Functional Characterization of Three GlnB Homologs in the Photosynthetic Bacterium *Rhodospirillum rubrum*: Roles in Sensing Ammonium and Energy Status

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The GlnB (P_{II}) protein, the product of *glnB*, has been characterized previously in the photosynthetic bacterium *Rhodospirillum rubrum*. Here we describe identification of two other P_{II} homologs in this organism, GlnK and GlnJ. Although the sequences of these three homologs are very similar, the molecules have both distinct and overlapping functions in the cell. While GlnB is required for activation of NifA activity in *R. rubrum*, GlnK and GlnJ do not appear to be involved in this process. In contrast, either GlnB or GlnJ can serve as a critical element in regulation of the reversible ADP ribosylation of dinitrogenase reductase catalyzed by the dinitrogenase reductase ADP-ribosyl transferase (DRAT)/dinitrogenase reductase-activating glycohydrolase (DRAG) regulatory system. Similarly, either GlnB or GlnJ is necessary for normal growth on a variety of minimal and rich media, and any of the proteins is sufficient for normal posttranslational regulation of glutamine synthetase. Surprisingly, in their regulation of the DRAT/DRAG system, GlnB and GlnJ appeared to be responsive not only to changes in nitrogen status but also to changes in energy status, revealing a new role for this family of regulators in central metabolic regulation.

In enteric bacteria, GlnB (or P_{II} protein), the product of *glnB*, plays a very important role in signal transduction of carbon and nitrogen status. The general nitrogen regulation (*ntr*) system, which has been most intensively studied in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and the nitrogen-fixing bacterium *Klebsiella pneumoniae* (39, 45, 49), controls the transcription of many genes involved in nitrogen fixation and assimilation, such as *glnA* (encoding glutamine synthetase [GS]) and *nifA* (encoding the transcriptional activator for the other *nif* genes). The activity of GlnB is regulated by a bifunctional, uridylyltransferase/uridylyl-removing enzyme, encoded by *glnD*. The uridylyltransferase/uridylyl-removing enzyme is believed to be a sensor of the intracellular nitrogen status (glutamine level) in the cell, and it reversibly controls the activity of GlnB by uridylylation or deuridylylation. GlnB also senses α -ketoglutarate (α -KG) levels in *E. coli* as an indicator of carbon status and controls NtrB (NRII) activity in response to the level of α -KG in the cell (20, 26). NtrB (NRII) and NtrC (NRI) (the products of *ntrB* and *ntrC*, respectively) belong to the family of two-component regulators and have been extensively studied in *E. coli* and *S. enterica* serovar Typhimurium (45, 49, 53). NtrB acts as a histidine kinase that phosphorylates NtrC (NRI) or as a phosphatase to dephosphorylate NtrC, depending on the nitrogen or carbon status. At a low α -KG concentration, GlnB trimers bind only one molecule of α -KG and can interact with NtrB, inhibiting its kinase activity and activating its phosphatase activity to dephosphorylate NtrC. However, at higher α -KG concentrations, GlnB binds additional molecules of α -KG and is unable to interact with NtrB, so that NtrB acts as a kinase to phos-

phorylate NtrC (20). Similarly, under N-limiting conditions, uridylylation of GlnB prevents its interaction with NtrB, so that NtrC is accumulated in the phosphorylated form (3). In these enteric bacteria, the phosphorylated form of NtrC acts as a transcriptional activator of *nifLA*, *glnA ntrBC*, *glnK amtB*, *nac*, and other operons involved in nitrogen fixation and assimilation (11, 16, 17, 39, 41, 52, 60, 63, 74). GlnB, together with adenylyltransferase (ATase), also controls GS activity by adenylylation or deadenylylation (22).

Besides the transcriptional regulation of *nifA* expression by the *ntr* system, NifA activity is also regulated. In *K. pneumoniae* and *Azotobacter vinelandii*, NifA activity is inhibited by NifL in response to NH₄⁺ and oxygen, probably by means of a direct interaction (32, 40). A P_{II} homolog, GlnK, has been identified and has been found to be involved in the relief of NifL inhibition of NifA in *K. pneumoniae* under N₂-fixing conditions (15, 18), resulting in the Nif⁺ phenotype of the *glnK* mutant.

Homologs of GlnK have been found in many eubacteria and archaea (44, 61), and in *E. coli* GlnK is very similar to GlnB in terms of sequence (18, 63) and structure (7, 36, 67). A hypothesis has been proposed to distinguish these two classes of homologs based on five specific residues (18). Although both proteins in *E. coli* can interact with NtrB and interact with ATase to adenylylate GS (4, 5, 64), they also have distinct functions in the cell. It is believed that only GlnK is involved in the relief of NifL inhibition in *K. pneumoniae* (15), although overexpressed GlnB can substitute for GlnK in this role (2). Additionally, GlnB-UMP can stimulate ATase activity to deadenylylate GS, but GlnK-UMP cannot (65). GlnB and GlnK can form heterotrimers in vivo and in vitro (13, 65), and in *E. coli* the uridylylated form of heterotrimers can also stimulate ATase activity, although less well than GlnB-UMP stimulates this activity (65). However, *A. vinelandii* apparently contains

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only one P_{II} homolog, and only the unmodified form of P_{II} stimulates NifL to inhibit NifA activity (34).

In *Rhodospirillum rubrum* and *Azospirillum brasilense*, the role of GlnB is quite different. In *A. brasilense*, a *glnB* mutant is Nif[−] (9) and excretes ammonium when it is grown in minimal medium with nitrate (8), indicating that the other P_{II} homolog in the cell (termed P_z , the product of *glnZ*) is unable to compensate for defects caused by the *glnB* mutation. Similarly, in a *R. rubrum glnB* mutant, *nif* expression is completely absent because GlnB is required for activation of NifA activity under NH₄⁺-limiting conditions (73). No NifL homolog has been identified in *R. rubrum* or *A. brasilense*.

