or *glnK* was inactivated and which expressed GlnK or GlnB, respectively (39), reacted like the wild-type strain (Table 2). No acetylene reduction activity was detected in the presence of nitrate or ammonium, and in Western blot analysis using specific antibodies, the iron protein of nitrogenase was not detected in cell extracts (not shown), corroborating a strong repression of *nifH*.

In contrast, in a *glnBK* double mutant which expresses GlnY (39), *nifH*::*gusA* expression was not completely repressed by nitrate and was still present at lower levels (10-fold repression) in the presence of ammonium (Table 2). Accordingly, acetylene reduction activity was high in this double mutant grown in the presence of nitrate and still detectable although at much lower levels during growth on ammonium (Table 2), when GlnY expression is very low (Fig. 1) (39). These results indicated that GlnB and GlnK were involved in efficient signal transduction leading to full *nif* gene repression in single mutants, while in the double mutant the presence of GlnY alone did not allow full repression.

Additionally, Western blot analyses were carried out with antibodies specific to GlnY (39) or NifH (Fig. 1). As expected, in cells grown microaerobically on nitrate, both, GlnY and the iron protein of nitrogenase occurred in significant amounts

TABLE 2. Nitrogenase activity and *nifH*::*gusA* expression of *Azoarcus* sp. strain BH72 wild-type, BHGLBS (*glnB*), BHGLKK (*glnK*), and BHGLKKBS (*glnBK*) strains grown microaerobically in SM medium supplemented with 10 mM NH_4Cl or KNO_3[a]

Genotype	N source	Nitrogenase activity (nmol of C_2H_4/mg of protein)	*nifH*::*gusA* expression (Miller units) in 1%/20% O_2
Wild type	NH_4Cl	n.d.	80 ± 10/<50
	KNO_3	n.d.	120 ± 10/<50
	N_2	10,430 ± 530	7,200 ± 200/n.dm.
glnB	NH_4Cl	n.d.	100 ± 10/<50
	KNO_3	n.d.	150 ± 20/<50
	N_2	9,240 ± 150	6,320 ± 410/n.dm.
glnK	NH_4Cl	n.d.	90 ± 10/<50
	KNO_3	n.d.	140 ± 10/<50
	N_2	9,460 ± 460	6,910 ± 390/n.dm.
glnBK	NH_4Cl	70 ± 10	710 ± 30/<50
	KNO_3	4,010 ± 360	2,860 ± 60/700 ± 60
	N_2	7,320 ± 410	7,100 ± 450/n.dm.

[a] Data were obtained from three parallel measurements from two independent experiments and are given with standard deviations. n.d., not detected; n.dm., not determined (cells did not grow under atmospheric O_2 concentrations (20%) with N_2 as the sole nitrogen source).

(lane 1), in contrast to cells grown on ammonium (lanes 4 and 5). Surprisingly, cells grown aerobically on nitrate also showed nitrogenase expression (lane 2). Accordingly, *nifH*::*gusA* reporter gene activity was significantly higher in this strain compared to the wild type (Table 2), suggesting circumvention of the O_2^- control of *nif* gene expression.

GlnK and AmtB are involved in nitrogenase inhibition (switch-off) by ammonium. Previously we have shown that upon addition of ammonium to nitrogen-fixing cells, nitrogenase activity is rapidly inhibited (14). We observed that upon ammonium addition to an N_2-fixing culture in a biofermenter with mass-flow-controlled oxygen supply (39), oxygen consumption was dramatically decreased after addition of ammonium (not shown). To assess whether P_{II}-like proteins are involved in the signaling pathway leading to nitrogenase inhibition, knockout mutants were tested in switch-off experiments using 2 mM NH_4Cl (Fig. 2B). The *glnB* mutant expressing only GlnK showed a wild-type level inhibition of nitrogenase activity, while the *glnK* mutant expressing GlnB and the *glnBK* double knockout mutant expressing GlnY were not rapidly inhibited (Fig. 2A). N_2 fixation of the latter mutant was almost not affected within 2 h. In the *glnK* mutant, acetylene reduction decreased slowly within 2 h, which can be explained by a decreased synthesis of nitrogenase due to strong repression of *nifHDK* transcription by ammonium, which is still observed in this mutant (see Table 2).

