Effect of AmtB homologues on the post-translational regulation of nitrogenase activity in response to ammonium and energy signals in *Rhodospirillum rubrum*

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The AmtB protein transports uncharged NH_3 into the cell, but it also interacts with the nitrogen regulatory protein P_{II} , which in turn regulates a variety of proteins involved in nitrogen fixation and utilization. Three P_{II} homologues, GlnB, GlnK and GlnJ, have been identified in the photosynthetic bacterium *Rhodospirillum rubrum*, and they have roles in at least four overlapping and distinct functions, one of which is the post-translational regulation of nitrogenase activity. In *R. rubrum*, nitrogenase activity is tightly regulated in response to NH_4^+ addition or energy depletion (shift to darkness), and this regulation is catalysed by the post-translational regulatory system encoded by *draTG*. Two *amtB* homologues, *amtB*₁ and *amtB*₂, have been identified in *R. rubrum*, and they are linked with *glnJ* and *glnK*, respectively. Mutants lacking AmtB₁ are defective in their response to both NH_4^+ addition and darkness, while mutants lacking AmtB₂ show little effect on the regulation of nitrogenase activity. These responses to darkness and NH_4^+ appear to involve different signal transduction pathways, and the poor response to darkness does not seem to be an indirect result of perturbation of internal pools of nitrogen. It is also shown that AmtB₁ is necessary to sequester detectable amounts GlnJ to the cell membrane. These results suggest that some element of the AmtB₁- P_{II} regulatory system senses energy deprivation and a consistent model for the integration of nitrogen, carbon and energy signals by P_{II} is proposed. Other results demonstrate a degree of specificity in interaction of AmtB₁ with the different P_{II} homologues in *R. rubrum*. Such interaction specificity might be important in explaining the way in which P_{II} proteins regulate processes involved in nitrogen acquisition and utilization.

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INTRODUCTION

Nitrogen is a major nutrient for the living cell and its availability is often the growth-limiting factor. Therefore, all organisms have evolved highly effective but complicated regulatory systems for nitrogen acquisition and utilization. The first step in the acquisition of ammonium, the transport of ammonium through the membrane, is facilitated by the ammonium/methylammonium transport (Amt) proteins, which serve as transmembrane channels (Khademi *et al.*, 2004; Soupene *et al.*, 1998). The Amt protein family [also called methylammonium permease (Mep)] has been found in all three domains of life (Howitt & Udvardi, 2000; Marini *et al.*, 1997; Saier *et al.*, 1999). AmtB in enteric bacteria has

been studied in the most detail (Blakey *et al.*, 2002; Soupene *et al.*, 2002a, 1998; Thomas *et al.*, 2000b) and X-ray crystal structures of AmtB from *Escherichia coli* and *Archaeoglobus fulgidus* have been solved at a high resolution (Andrade *et al.*, 2005; Khademi *et al.*, 2004; Zheng *et al.*, 2004). It appears that AmtB is a passive gas channel, rather than an active transporter (Javelle *et al.*, 2005; Khademi *et al.*, 2004; Soupene *et al.*, 2001, 2002a, 1998, 2002b).

After NH_3 is transported into the cell, it is assimilated into glutamine by either the glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway or the glutamate dehydrogenase (GDH) pathway. Glutamine synthetase (GS) activity is particularly critical and it is closely regulated transcriptionally and post-translationally, based on the nitrogen status of the cell (Arcondéguy *et al.*, 2001; Stadtman, 2001). There are two sensors of nitrogen status in the enterics, reflecting the pools of two small molecules: nitrogen-rich glutamine and carbon-rich 2-oxoglutarate (α -ketoglutarate, α -KG). The primary nitrogen sensor is GlnD, a bifunctional

Abbreviations: Amt, ammonium/methylammonium transport; DRAG, dinitrogenase reductase activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyl transferase; GS, glutamine synthetase; α -KG, 2-oxoglutarate; MSX, methionine sulfoximine; Gm, gentamycin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.

uridylyltransferase/uridylyl-removing enzyme (UTase/UR, the product of *glnD*), which uridylylates or deuridylylates the other central protein in nitrogen sensing, P_{II} (Ikeda *et al.*, 1996; Jiang *et al.*, 1998a, b). GlnD directly senses the level of glutamine in the cell, while P_{II} binds both α -KG and ATP (Jiang *et al.*, 1998b; Kamberov *et al.*, 1995; Ninfa & Jiang, 2005). Under nitrogen-deficient conditions (low glutamine and high α -KG) GlnD acts as uridylyltransferase to uridylylate P_{II}. P_{II}-UMP then binds to a set of appropriate receptor proteins and affects their activities. In nitrogen-excess conditions (high glutamine and low α -KG), GlnD acts as a uridylyl-removing enzyme to deuridylylate P_{II}, which interacts with a different but overlapping set of receptor proteins to regulate them appropriately. In many bacteria the receptor proteins for P_{II} interaction include NtrB, which is part of a two-component regulatory system (Ninfa *et al.*, 1995; Reitzer, 2003), and GlnE, which controls the reversible adenylation of GS (Jiang *et al.*, 1998a; Stadtman, 2001). In diazotrophs P_{II} homologues interact with either NifA or NifL, which directly or indirectly regulates *nif* expression (He *et al.*, 1998; Jack *et al.*, 1999; Little *et al.*, 2002, 2000; Rudnick *et al.*, 2002; Stips *et al.*, 2004). Two P_{II} homologues, GlnB and GlnK, have been identified in *E. coli* and *Klebsiella pneumoniae* (Jack *et al.*, 1999; van Heeswijk *et al.*, 1996); however, the number of homologues varies according to the organism (Arcondéguy *et al.*, 2001; Ninfa & Atkinson, 2000; Zhang *et al.*, 2003).