In *R. rubrum*, *A. brasilense*, and *Rhodobacter capsulatus*, nitrogen fixation is also regulated posttranslationally through reversible mono-ADP ribosylation of dinitrogenase reductase (35, 38, 70). Dinitrogenase reductase ADP-ribosyl transferase (DRAT) (the product of *draT*) transfers ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase homodimer, which results in inactivation of the enzyme. The ADP-ribose group attached to dinitrogenase reductase can be removed by another enzyme, dinitrogenase reductase-activating glycohydrolase (DRAG) (the product of *draG*), which restores nitrogenase activity. This system responds to fixed nitrogen or to energy limitation in the form of darkness shifts (in *R. rubrum* and *R. capsulatus*) or anaerobiosis shifts (in *A. brasilense*) (27, 33, 38, 48, 69). The activities of DRAT and DRAG are themselves subject to posttranslational regulation (35, 70). Under nitrogen-fixing conditions, DRAT is inactive and DRAG is active, so that dinitrogenase reductase is in its active form. Following a negative stimulus, such as addition of exogenous NH₄⁺ or energy depletion, DRAT is transiently activated and DRAG becomes inactive, which results in modification of dinitrogenase reductase and loss of nitrogenase activity. The precise mechanism of regulation of DRAT and DRAG activities is still unknown.

Heterologous expression of *R. rubrum draTG* in *K. pneumoniae* showed that regulation of DRAG activity is partially altered in a *glnB* mutant and completely absent in a *glnK* mutant (72), suggesting that P_{II} homologs might play a significant role in regulation of nitrogenase activity in *R. rubrum*. Here we describe identification of two additional *glnB* homologs, *glnK* and *glnJ*, in *R. rubrum* and the roles of their products in regulation of nitrogenase activity in response to nitrogen and energy status.

Because we are concerned that use of the term P_{II} for the *R. rubrum* homologs implies functional properties that may not be precisely correct, we refer to the proteins studied as GlnB, GlnK, and GlnJ and use the term P_{II} for the family of these homologs.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Antibiotics were used as necessary at the levels described previously (73).

Growth conditions and whole-cell nitrogenase activity assay. *R. rubrum* was grown in rich SMN medium or minimal medium, as described previously (12, 31). For derepression for nitrogenase *R. rubrum* was grown in malate-glutamate (MG) medium (31) or MN[−] medium. MN[−] medium was the same as minimal medium except that it lacked fixed nitrogen (NH₄Cl). The whole-cell nitrogenase activity assay and darkness and NH₄Cl treatments used have been described previously (68).

Cloning of *glnK*. Two oligonucleotides, *glnB*-P10 (5'-CATCAAGCCCTTCAAGCTC-3') and *glnB*-P11 (5'-CTCCCTGTACGGATGCG-3'), were designed from two conserved regions of *glnB* from *R. rubrum* and used as primers to PCR amplify *glnK* from a *glnB* deletion mutant of *R. rubrum* (UR171) (73) using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). A strong 100-bp band resulted from the partially deleted *glnB* allele, but a weaker 300-bp band was subcloned into pBSKS(−), yielding pYPZ232. The deduced amino acid sequence of the fragment from pYPZ232 exhibited high levels of similarity to GlnB from various bacteria, and the amplified gene was designated *glnK*.

To clone the entire *glnK* gene, the 300-bp *glnK'* fragment was subcloned into pUX19 (a suicide vector for *R. rubrum* [D. P. Lies and G. P. Roberts, unpublished data]), yielding pYPZ234. pYPZ234 was integrated into the chromosome of the *R. rubrum* wild-type strain, and total DNA was isolated, digested with *Bam*HI or *Xho*I, ligated, and transformed into *E. coli* DH5α. Km^r colonies were selected, and plasmids were isolated from each transformant. Two new plasmids, pYPZ236 from the *Xho*I digestion and pYPZ237 from the *Bam*HI digestion, contained overlapping portions of *glnK*; pYPZ236 had a 2.9-kb insert that began in *glnK* and extended 5', while pYPZ237 had a 3.6-kb insert that started in *glnK* and extended 3'. Both plasmids were used for DNA sequencing of the entire *glnK* gene.

Construction of *glnK* deletion mutants. Fragments of the 3' and 5' regions of *glnK* were amplified by PCR and ligated into pUX19. *aacC1*, encoding gentamicin resistance (Gm^r) (54), was inserted between these two fragments, yielding pYPZ249, such that 310 bp of *glnK* was replaced by the 800-bp *aacC1* fragment. After pYPZ249 was conjugated into *R. rubrum* UR2 (wild type) and UR717 (Δ *glnB3*), Sm^r Nx^r Gm^r colonies were selected and then screened for Km^r (Km^r is encoded by pUX19) resulting from double-crossover recombination events. The mutation was designated Δ *glnK1::aacC1*. The strains were designated UR755 (Δ *glnK1*) and UR757 (Δ *glnB3 glnK1*).

Cloning of *glnJ*. A *glnK*-P7 oligonucleotide (5'-CGTAGACGAAGACCTTGCCATCGCC-3') was designed based on conserved regions of *glnK* and was used with *glnB*-P10 to PCR amplify *glnJ* from the Δ *glnBK* mutant (UR757). No *glnB* or *glnK* amplification occurred with DNA from UR757 because of deletion of either one or both primer annealing regions in *glnB* and *glnK*. However, a 270-bp PCR product was detected and cloned into pBSKS(−), yielding pUX255. The deduced amino acid sequence of this fragment from pUX255 showed high similarity to the sequences of GlnB and GlnK from *R. rubrum*, indicating the presence of a third homolog of *glnB*, designated *glnJ*.

Similar to the cloning approach used with *glnK*, the 270-bp *glnJ'* fragment from pUX255 was subcloned into pUX19, yielding pUX257, which was integrated into the *R. rubrum* chromosome. Total DNA was isolated, digested with *Eco*RI or *Xho*I, and ligated and transformed into *E. coli* DH5α. The resulting plasmids, pUX268 and pUX269, were used for DNA sequencing of the entire *glnJ* gene.