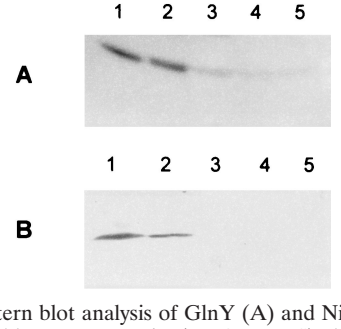

FIG. 1. Western blot analysis of GlnY (A) and NifH (B) synthesis of a *glnBK* double mutant strain (BHGLKKBS) during growth on ammonium or nitrate. Equal amounts (20 μg) of protein extracts from cells grown microaerobically on 10 mM nitrate (lane 1), aerobically on 10 mM nitrate (lane 2), microaerobically on 10 mM ammonium (preculture on nitrate) (lane 3), aerobically on 10 mM ammonium (preculture on ammonium) (lane 4), or microaerobically on 10 mM ammonium (preculture on ammonium) (lane 5) in SM medium were separated by SDS-PAGE, followed by immunodetection of GlnY and NifH.

The *glnK* gene is organized in an operon with *amtB*, encoding a putative membrane protein with high homology to ammonium transporters (50% identity to *E. coli amtB*) (39); typically, these proteins have 11 to 12 transmembrane helices; 12 membrane helices are also predicted for AmtB of strain BH72 (using programTopPred2). To rule out a polar effect of the *glnK* mutation on *amtB* function, the *glnK* gene including the upstream promoter region was complemented in *trans* on a plasmid (pKKOM). The resulting strain, BHGLKK(pKKOM), was functionally complemented, since nitrogenase activity was rapidly inhibited as in the wild-type *Azoarcus* sp. strain (Fig. 2A and B). Additionally, an insertion mutation inactivating the *amtB* gene (strain BHABK) resulted in loss of the rapid ammonium switch-off, similar to the *glnK* mutation (Fig. 2B). Therefore, both proteins, AmtB and GlnK, were essential for the signaling pathway leading to physiological nitrogenase switch-off by ammonium in *Azoarcus* sp. strain BH72.

The rapid nitrogenase switch-off of N_2-fixing cells was not observed upon addition of the alternative nitrogen source nitrate (Fig. 2C) or glutamine (not shown), revealing that this response is specific to ammonium. Addition of nitrate led to a slow decrease in acetylene reduction activity in the wild type as well as in single *glnB* or *glnK* mutants (not shown), probably resulting from repression of *nifHDK* transcription by nitrate (see Table 2). In the *glnBK* double mutant, which expresses GlnY and is not subject to nitrogenase gene repression (see above), acetylene reduction activity remained at a high level (Fig. 2C).

Since *amtB* encodes a putative ammonium transporter, the *glnK* knockout mutation may affect ammonium transport. Therefore, uptake of ammonium from the medium was monitored in the wild-type and in the mutant strains BHABK (*amtB*) and BHGLKK (*glnK*) after addition of 2 mM and 0.2 mM ammonium under the same conditions applied for switch-off experiments (Fig. 3). Wild-type and mutant strains assimilated ammonium from the medium at approximately the same rate at an initial concentration of 2 mM (70 to 75 nmol/min/mg of protein), and thus overall NH_4^+ transport was not affected by the *amtB* mutation under these conditions. However, ammonium uptake at low concentrations of ammonium (0.2 mM) was slightly higher in the wild-type strain and the *glnK* strain (10 nmol/min/mg of protein) compared to the *amtB* mutant (5 nmol/min/mg protein), which might indicate a function of AmtB for efficient NH_4^+ uptake during exposure to low ammonium concentrations.

Since GlnK and AmtB were shown to be essential for ammonium-induced switch-off and GlnK did not affect NH_4^+ transport via AmtB or other uptake systems under the conditions investigated, it is likely that AmtB fulfills a sensory function in detection of changes in the external ammonium concentration. These results suggest a dual role of AmtB as an ammonium transporter and ammonium sensor.