AmtB has been shown to be another receptor for P_{II} interaction (Blauwkamp & Ninfa, 2003; Coutts *et al.*, 2002; Javelle *et al.*, 2004). In a variety of diverse bacteria, AmtB interacts directly with unmodified GlnK to form a membrane-bound complex under NH₄⁺-excess conditions (Coutts *et al.*, 2002; Detsch & Stülke, 2003; Javelle *et al.*, 2004; Klopprogge *et al.*, 2002; Strösser *et al.*, 2004). This interaction is reversible and is thought to block the NH₃-transporting function of AmtB (Coutts *et al.*, 2002; Javelle *et al.*, 2004), and a docking model for the interaction of these two proteins from *A. fulgidus* has been proposed (Andrade *et al.*, 2005). However, overexpression of AmtB affects other P_{II} targets in *E. coli*, such as NtrB (Blauwkamp & Ninfa, 2003), which strongly suggests that the AmtB-P_{II} interaction can affect P_{II} function as well. It has been proposed that the co-production of GlnK and AmtB could prevent AmtB from sequestering GlnB under nitrogen-deficient conditions, allowing GlnB to play its necessary roles in the cell (Blauwkamp & Ninfa, 2003). Mutations in *amtB* of *Rhodobacter capsulatus* and *Azoarcus* also show dramatic effects on the regulation of nitrogenase activity by NH₄⁺, and it has been proposed that AmtB may be an ammonium sensor (Javelle *et al.*, 2004; Martin & Reinhold-Hurek, 2002; Yakunin & Hallenbeck, 2002), but the effect might be indirect, since mutants lacking P_{II} also showed an altered NH₄⁺ response (Drepper *et al.*, 2003; Martin & Reinhold-Hurek, 2002). Given the centrality of P_{II} to nitrogen regulation, the notion that AmtB might exert its effects through P_{II} interaction is certainly attractive. It is notable that the darkness response to regulate nitrogenase activity is normal in these *amtB* mutants of *R. capsulatus* (Yakunin & Hallenbeck, 2002).

The issue of P_{II} interacting with different receptor proteins raises another basic, but understudied question. If P_{II} functions by interacting with different receptor proteins, including AmtB, under different conditions, is there any competition for available P_{II} among these receptors, which must in turn affect P_{II} function? This competition would certainly be influenced by the level of each protein in the cell and by the relative affinity between each P_{II} protein and each receptor. While different interaction specificities of GlnB and GlnK in *E. coli* have been suggested (Atkinson & Ninfa, 1998, 1999; Atkinson *et al.*, 2002; Blauwkamp & Ninfa, 2002), there are only two clear examples of P_{II} interaction specificity. In *K. pneumoniae*, GlnK but not GlnB interacts with NifL to relieve its inhibition of NifA activity (He *et al.*, 1998; Jack *et al.*, 1999). The substitution of residues 43 and 54 allows GlnB of *K. pneumoniae* to perform the 'GlnK function' of interacting with NifL when the altered GlnB is overexpressed (Arcondéguy *et al.*, 2000). In *Rhodospirillum rubrum*, GlnB-UMP, but not the other two P_{II} homologues, interacts directly with NifA to activate it. Specific residues of GlnB that define this specific interaction have been analysed (Zhang *et al.*, 2004).

R. rubrum has three P_{II} homologues, GlnB, GlnK and GlnJ, which play both distinct and overlapping functions by interacting with multiple target proteins in the cell (Zhang *et al.*, 2005, 2001a). All of these homologues can properly regulate GlnE to regulate GS activity (Zhang *et al.*, 2001a). However, GlnB is more effective in the regulation of NtrB activity than are the other P_{II} homologues (Zhang *et al.*, 2005). *R. rubrum* strains lacking both *glnB* and *glnJ* grow very poorly on a variety of media, irrespective of the presence of a functional *glnK* (Zhang *et al.*, 2001a).