Construction of *glnJ* deletion mutants. A 5-kb fragment containing *glnJ* was ligated into pSUP202 (another suicide vector for *R. rubrum*) (56), yielding pUX284. After *Bam*HI digestion, the 1.4-kb Km^r gene from pUC-4K (66) was inserted, yielding pUX306, such that 230 bp of *glnJ* was replaced by *kan*, and the mutation was designated Δ *glnJ1::kan*. pUX306 was conjugated into *R. rubrum* UR2 (wild type), UR717 (Δ *glnB3*), UR755 (Δ *glnK1*), and UR757 (Δ *glnB3 glnK1*), and Sm^r Nx^r Km^r colonies were selected and replica printed to screen for Cm^r (Cm^r is encoded by pSUP202) resulting from double-crossover events. The mutants were designated UR806 (Δ *glnJ1*), UR808 (Δ *glnB3 glnJ1*), UR810 (Δ *glnK1 glnJ1*), and UR812 (Δ *glnB3 glnK1 glnJ1*).

Protein immunoblotting. A trichloroacetic acid precipitation method was used to extract protein quickly, as described previously (69). Low-cross-linker sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein separation to obtain better resolution of the modified and unmodified subunits of dinitrogenase reductase or GS, since the modified subunit migrated more slowly (69, 73). Proteins from SDS-PAGE gels were electrophoretically transferred onto a nitrocellulose membrane, and then they were immunoblotted with polyclonal antibody against dinitrogenase reductase or GS and visualized with horseradish peroxidase color detection reagents (Bio-Rad, Richmond, Calif.).

DNA sequencing. DNA sequences were determined with an ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.) and were analyzed with software from DNASTAR (Madison, Wis.) and Genetics Computer Group (Madison, Wis.).

Nucleotide sequence accession numbers. The nucleotide sequences of the *R. rubrum glnK* and *glnJ* regions have been deposited in the GenBank database under accession numbers AF207908 and AF329498, respectively.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and description	Reference or source
<i>R. rubrum</i> strains		
UR2	Wild type, Sm ^r	27
UR687	<i>glnA2</i> (GS-Y398F) mutant, Sm ^r Km ^r	73
UR694	Transconjugant of UR2 with pCK3, Sm ^r Tc ^r	73
UR717	<i>ΔglnB3</i> (in-frame deletion) mutant, Sm ^r	73
UR720	Transconjugant of UR717 with pCK3, Sm ^r Tc ^r	73
UR755	<i>ΔglnK1::aacC1</i> mutant, Sm ^r Gm ^r	This study
UR757	<i>ΔglnB3 ΔglnK1::aacC1</i> double mutant, Sm ^r Gm ^r	This study
UR758	Transconjugant of UR755 with pCK3, Sm ^r Gm ^r Tc ^r	This study
UR760	Transconjugant of UR757 with pCK3, Sm ^r Gm ^r Tc ^r	This study
UR806	<i>ΔglnJ1::kan</i> mutant, Sm ^r Km ^r	This study
UR808	<i>ΔglnB3 ΔglnJ1::kan</i> double mutant, Sm ^r Km ^r	This study
UR810	<i>ΔglnK1::aacC1 ΔglnJ1::kan</i> double mutant, Sm ^r Gm ^r Km ^r	This study
UR812	<i>ΔglnB3 ΔglnK1::aacC1 ΔglnJ1::kan</i> triple mutant, Sm ^r Gm ^r Km ^r	This study
UR816	UR808 with an unknown <i>sgn-1</i> suppressor mutation (fast growing), Sm ^r Km ^r	This study
UR818	UR812 with an unknown <i>sgn-2</i> suppressor mutation (fast growing), Sm ^r Gm ^r Km ^r	This study
UR820	Transconjugant of UR816 with pCK3, Sm ^r Km ^r Tc ^r	This study
UR822	Transconjugant of UR818 with pCK3, Sm ^r Gm ^r Tc ^r	This study
UR824	Transconjugant of UR808 with pCK3, Sm ^r Km ^r Tc ^r	This study
UR826	Transconjugant of UR812 with pCK3, Sm ^r Gm ^r Km ^r Tc ^r	This study
Plasmids		
pUX19	Suicide vector for <i>R. rubrum</i> , Km ^r	Lies and Roberts ^a
pSUP202	Suicide vector for <i>R. rubrum</i> , Ap ^r Cm ^r Tc ^r	56
pCK3	pRK290 derivative containing <i>K. pneumoniae nifA</i> , Tc ^r	28
pYPZ232	300-bp PCR fragment of <i>R. rubrum glnJ'</i> was cloned into pBSKS(−), Ap ^r	This study
pYPZ234	300-bp fragment of <i>R. rubrum glnK'</i> from pYPZ232 was subcloned into pUX19, Km ^r	This study
pYPZ236	2.9-kb fragment of <i>R. rubrum glnK'</i> and 5' region were cloned into pUX19, Km ^r	This study
pYPZ237	3.6-kb fragment of <i>R. rubrum glnK'</i> and 3' region were cloned into pUX19, Km ^r	This study
pYPZ249	6.5-kb fragment of <i>R. rubrum glnK</i> region was cloned into pUX19, and 850-bp fragment of <i>aacC1</i> (Gm ^r gene) from pUCGM was inserted into <i>glnK</i> and replaced a 300-bp <i>glnK</i> gene, Km ^r	This study
pUX255	260-bp PCR fragment of <i>R. rubrum glnJ'</i> was cloned into pBSKS(−), Ap ^r	This study
pUX257	260-bp fragment of <i>R. rubrum glnJ'</i> from pYPZ232 was subcloned into pUX19, Km ^r	This study
pUX268	5.0-kb fragment of <i>R. rubrum glnJ'</i> and 3' region were cloned into pUX19, Km ^r	This study
pUX269	6.0-kb fragment of <i>R. rubrum glnJ'</i> and 5' region were cloned into pUX19, Km ^r	This study
pUX284	5.0-kb fragment of <i>R. rubrum glnJ</i> region was cloned into pSUP202, Tc ^r Cm ^r	This study
pUX306	1.4-kb fragment of Km ^r gene from pUC-4K was inserted into <i>glnJ</i> in pUX284 and replaced a 230-bp <i>glnJ</i> gene, Tc ^r Cm ^r Km ^r	This study

^a D. P. Lies and G. P. Roberts, unpublished data.