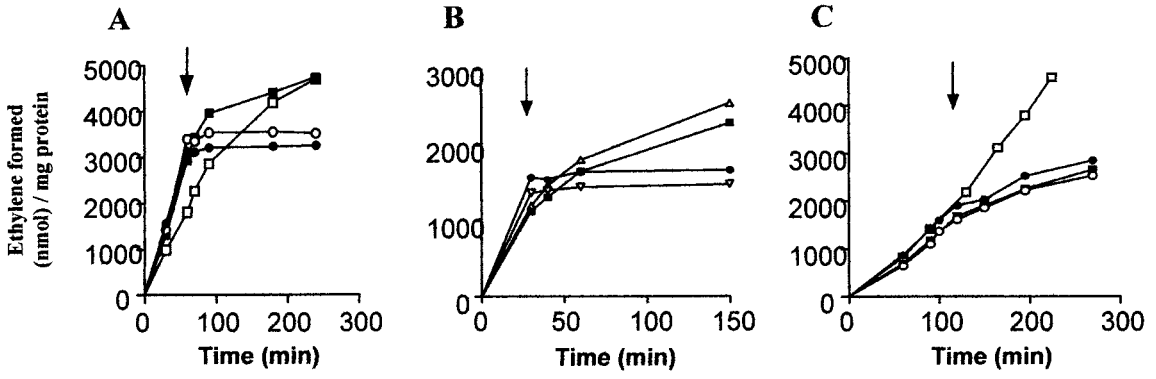

FIG. 2. Effects of ammonia and nitrate addition on nitrogenase activity of N_2-fixing cultures of wild-type *Azoarcus* sp. strain BH72 and *glnB*, *glnK*, *glnBK*, and *amtB* knockout mutants. Data are based on at least two independent experiments for all conditions tested. Arrows indicate time of ammonia or nitrate addition. (A and B) Influence of ammonium addition (2 mM NH_4Cl) on acetylene reduction (nitrogenase activity) of wild-type *Azoarcus* sp. strain BH72 (●), *glnB* (○), *glnK* (■), *glnBK* (□), and *amtB* (△) mutant strains, and *glnK* complementation strain BHGLKK/pKKOM (▽). (C) Effect of nitrate addition (2 mM KNO_3) on acetylene reduction (nitrogenase activity) of wild-type *Azoarcus* sp. strain BH72 (●) and *glnB* (○), *glnK* (■) and *glnBK* (□) mutant strains.

Effect of P_{II}-like proteins and AmtB on posttranslational modification of dinitrogenase reductase. To analyze the effect of mutations on the putative covalent modification of dinitrogenase reductase in *Azoarcus* sp. strain BH72, protein extracts of cells were subjected to Western blot analysis using antibodies against NifH (Fig. 4). Previously it was shown that the NifH protein of *Azoarcus* sp. strain BH72 was covalently modified in response to oxygen deficiency: a NifH protein of lower electrophoretic mobility accumulated (difference of approximately 1.5 kDa) which had identical amino acid sequences in the N terminus with the unmodified NifH protein usually observed (25).

After addition of ammonium, a modified NifH protein of higher apparent molecular weight accumulated (Fig. 4, lanes 2 and 3). To determine the type of NifH modification, protein extracts from an ammonium switch-off experiment were treated with either phosphodiesterase I or neutral hydroxylamine (NH_2OH), which both led to the disappearance of the higher molecular weight protein in contrast to untreated controls in Western blots (not shown) and thus to a complete removal of the modifying group. Incubation with RNase A, alkaline phosphatase, 10 mM $HgCl_2$, or 1 M HCl had no effect on NifH modification, whereas incubation with 1 M NaOH abolished the antigenicity of both the modified and the unmodified form of NifH (data not shown).

Phosphodiesterase I is known to cleave substrates harboring a phosphodiester bond and is therefore in general able to cleave protein-bound adenyl-, uridylyl-, or ADP-ribosyl residues (8, 36, 55). NH_2OH has been shown to specifically hydrolyze ADP-ribose from arginine, whereas $HgCl_2$ cleaves only cysteine-bound ADP-ribose (7). Therefore, it is very likely, by analogy to other reports, e.g., in *R. rubrum* and *Rhodobacter capsulatus* (31, 47), that the modifying group of NifH under conditions of ammonium switch-off represented an ADP-ribosyl moiety covalently linked to an arginine residue.

Inactivation of single genes *glnB*, *glnK*, or *amtB* abolished the alteration of the electrophoretic mobility and thus the modification (Fig. 4), indicating that the presence of both P_{II}-like proteins as well as AmtB was necessary for this function.