RESULTS

Cloning and sequencing of *glnK* from *R. rubrum*. Many nitrogen-fixing bacteria related to *R. rubrum*, such as *A. brasilense* and *Rhodobacter sphaeroides*, have two copies of *glnB* (10, 50). It was our hypothesis that *glnK* also is present in *R. rubrum* and might be involved in regulation of GS and the DRAT/DRAG regulatory system (73).

The sequences of GlnB and GlnK from different bacteria are very similar (18), so the internal region of the putative *glnK* gene of *R. rubrum* was cloned by PCR with two primers designed from two conserved regions of *R. rubrum glnB*. The 1.5-kb *glnK* region was sequenced and contains three open reading frames (ORFs) with reasonable codon usage for *R. rubrum*.

The putative GlnK protein has 112 amino acid residues and is 64% identical to GlnB of *R. rubrum*. We designated the gene *glnK* based primarily on previously published comparisons of GlnB and GlnK homologs, which were identified by five positions (residues 3, 5, 52, 54, and 64) that distinguish the two classes of homologs (18). We also noticed that residue 112 was characteristic; all GlnK homologs have leucine at this position, while most GlnB homologs have isoleucine or valine. As de-

termined by this analysis, the putative *R. rubrum* GlnK protein clearly falls into the GlnK class, because it has residues identical to those of other GlnK homologs at all positions except position 52, where it has a unique glutamine residue.

An ORF starting 14 bp from the 3' end of *glnK* was partially sequenced, which revealed that it had about 20% identity to *amtB* from *E. coli* and other bacteria. *amtB* encodes a predicted membrane-bound ammonium transport protein (62) and has been found to be cotranscribed with *glnK* in many bacteria (61). We designated this ORF *amtB*₂, because another *R. rubrum amtB* homolog was cloned, which is described below. An ORF in the 5' region of *glnK* was also partially sequenced, and this ORF exhibited 60 to 70% identity to *purU* from other bacteria, which encodes a formyltetrahydrofolate deformylase (hydrolase) (42).

Effect of a *glnK* mutation on nitrogenase activity. In *K. pneumoniae*, a *glnK* mutant is Nif[−] (18, 72) because of the failure to relieve NifL inhibition of NifA under N-limiting conditions (15, 18). Using heterologous expression of *R. rubrum draTG* in *K. pneumoniae*, we recently found that the GlnK of this organism is also involved in regulation of DRAG activity (72). To study the role of GlnK in *R. rubrum*, a *ΔglnK*

TABLE 2. Nitrogenase activity and its response to dark-light shifts in *R. rubrum* wild type and mutants in the presence or absence of *K. pneumoniae nifA* (in pCK3) when cells were grown in MG medium

Strain	Chromosomal genotype	Gene(s) on plasmid	Nitrogenase activity (nmol h ⁻¹ ml ⁻¹) ^a		
			Initial	40 min after shift to dark	10 min after reillumination
UR2	Wild type	None	1,000	70 (7) ^b	890 (89)
UR694	Wild type	<i>K. pneumoniae nifA</i>	850	50 (6)	800 (94)
UR717	Δ <i>glnB</i>	None	<2	NT ^c	NT
UR720	Δ <i>glnB</i>	<i>K. pneumoniae nifA</i>	550	66 (12)	470 (85)
UR755	Δ <i>glnK</i>	None	850	70 (8)	860 (101)
UR757	Δ <i>glnB</i> Δ <i>glnK</i>	None	<2	NT	NT
UR758	Δ <i>glnK</i>	<i>K. pneumoniae nifA</i>	900	90 (10)	910 (101)
UR760	Δ <i>glnB</i> Δ <i>glnK</i>	<i>K. pneumoniae nifA</i>	800	100 (13)	840 (105)
UR806	Δ <i>glnJ</i>	None	1,000	70 (7)	970 (97)
UR808	Δ <i>glnB</i> Δ <i>glnJ</i>	None	<2	NT	NT
UR810	Δ <i>glnK</i> Δ <i>glnJ</i>	None	900	90 (10)	770 (86)
UR812	Δ <i>glnB</i> Δ <i>glnK</i> Δ <i>glnJ</i>	None	<2	NT	NT
UR820	Δ <i>glnB</i> Δ <i>glnJ</i> <i>sgn-1</i>	<i>K. pneumoniae nifA</i>	1,000	1,050 (105)	1,060 (106)
UR822	Δ <i>glnB</i> Δ <i>glnK</i> Δ <i>glnJ</i> <i>sgn-2</i>	<i>K. pneumoniae nifA</i>	950	1,100 (116)	1,120 (118)
UR824	Δ <i>glnB</i> Δ <i>glnJ</i>	<i>K. pneumoniae nifA</i>	700	720 (103)	710 (101)
UR826	Δ <i>glnB</i> Δ <i>glnK</i> Δ <i>glnJ</i>	<i>K. pneumoniae nifA</i>	700	740 (106)	720 (103)

^a Nitrogenase activity was assayed before a darkness shift (initial), 40 min after cells were shifted to darkness, and 10 min after cells were returned to light. Nitrogenase activity is expressed in nanomoles of ethylene produced per hour per milliliter of cells at an optical density at 600 nm of 1. Each activity value is based on at least 10 replicate assays performed with different individually grown cultures. The standard deviations were between 5 and 15%.

^b The values in parentheses are the percentages of the initial nitrogenase activity remaining after the shift to darkness or reillumination.

^c NT, not tested.

single mutant (UR755) and a Δ *glnB* Δ *glnK* double mutant (UR757) were constructed as described above. As shown in Table 2, UR755 showed high nitrogenase activity when it was derepressed in MG medium, similar to the activity in UR2 (wild type), indicating that GlnK is not essential for nitrogen fixation in *R. rubrum*. Because of the lack of GlnB, which has been shown to be essential for activation of NifA activity in *R. rubrum* (73), UR757 showed little nitrogenase activity (Table 2), and the activity was similar to the activity seen in *glnB* in-frame deletion mutant UR717 (73).