We also analyzed the effect of oxygen limitation on NifH modification. All mutant strains investigated reacted with a fast and complete inhibition of nitrogenase activity upon transfer to anaerobic conditions (not shown). Since *Azoarcus* sp. strain BH72 has a strictly respiratory type of metabolism, nitrogenase

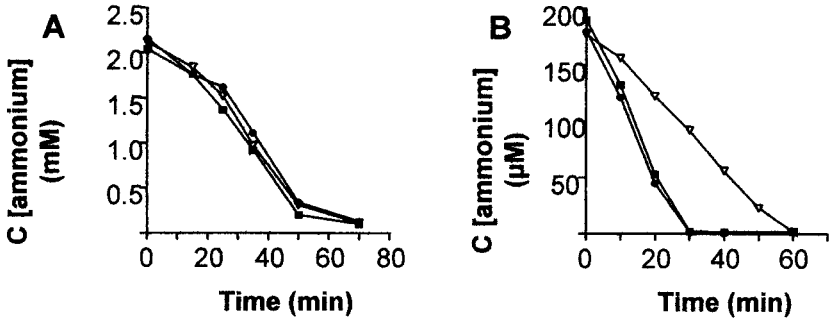

FIG. 3. Ammonium assimilation of *Azoarcus* sp. wild-type strain BH72, BHGLKK (*glnK*), and BHABK (*amtB*). Decrease of ammonium concentrations in supernatants after an initial addition of 2 mM NH_4Cl (A) or 0.2 mM NH_4Cl (B) to N_2-fixing cultures of *Azoarcus* sp. wild-type strain BH72 (●) and *glnK* (■) and *amtB* (▽) mutant strains.

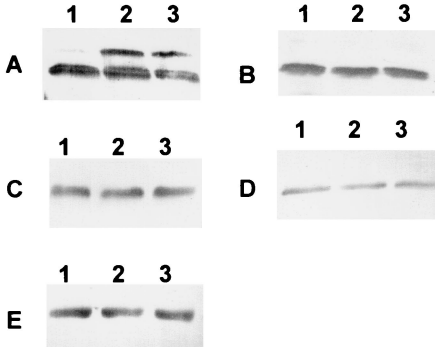

FIG. 4. Western blot analysis of NifH modification after ammonium addition to nitrogen-fixing cultures of *Azoarcus* sp. wild-type strain BH72 (A) and BHGLBS (*glnB*) (B), BHGLKK (*glnK*) (C), BHGLKKBS (*glnBK*) (D), and BHABK (*amtB*) (E). Samples were taken before (lane 1) or 5 min (lane 2) or 15 min (lane 3) after addition of 2 mM NH_4Cl. Experiments were performed at least two times.

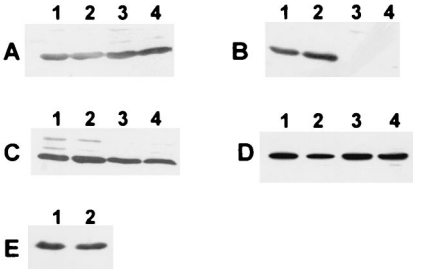

FIG. 6. Determination of cellular localization of GlnB, GlnK, and GlnY by Western blot analysis. Cytoplasmic (lanes 1 and 2) and membrane fractions (lanes 3 and 4) (20 μg of protein per lane) of *Azoarcus* sp. strain BH72 wild-type (A and B), strain BHGLKKBS (*glnBK*) (C), and strain BHABK (*amtB*) (D) grown on SM medium with 10 mM NH_4Cl (lanes 1 and 3) or grown under conditions of nitrogen fixation on N-free SM medium (lanes 2 and 4) were analyzed using specific antisera against GlnK (A, D, and E), GlnB (B), and GlnY (C). (E) Membrane fractions of N_2-fixing wild-type cells before (lane 1) and 15 min after (lane 2) ammonium switch-off.

inhibition was most likely due to energy limitation. Western blot analyses showed that nitrogenase modification upon oxygen limitation was not affected in single *glnB*, *glnK*, or *amtB* mutants, but was abolished in the *glnBK* double mutant (Fig 5). These results suggested that the signal transduction pathways leading to modification of NifH were different for the two stimuli tested, ammonium excess and oxygen limitation.

Apparently the presence of both P_{II} paralogues, GlnB and GlnK, and AmtB was required for the response to ammonium, while for the response to anaerobiosis, AmtB was not required at all, and the presence of either GlnK or GlnB was necessary. Since nitrogenase inhibition still occurred in the double mutant, which expresses only GlnY and does not show a NifH modification, the modification is apparently not a prerequisite for inhibition.

GlnK and GlnY occur membrane associated, in contrast to GlnB. As GlnK and the putative membrane protein AmtB were both found to be part of the signal transduction cascade for physiological nitrogenase switch-off upon ammonium addition, we speculated that GlnK, albeit a cytoplasmic protein, might occur in association with membranes due to interactions with integral membrane proteins. Therefore, membrane and cytoplasmic fractions were extracted from *Azoarcus* sp. strain BH72 cells, and the membranes were treated with salt (600 mM NaCl) to remove loosely attached proteins. Both fractions were compared in Western blot analysis using specific antibodies.

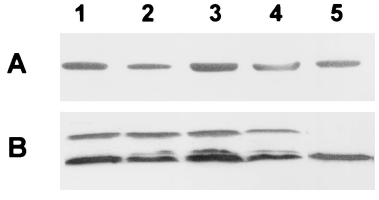

FIG. 5. Western blot analysis of NifH modification upon anaerobiosis. N_2-fixing cultures of the *Azoarcus* sp. were transferred from microaerobic conditions (0.8% oxygen) (A) to anaerobic conditions. Samples were taken after 5 min of anaerobic incubation (B). Lanes: wild-type strain BH72 (lane 1), *glnB* (lane 2), *glnK* (lane 3), *amtB* (lane 4), and *glnBK* (lane 5) mutant strains.

GlnB was detected exclusively in the cytoplasmic fractions (Fig. 6B, lanes 1 and 2), whereas GlnK and GlnY also occurred membrane associated: antibodies reacting with GlnK and GlnY localized a protein in the membrane as well as cytoplasmic fractions, which was GlnK in wild-type cells (Fig. 6A), as GlnY cannot be detected in wild-type *Azoarcus* sp. strain BH72 (39). In the *glnBK* double mutant, the reacting protein was GlnY (Fig. 6C). Membrane fractions of cells grown on SM medium with 10 mM NH_4Cl (Fig. 6A, lanes 1 and 3), on complex medium (not shown), at conditions of N_2 fixation (lanes 2 and 4), or at N_2-fixing conditions after ammonium switch-off (Fig. 6E, lanes 1 and 2) contained GlnK, indicating that the nitrogen status of the cell did not strongly affect the binding of these proteins. Moreover, GlnK was still detectable in association with membranes in the *amtB* mutant strain BHABK (Fig. 6D).

DISCUSSION

Here we report evidence that the three paralogous P_{II}-like proteins of *Azoarcus* sp. strain BH72 are involved differently in regulation of *nif* gene expression, nitrogenase activity and post-translational modification of dinitrogenase reductase (NifH).

In several diazotrophic bacteria, one P_{II} paralogue is essential for nitrogen fixation, however, the identity may differ. In *Azotobacter vinelandii*, which harbors only one gene encoding a P_{II}-like protein (GlnK), a *glnK* null mutant could not be obtained up to now (40). In *Azospirillum brasilense* (9) and *Herbaspirillum seropedicae* (5), a null mutation of *glnB* results in a Nif^- phenotype. In *Azorhizobium caulinodans* only a *glnK glnB* mutant is Nif^-, since GlnK and GlnB can complement each other for free-living N_2 fixation; however, symbiotic N_2 fixation requires GlnB (43). In contrast, neither GlnB nor GlnK was essential for nitrogen fixation in *Azoarcus* sp. strain BH72; in the *glnBK* double mutant, GlnY is expressed, which might be sufficient to allow N_2 fixation (39).