The effects of the *glnK* and *glnB* mutations on regulation of nitrogenase activity in response to NH₄⁺ and darkness were tested. In previous studies, cultures grown in MG medium were used, but a slow NH₄⁺ response was observed with UR2 (wild type) and a high residual nitrogenase activity (40 to 50%)

remained 45 min after NH₄⁺ addition (68). However, use of MN⁻ medium, which lacks fixed nitrogen, resulted in a more striking response to NH₄⁺. As shown in Table 3, MN⁻ medium-grown UR2 (wild type) showed an almost complete loss of nitrogenase activity by 10 min after NH₄⁺ addition, as did UR755 (*glnK*). To study regulation of nitrogenase activity in a *glnBK* mutant, we introduced plasmid pCK3 (28), expressing *K. pneumoniae nifA* from a constitutive promoter (creating UR760), as we did previously in the *glnB* background (73). The constitutively expressed *K. pneumoniae nifA* gene restored *nif* expression and nitrogenase activity in *R. rubrum glnB* mutants UR720 and UR760 (Tables 2 and 3), as *K. pneumoniae* NifA did not require activation by GlnB. Both the *glnB* and *glnBK* mutants (UR720 and UR760) responded to NH₄⁺, but with significantly greater residual activity (Table 3), indicating that

TABLE 3. Nitrogenase activity and its response to NH₄⁺ in *R. rubrum* wild type and mutants in the presence or absence of *K. pneumoniae nifA* (in pCK3) when cells were grown in MN⁻ medium

Strain	Chromosomal genotype	Gene(s) on plasmid	Nitrogenase activity (nmol h ⁻¹ ml ⁻¹) ^a		
			Initial	10 min after addition	40 min after addition
UR2	Wild type	None	500	5 (1) ^b	5 (1)
UR694	Wild type	<i>K. pneumoniae nifA</i>	350	10 (3)	5 (1)
UR720	Δ <i>glnB</i>	<i>K. pneumoniae nifA</i>	400	80 (20)	20 (5)
UR755	Δ <i>glnK</i>	None	400	5 (1)	5 (1)
UR758	Δ <i>glnK</i>	<i>K. pneumoniae nifA</i>	500	10 (2)	5 (1)
UR760	Δ <i>glnB</i> Δ <i>glnK</i>	<i>K. pneumoniae nifA</i>	500	120 (24)	50 (10)
UR806	Δ <i>glnJ</i>	None	350	5 (1)	5 (1)
UR810	Δ <i>glnK</i> Δ <i>glnJ</i>	None	400	5 (1)	5 (1)
UR820	Δ <i>glnB</i> Δ <i>glnJ</i> <i>sgn-1</i>	<i>K. pneumoniae nifA</i>	500	445 (89)	420 (84)
UR822	Δ <i>glnB</i> Δ <i>glnK</i> Δ <i>glnJ</i> <i>sgn-2</i>	<i>K. pneumoniae nifA</i>	400	350 (88)	320 (80)
UR824	Δ <i>glnB</i> Δ <i>glnJ</i>	<i>K. pneumoniae nifA</i>	300	210 (70)	180 (60)
UR826	Δ <i>glnB</i> Δ <i>glnK</i> Δ <i>glnJ</i>	<i>K. pneumoniae nifA</i>	300	225 (75)	210 (70)

^a Nitrogenase activity was assayed before NH₄⁺ addition (initial) and 10 and 40 min after NH₄Cl addition to a final concentration of 10 mM. Nitrogenase activity is expressed in nanomoles of ethylene produced per hour per milliliter of cells at an optical density at 600 nm of 1. Each activity value is based on at least 10 replicate assays performed with different individually grown cultures. The standard deviations were between 5 and 15%.

^b The values in parentheses are the percentages of the initial nitrogenase activity that remained after the NH₄Cl treatment.

ADP ribosylation of dinitrogenase reductase was slightly altered in the *glnB* mutants compared to the wild type under these conditions.

In *R. rubrum*, the DRAT/DRAG regulatory system also responds to changes in energy status, such as that resulting from a shift of cultures from light to darkness. The darkness response was tested in these mutants when they were derepressed in MG medium, since a similar residual activity was seen in both MG and MN⁻ media under dark conditions (data not shown). All of the *glnB*, *glnK*, and *glnBK* mutants (UR720, UR755, UR758, and UR760) exhibited a response to darkness similar to that of the wild-type controls (UR2 and UR694) (Table 2).

Identification of a third *glnB* homolog, *glnJ*, in *R. rubrum*. Three or more *glnB* homologs have been found in some eubacteria and archaea (30, 37, 57). The small effects of the *glnBK* mutations on regulation of nitrogenase activity suggested that *R. rubrum* might have a third *glnB* homolog. As described above, a different PCR primer based on a very conserved region of *glnK* allowed isolation of a third *glnB* homolog gene, which we designated *glnJ*.

The *glnJ* region was sequenced, and three ORFs were identified. The deduced amino acid sequence of GlnJ was about 69% identical to both the GlnB and GlnK sequences of *R. rubrum*. GlnJ is more similar to the GlnK family, although it has a unique phenylalanine at residue 3 and a unique isoleucine at residue 5. Only one GlnB-GlnK homolog has a phenylalanine at residue 3 (37), and three other GlnB-GlnK homologs have an isoleucine residue at residue 5 (43, 57, 59). However, at residue 52 GlnJ has a valine residue, which is common in GlnB.

An ORF at the 5' end of *glnJ* was partially sequenced, and it is similar to *ilvE*, which encodes a branched-chain amino acid aminotransferase. Immediately 3' of *glnJ*, another *amtB* homolog was found, and its predicated protein is 67% identical to AmtB of *A. brasilense*. We designated this gene *amtB₁*, since it exhibited much greater similarity to other *amtB* genes than did *amtB₂*, which is located 3' of *glnK*.

Either GlnB or GlnJ is necessary for regulation of nitrogenase activities by the DRAT/DRAG regulatory system in response to NH₄⁺ and darkness. A $\Delta glnJ$ mutant, a $\Delta glnB \Delta glnJ$ double mutant, a $\Delta glnK \Delta glnJ$ double mutant, and a $\Delta glnB \Delta glnK \Delta glnJ$ triple mutant were constructed as described above. Both *glnJ* (UR806) and *glnKJ* (UR810) mutants grew normally in SMN and MG media and exhibited substantial nitrogenase activity under derepression conditions (MG medium), similar to the activity of UR2 (wild type) (Table 2). This indicates that only GlnB is required for activation of NifA activity in *R. rubrum*. Furthermore, neither GlnJ nor GlnK could substitute for GlnB in activating NifA in *R. rubrum*. The strains examined also responded to darkness and NH₄⁺ treatment (Tables 2 and 3), indicating that mutation of *glnJ* alone or *glnJK* does not significantly affect the DRAT/DRAG regulatory system.