In accordance with these previous results, transcriptional activity of the *nifH* promoter was not affected by null mutations of *glnK*, *glnB*, or a *glnBK* double mutation under N-limiting conditions. However, the presence of GlnB or GlnK was nec-

essary to confer full repression of nitrogenase gene transcription in response to combined nitrogen sources. In members of the gamma *Proteobacteria* such as *Klebsiella pneumoniae* (21, 42), in addition to the GlnB-dependent NtrBC pathway regulating *nifA* expression, NifL encoded in the *nifLA* operon inhibits NifA activity in the presence of high oxygen or ammonium concentrations. In *K. pneumoniae*, only GlnK and not GlnB is able to relieve this inhibition when ammonium is limiting (20, 27). In *Azoarcus* sp. strain BH72, *nifLA* homologues were also detected, their transcription being only moderately repressed in the presence of ammonium (Egener, Martin, and Reinhold-Hurek, submitted). NifLA might thus be an additional regulatory target for GlnK or GlnB.

The third P_{II} paralogue in *Azoarcus* spp., GlnY, is unusual in several aspects. In a *glnBK* double mutant expressing only GlnY, the *nifH* gene transcription was only weakly repressed in the presence of nitrate. This may be due to the uridylylation state of GlnY. While both GlnK and GlnB are differentially uridylylated in response to changing ammonium concentrations, the third paralogue, GlnY, is not deuridylylated upon addition of ammonium or in the presence of nitrate but can only be detected in the uridylylated form in vivo (39). Therefore, GlnY is apparently not able to serve as a signal transmitter for changing nitrogen conditions.

Since GlnY is only detectable in a *glnBK* double mutant background, it cannot be excluded that deuridylylation might occur in the presence of another paralogue; however, such a dependence on a second paralogue would be very unusual. In single *glnK* or *glnB* mutants of *A. brasilense* (9) or *E. coli* (58), both proteins were modified or demodified in response to nitrogen in vivo. The structural reason for this unusual feature of GlnY still needs to be elucidated.

Transcription of *nifH* or nitrogenase protein synthesis was only observed under conditions which allowed high levels of GlnY expression, e.g., on nitrate (see Fig. 1 and Table 2). Surprisingly, in the *glnBK* mutant strain, the repression of nitrogenase synthesis by high oxygen concentrations was also relieved (see Fig. 1, lane 1, and Table 2). This indicates that either directly or indirectly by regulating expression of other genes involved, P_{II}-like proteins may also play a role in the signal transduction pathway controlling *nif* gene expression in response to oxygen, presumably by interaction with the NifLA complex, which was also detected in strain BH72 (Egener, Martin, and Reinhold-Hurek, submitted).

In addition to control of *nif* gene expression, novel roles of P_{II}-like proteins were identified with respect to the control of nitrogenase activity. In *Azoarcus* sp. strain BH72 a fast and reversible physiological switch-off mechanism was observed, similar to those in *Rhodobacter capsulatus* and *Azospirillum brasilense* in terms of speed and extent of nitrogenase inhibition (14). Previously we have shown that the ferredoxin FdxN may be involved in this process (14). As additional components involved in ammonium-induced nitrogenase switch-off, we identified AmtB and GlnK, whereas GlnB was not involved. Since at switch-off conditions (2 mM NH_4Cl) the *amtB* and *glnK* mutants showed similar rates of NH_4^+ uptake as the wild type, the NH_4^+ transport via AmtB or other uptake systems was apparently not affected. Therefore, it is likely that AmtB fulfills a sensory function in detection of changes in the external ammonium concentration, rather than playing a mere role in the import of ammonium into the cell.

It is tempting to speculate that the GlnK and AmtB proteins interact physically. It is remarkable that the hydrophilic P_{II}-like proteins showed association to membrane fractions at all, moreover, this was differential in *Azoarcus*. Only GlnK and GlnY, which are encoded in an operon with *amtB* homologues, occurred in association with membranes, but not GlnB. Previously it has been proposed that AmtB and GlnK might physically and functionally interact, based on conservation of an operon-like gene order of *glnK* and *amtB* in many different prokaryotes (57).

The cellular localization of the hydrophilic proteins at membranes in addition to the cytoplasmic fraction indeed suggests the existence of specific interactions with membrane proteins. Since we still observed membrane association of GlnK in *amtB* mutant strain BHABK, in which the gene is interrupted between putative transmembrane helices 4 and 5, GlnK might interact either with the N-terminal part or with as yet unidentified membrane proteins. Additionally, our observation implies that heterotrimers of GlnB and GlnK subunits, which have been proposed to fine tune nitrogen sensing in *E. coli* (59), are apparently not formed in vivo in *Azoarcus* spp., at least not at the cytoplasmic membrane.