However, the $\Delta glnBJ$ double mutant (UR808) and the $\Delta glnBKJ$ triple mutant (UR812) had lower growth rates in SMN medium (generation times, about 8 h) than UR2 (generation time, 5 h). These mutants also had significantly less red pigmentation. During purification of single colonies of these strains, colonies that grew faster appeared at a low frequency

(10⁻⁵ to 10⁻⁶), suggesting the presence of suppressor mutations, which we designated *sgn* (suppressor of *glnBK*). The strains with suppressor mutations were UR816 (*glnBJ* with *sgn-1*) and UR818 (*glnBKJ* with *sgn-2*). Both UR816 and UR818 appeared to be stable and to grow as well as UR2 in SMN medium, except that less red pigment accumulated in UR818.

We strongly doubt that the growth problems mentioned above are affected by mutation polarity. The *glnB* mutation is an in-frame deletion, and although *glnK* and *glnJ* each appears to be cotranscribed with an *amtB* homolog, the *glnK glnJ* double mutant (UR810) showed no obvious defect in growth. This mutant also showed no obvious defect in NH₄⁺ uptake, since it responded to NH₄⁺ normally (Table 3). Furthermore, polarity does not readily explain any of the important differences detected in apparent protein function or the response to energy status.

Because of the *glnB* mutation, the *glnBJ* (UR808) and *glnBKJ* (UR812) mutants exhibited little nitrogenase activity under derepression conditions (Table 2), so *K. pneumoniae nifA* (pCK3) was introduced into these strains. As shown in Table 2, UR820 ($\Delta glnB \Delta glnJ$ *sgn-1*, with *K. pneumoniae nifA*) and UR822 ($\Delta glnB \Delta glnK \Delta glnJ$ *sgn-2*, with *K. pneumoniae nifA*) exhibited substantial nitrogenase activity, similar to that seen in UR2. However, these two strains responded poorly to NH₄⁺ when they were derepressed for nitrogenase in MG or MN⁻ medium (Table 3; data not shown). More surprisingly, no response to darkness was seen in these mutants (Table 2). These results indicate that the simultaneous absence of *glnB* and *glnJ* dramatically alters regulation of the DRAT/DRAG regulatory system in response to both NH₄⁺ and darkness (energy depletion).

To verify that the effects on nitrogenase activity were actually due to changes in the DRAT/DRAG system and not due to some other effect, such as limitation of the reductant reaching dinitrogenase reductase, modification of dinitrogenase reductase was monitored directly by SDS-PAGE and Western blotting; such modification was revealed by a diagnostic shift in migration of one modified subunit of dinitrogenase reductase (68, 71). The *glnB*, *glnK*, and *glnBK* mutants (UR720, UR755, UR758, and UR760) exhibited modification of dinitrogenase reductase in response to both NH₄⁺ and darkness, like wild-type controls (UR2 and UR694), but little modification of dinitrogenase reductase was found in *glnBJ* and *glnBKJ* mutants (UR820 and UR822) (data not shown). This analysis showed that there was an excellent correlation between loss of nitrogenase activity in vivo and the appearance of ADP-ribosylated dinitrogenase reductase.

Altered DRAT/DRAG regulation is caused by the *glnBJ* mutations rather than the *sgn* mutations. To rule out the possibility that the altered regulation of nitrogenase activity is caused by *sgn* suppressor mutations rather than the *glnJK* mutations, *K. pneumoniae nifA* (pCK3) was introduced into UR808 and UR812 (*glnBJ* and *glnBKJ* mutants without a *sgn* mutation), yielding UR824 and UR826. As expected, UR824 and UR826 grew slowly on SMN medium plates and were not stable, but more slowly growing colonies of each were picked and derepressed for nitrogenase activity in MG and MN⁻ media. As shown in Tables 2 and 3, UR824 and UR826 showed no darkness response and poor responses to NH₄⁺, like the

TABLE 4. Effect of NAD on nitrogenase activity in *R. rubrum* wild type and *glnBJ* and *glnBKJ* mutants when cells were grown in MN⁻ medium

Strain	Chromosomal genotype	Gene(s) on plasmid	Nitrogenase activity (nmol h ⁻¹ ml ⁻¹) ^a		
			Initial	10 min after addition	40 min after addition
UR2	Wild type	None	500	5 (1) ^b	495 (97)
UR820	$\Delta glnB \Delta glnJ sgn-1$	<i>K. pneumoniae nifA</i>	500	465 (93)	485 (99)
UR822	$\Delta glnB \Delta glnJ \Delta glnK sgn-2$	<i>K. pneumoniae nifA</i>	400	400 (100)	460 (115)

^a Nitrogenase activity was assayed before NAD addition (initial) and 10 and 40 min after NAD addition at a final concentration of 2.5 mM. Nitrogenase activity is expressed in nanomoles of ethylene produced per hour per milliliter of cells at an optical density at 600 nm of 1. Each activity value is based on at least 10 replicate assays performed with different individually grown cultures. The standard deviations were between 5 and 15%.

^b The values in parentheses are the percentages of the initial nitrogenase activity that remained after the NAD treatment.

strains with the suppressor mutations (UR820 and UR822). Immediately after the assay for nitrogenase activity, the frequencies of suppressors in these cultures were estimated on the basis of colony size on SMN plates, and fewer than 10% of the cells contained suppressors. This result strongly supports the hypothesis that the altered regulation of nitrogenase activity is caused by the *glnBJ* mutations. The *sgn* mutations suppress the growth defect of *glnBJ* and *glnBKJ* mutants but are not the main cause of the drastically altered posttranslational regulation of nitrogenase activity.

Effect of NAD on nitrogenase activity is also absent in *glnBJ* and *glnBKJ* mutants. NAD serves as the donor of the ADP-ribose group for modification of dinitrogenase reductase (35) and is also required for the interaction between DRAT and dinitrogenase reductase in vitro (14). Exogenous NAD can also stimulate modification of dinitrogenase reductase in *R. rubrum* (46, 47, 58). It was therefore possible that the *glnB* and *glnJ* mutations had a direct effect on the NAD pool. As shown in Table 4, exogenous NAD (2.5 mM) added to MN⁻ medium-grown cultures completely inhibited nitrogenase activity in UR2 (wild type) in 10 min, and recovery of nitrogenase activity occurred after 40 min. However, no significant effect of NAD on nitrogenase activity was seen with UR820 ($\Delta glnB \Delta glnJ sgn-1$ with *K. pneumoniae nifA*) and UR822 ($\Delta glnB \Delta glnK \Delta glnJ sgn-2$ with *K. pneumoniae nifA*) (Table 4), indicating that the DRAT/DRAG regulatory system in these *glnBJ* and *glnBKJ* mutants is also unable to respond to exogenous NAD. This result suggests that the failure to respond to negative stimuli in these mutants is due to altered regulation of DRAT and/or DRAG activity rather than to the availability of NAD.

Effect of GlnB, GlnK, and GlnJ on modification of GS. Both GlnB and GlnK of *E. coli* can stimulate ATase to modify GS both in vivo and in vitro (5, 21, 63, 65). GlnB of *R. rubrum* has also been shown to stimulate modification of GS in vitro (24), although a *glnB* mutation had no effect on modification of GS (73). Therefore, we compared GS modification in *glnBK* (UR757), *glnKJ* (UR810), *glnBJ* (UR816), and *glnBKJ* (UR818) mutants when they were grown in MG medium. As shown in Fig. 1, in the *glnBJ* mutant (UR816) GS was modified after NH₄⁺ was added, like the results obtained previously for the wild type (UR2) (73). Similar GS modifications were seen in *glnBK* (UR757) and *glnKJ* (UR810) mutants (data not shown). However, very little GS was modified in the *glnBKJ* triple mutant (UR818) after NH₄⁺ treatment (Fig. 1). These results indicate that any of the P_{II} homologs is sufficient to support proper GS modification. They also show that GlnK is functional in *R. rubrum*, and therefore, the inability of GlnK to

support other roles of GlnB described above has functional significance.

DISCUSSION

In this report we describe cloning and functional characterization of two P_{II} homologs, GlnK and GlnJ, from *R. rubrum*. Unlike GlnB, GlnK and GlnJ have no significant effect on *nif* expression. However, GlnB and GlnJ play an important role in posttranslational regulation of nitrogenase activity and, of particular interest, are involved in the responses to both NH₄⁺ and darkness.

Two P_{II} homologs (GlnB and GlnK) have been found in many eubacteria and archaea (44, 61), and three or more P_{II} homologs have been found in some archaea, such as *Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum*, and *Methanosarcina barkeri* (30, 55, 57); the functions of these P_{II} homologs are unknown in most of these archaea. In *Methanococcus maripaludis*, there are two *glnB* alleles in the *nif* cluster, and deletion of these two genes affects posttranslational regulation of nitrogenase activity in response to NH₄⁺ but not *nif* expression (29). However, the mechanism for posttranslational regulation of nitrogenase activity in *M. maripaludis* is not

FIG. 1. Western immunoblot of GS in *glnBJ* (UR816) (lanes 5 and 6) and *glnBKJ* (UR818) (lanes 7 and 8) mutants. UR2 (wild type) (lanes 1 and 2) and UR687 (*glnA*, GS-Y398F) (lanes 3 and 4) were used as controls. Samples of GS were obtained from cultures grown in MG medium before (lanes 1, 3, 5, and 7) and after (lanes 2, 4, 6, and 8) treatment with 10 mM NH₄Cl for 60 min. Similar amounts of total protein were loaded on SDS-PAGE gels and immunoblotted with antibody against *R. rubrum* GS. Arrow M indicates the position of the modified subunit, and arrow U indicates the position of the unmodified subunit. As seen previously (73), GS in UR2 (wild type) becomes modified after NH₄⁺ treatment, and no modification of GS was seen in UR687 because the site of adenylation (Tyr-398) was altered.

known, and a *draG* homolog has not been identified, although *draG* homologs have been found in some other archaea (6, 30). Recently, three P_{II} homologs have been identified in an associative N_2 -fixing bacterium, *Azoarcus* sp. strain BH72; these homologs are encoded by *glnB*, *glnK*, and *glnY* (37). In *Azoarcus*, GlnB and GlnK are present under both N-limiting and N-excess conditions, but GlnY is present only in the *glnBK* double mutant (37). Although neither GlnB nor GlnK is essential for nitrogen fixation, the *glnBK* mutant grew significantly more slowly than the wild type or single mutants when N_2 , NH_4Cl , or KNO_3 was used as the sole nitrogen source (37).

X-ray crystallographic analysis revealed that GlnB and GlnK of *E. coli* have very similar core structures but different conformations of the T, C, and B loops (67). These loops are on the external surface, and the T loop (residues 37 to 55) is thought to interact with target proteins (19, 23). Consistent with the importance of the T loop, substitution for two residues (residues 43 and 54) in GlnB of *K. pneumoniae* allows it to function like GlnK to completely relieve NifL inhibition of NifA activity under N-limiting conditions (1). Attractive as the hypothesis that the T loop is on the P_{II} surface and interacts with other proteins is, our data seems inconsistent with this T loop being the only critical region for two reasons. First, there are very few differences in the *R. rubrum* P_{II} homologs in the T loop region even though we have demonstrated that there are functional differences. Second, when *R. rubrum draTG* was heterologously expressed in *K. pneumoniae*, GlnK of *K. pneumoniae* was required for DRAG activity. Unlike *R. rubrum* GlnB, *K. pneumoniae* GlnB could not replace GlnK in this role (72). The only difference in the T loop between GlnB of *R. rubrum* and GlnB of *K. pneumoniae* is residue 52 (M in *K. pneumoniae* GlnB and V in *R. rubrum* GlnB). This suggests that other differences outside the T loop region, especially in the vicinity of the B and C loops, are much more likely to be the structural basis for the functional differences that we describe.