In *Azoarcus* sp. wild-type strain BH72, inhibition of nitrogenase activity coincided with a covalent modification of NifH. Reversible modification of NifH is known to be catalyzed by the DraT/G system in several N_2-fixing bacteria, which is controlled by ammonium and darkness (in phototrophs) or oxygen limitation and anaerobiosis (in *A. brasilense*). Chemical analyses of the nature and covalent linkage of the modifying group and the presence of a *draT* homologue upstream of the *nifHDK* operon (Junker and Reinhold-Hurek, unpublished) indicated the presence of NifH-ADP-ribosylation also in *Azoarcus* sp. strain BH72.

We found that P_{II}-like proteins are also involved in the signal transduction of this process. Interestingly, ammonium-induced NifH modification required the presence of both GlnB and GlnK. In contrast, the rapid nitrogenase inactivation, which we refer to as the physiological switch-off, required only GlnK. Thus, the differential roles of P_{II}-like proteins in *Azoarcus* spp. allow a clear distinction of these two mechanisms of nitrogenase inhibition: the *glnB* mutant showed rapid inactivation but lacked NifH modification. Also in *R. capsulatus* and *A. brasilense*, two mechanisms for nitrogenase inhibition were described, the physiological switch-off presumably accounting for the majority of nitrogenase inhibition, which is not the primary result of NifH modification (15, 16, 46, 60).

It has been proposed that this mechanism involves reduced electron flux to nitrogenase, resulting in fast inhibition of nitrogenase activity (46). The decrease in oxygen consumption which we observed after addition of ammonium to an N_2-fixing culture of strain BH72 indicates a reduced flow of electrons towards O_2 concomitant with decreased N_2 fixation, which might result in changes in the redox status of cellular electron carriers. A regulatory role of the electron flux in nitrogenase inactivation is also suggested, since the ferredoxin FdxN, an electron donor to NifH, is essential for ammonium inactivation of nitrogenase in strain BH72 (14).

In accordance with these results, Halbleib et al. (18) showed

that electron transport via NifF or NifJ and the redox state of NifH influences responses to NH_4^+ via ADP-ribosylation of NifH. Therefore, the sensory signals, e.g., the redox state of certain electron carriers, might be integrated, resulting in fast nitrogenase switch-off upon addition of ammonium or oxygen or energy limitation. However, cascades leading to nitrogenase modification in response to ammonium or anaerobiosis differ, since anaerobiosis-induced NifH modification did only require the presence of GlnB or GlnK, whereas ammonium-induced modification required the presence of both GlnB and GlnK as well as AmtB in strain BH72.

Involvement of P_{II}-like proteins in regulation of nitrogenase activity was also observed in the methanogenic archaeon *Methanococcus maripaludis* (33). Klipp and coworkers also proposed a model for regulation of nitrogenase activity by P_{II}-like proteins in *R. capsulatus* (34). Taken together, these results suggest that P_{II}-protein-mediated regulation of nitrogenase activity might be widespread among diazotrophs.

In conclusion, our studies demonstrated that P_{II}-like proteins in *Azoarcus* sp. strain BH72 are paralogues with distinct, characteristic functional properties, although they can complement some functions of each other. The most distantly related protein appears to be the third novel paralogue, GlnY, which cannot participate in certain signal transduction pathways in the absence of the other two proteins. Its specific functions and structure-function relationships still need to be elucidated. Moreover, our studies demonstrate the intriguing phenotypic diversity of P_{II}-like proteins: the role of a particular paralogue in fine-tuning of nitrogen metabolism may vary considerably depending on the organisms studied.

Using mutant analyses, GlnK especially was shown to be involved in multiple signal transduction cascades. This involvement might be indirect, via transcriptional control of novel constituents of the cascade, or direct, by protein-protein interactions with novel receptors, for example, with AmtB (in physiological nitrogenase switch-off) or DraT/G and/or nitrogenase (in covalent modification of the iron protein of nitrogenase). In any case, the picture is emerging that P_{II}-like proteins show a remarkable diversity of functions despite their small size.

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