We assume that the various P_{II} homologs of *R. rubrum* have different roles in the cell because of structural differences that affect interactions with other proteins, but it is possible that some effects might instead reflect the level of a given homolog in the cell. While we cannot rule out this possibility entirely, we used antibody raised to GlnB peptide of *R. rubrum* in a Western blot analysis of *R. rubrum* extracts and found that GlnJ is more abundant than GlnB (data not shown). Since the antibody was raised to a protein that is more similar to GlnB of *R. rubrum* than to GlnJ, we feel that it is unlikely that the results obtained reflect better cross-reaction with the latter protein. This finding strongly suggests that at least the failure of GlnJ to support NifA activation is not a result of lower levels of this protein than of GlnB.

While no growth phenotype was associated with a *glnK* mutation, GlnK is expressed in *R. rubrum* and supports normal modification of GS in response to exogenous NH_4^+ . We have not determined the levels of accumulation of this protein, however, and it is possible that its level affects its apparent inability to perform some of other functions analyzed. To completely rule out the possibility that some effects might be due to the levels of these P_{II} homologs or to the timing of their expression, further studies need to be done, such as studies to

examine expression of all of the genes under the same promoter and subsequent analysis of their functional differences.

In *R. rubrum*, GlnB and GlnJ respond not only to nitrogen status but also to energy status. We were surprised that GlnB and GlnJ have a role in energy signal transduction, because P_{II} homologs have typically been assigned roles in nitrogen regulation and carbon-nitrogen balance. This indicates that the signal transduction pathways for nitrogen and energy must merge at some point, and P_{II} homologs play a critical role in the transfer of the signals to different targets, including NifA, DRAT/DRAG, and GS. In *R. sphaeroides* and *R. rubrum* regulation of photosynthesis, CO_2 assimilation, and N_2 fixation processes is affected by a *cbbM* mutation (lacking ribulose 1,5-bisphosphate carboxylase/oxygenase or RubisCO) (25). RubisCO is a key enzyme in the Calvin-Benson-Bassham cycle, and in *R. sphaeroides* expression of *cbbM* and other *cbb* genes is regulated by the RegB-RegA regulatory system (25, 51). Like *R. sphaeroides*, which lacks the DRAT/DRAG regulatory system, *R. rubrum cbbM* mutants also exhibit high nitrogenase activity in the presence of NH_4^+ (25), indicating that *nif* expression and the DRAT/DRAG regulatory system are altered in these mutants, as is expression of *glnB* and *glnK* (50). The altered regulation of *nif* expression and DRAT/DRAG regulation in *cbbM* mutants of *R. rubrum* are likely effected through GlnB and its homologs, and this hypothesis is consistent with our previous observation that GlnB is involved in *nif* expression (73) and our observation in this study that GlnB and GlnJ are involved in DRAT/DRAG regulation. Although the mechanism for the effects of GlnB and GlnJ on the DRAT/DRAG regulatory system is unknown, these effects are probably caused by altered posttranslational regulation of DRAT/DRAG activities rather than by alteration of the levels of expression of these proteins, based on our previous heterologous studies of this system (72).

The molecular basis for the response of GlnB and GlnJ to energy status remains unclear, and it could be hypothesized that when we perturb the energy status, we are actually altering the nitrogen status (or carbon/nitrogen ratio) indirectly and that the GlnB-GlnJ response is actually a response to that status. However, this hypothesis is not supported by our previous observation that the response to energy limitation was more rapid and complete (i.e., the residual nitrogenase activity after the treatment was lower) than the response to exogenous fixed nitrogen when cells were derepressed in MG medium (68). While it is true that there are some unknown differences in the metabolism of each of these signals, it seems unlikely that perturbation of the nitrogen status through a direct effect on energy would have a more striking effect than a direct perturbation of nitrogen status itself would have. While we cannot speak to the specific signal pathway that transduces the energy signal, we feel that it is highly unlikely that this pathway is through the nitrogen system, and therefore it almost certainly represents a different type of signal than the P_{II} homologs have been thought to sense in *R. rubrum*. However, the mechanism of the GlnB-GlnJ response to energy status needs to be investigated further.

The *glnBJ* mutants grew more slowly than the wild type in SMN (rich) medium and are not stable. Suppressor mutations (*sgn*) for *glnBJ* mutants occur at a frequency of 10^{-5} to 10^{-6} , which is consistent with loss-of-function mutations. Our results

FIG. 2. Model for the roles of P_{II} homologs in *R. rubrum*. The model is meant to provide a general sense of the roles of the P_{II} homologs, but many specific aspects are unknown. For example, GlnB and GlnJ probably affect both DRAG and DRAT activities. Except for the specific role of GlnB-UMP in activation of NifA shown, we are unaware of specific roles of uridylylated forms of these homologs for GS modification, DRAT/DRAG regulation, and regulation of the unknown growth factor.

clearly show that the altered regulation of nitrogenase activity in response to NH₄⁺ and darkness is caused by GlnB and GlnJ rather than by these suppressor mutations, but the nature and roles of these suppressor mutations remain to be elucidated.

Although the terms P_{II} homolog and P_{II} paralog have been used for GlnK in *K. pneumoniae* and *E. coli*, they seem awkward for the P_{II}-like proteins in *R. rubrum*, as they share some functions. One of the important results presented here is the finding that the evolutionary relatedness of the various proteins from different organisms does not appear to closely follow function; it is the GlnK homolog in *K. pneumoniae* that affects DRAT/DRAG (when it is expressed heterologously in this organism), but either the GlnB or GlnJ (not GlnK) homolog affects DRAT/DRAG in *R. rubrum*. This underscores the problem of assignment of protein function based solely on sequence similarity and raises issues of how the distinct functions could have evolved.

In summary, there are three P_{II} homologs in *R. rubrum*, and they have distinct and overlapping functions in the cell (Fig. 2). Only GlnB is required for *nif* expression. However, either GlnB or GlnJ alone is sufficient for proper regulation of the

DRAT/DRAG regulatory system and for optimal growth on a variety of media. The observation that the P_{II} homologs are involved in responses to both nitrogen status and energy status is a particularly important observation that should have important implications for other bacteria and perhaps archaea.

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