Another role of P_{II} homologues in *R. rubrum* is to regulate the reversible mono-ADP ribosylation of dinitrogenase reductase to control nitrogenase activity (Zhang *et al.*, 2003). Under NH₄⁺-excess or energy-limitation (cells are shifted from light to dark) conditions, dinitrogenase reductase is ADP-ribosylated and thereby inactivated by DRAT (dinitrogenase reductase ADP-ribosyl transferase, the gene product of *draT*). However, when NH₄⁺ is exhausted or cells are returned to light, the ADP-ribose group can be removed by DRAG (dinitrogenase reductase activating glycohydrolase, the gene product of *draG*), thus restoring nitrogenase activity. The activities of DRAT and DRAG are themselves subject to post-translational regulation (Zhang *et al.*, 1997). Either GlnB or GlnJ is sufficient for approximately normal function of this regulatory system, though we cannot assume that their roles are mechanistically similar (Zhang *et al.*, 2001a).

In *R. rubrum* there are two *amtB* homologues: *amtB*₁ is linked to *glnJ*, while *amtB*₂ is linked to *glnK* (Zhang *et al.*, 2001a). The goal of this work was to better understand the role of these AmtB homologues in *R. rubrum* and their possible interactions with P_{II} homologues.

METHODS

Bacterial strains, growth conditions and whole-cell nitrogenase activity assay. The strains and plasmids used in this study are listed in Table 1. *R. rubrum* was grown in SMN (rich) medium, malate-glutamate (MG, minimal medium with glutamate as nitrogen source) medium or MN⁻ (nitrogen-free minimal) medium as described previously (Fitzmaurice *et al.*, 1989; Lehman & Roberts, 1991; Zhang *et al.*, 2001a). Antibiotics were used as necessary at the levels described previously (Zhang *et al.*, 2000). Whole-cell nitrogenase activity assay and darkness/NH₄Cl treatments have also been described previously (Zhang *et al.*, 1995).

To measure generation times, purified colonies of *R. rubrum* were used as inoculants into 5 ml aliquots of SMN medium and grown aerobically at 30 °C for 2 days. Cultures were diluted (25- to 100-fold) into 25 ml SMN in a 125 ml flask (with an initial OD₆₀₀ of 0.04–0.05) and then incubated at 30 °C on an orbital shaker with a speed of 250 r.p.m. One millilitre of culture was taken out every 4 h to measure OD₆₀₀ for 36 h and the generation times were determined from the exponential phase.

Construction of *amtB*₁ and *amtB*₂ mutants. A 5 kb *Eco*RI fragment of the *glnJ-amtB*₁ region from pUX268 (Zhang *et al.*, 2001a) was subcloned into pUX19 (Lies, 1994), yielding pUX277. To construct *amtB*₁ mutants, *aacC1* from pUCGM (Schweizer, 1993) was inserted into *Xcm*I sites, resulting in the deletion of 1.3 kb of the 3' portion of *amtB*₁ and the adjoining region, yielding pUX283. pUX283 was conjugated into *R. rubrum* wild-type (UR2) and a *glnB* mutant (UR717) as described previously (Liang *et al.*, 1991). Mutants resulting from a double-crossover recombination event were identified by drug phenotype and verified by PCR. Two *amtB*₁ mutants were designated UR794 (Δ *amtB*₁1::*aacC1*) and UR795 (Δ *glnB3* Δ *amtB*₁1::*aacC1*).

Similarly, a 3 kb *Bgl*II–*Hind*III fragment containing *glnK-amtB*₂ was cloned into pSUP202, yielding pUX1664. The *kan* gene from pUC4K (Tanigawa *et al.*, 2002) was inserted into a *Pst*I site in *amtB*₂, yielding pUX1974. This plasmid was transferred into *R. rubrum* wild-type and an *amtB*₁ mutant and mutants created by double crossovers were identified: UR1635 (*amtB*₂1::*kan*) and UR1790 (Δ *amtB*₁1::*aacC1* *amtB*₂1::*kan*).

Construction of *glnJ-amtB*₁ deletion mutants. An 8 kb *Eco*RI fragment containing *glnJ-amtB*₁ was amplified by PCR and cloned into pBSKS(–) yielding pUX281. The *Km*^r gene from pUC4K (Vieira & Messing, 1982) was inserted into a *Bgl*II site, yielding pUX952, so that about 2.2 kb of *glnJ-amtB*₁ region was deleted and replaced with the *kan* gene. The mutated *glnJ-amtB*₁ region was subcloned into pSUP202 (Simon *et al.*, 1983), yielding pUX954, where *kan* is transcribed in the same direction as *glnJ*. pUX954 was conjugated into *R. rubrum* wild-type (UR2), a *glnB* mutant (UR717) and a *glnB glnK* mutant (UR757) to create Δ *glnJ-amtB*₁2::*kan* (UR1147), Δ *glnB3* Δ *glnJ-amtB*₁2::*kan* (UR1149) and Δ *glnB3* Δ *glnK1*::*aacC1* Δ *glnJ-amtB*₁2::*kan* (UR1240). Strains with the opposite orientation of *kan* were constructed, but behaved identically, so that only data from the first set of mutants is reported here.

Construction of Δ *glnJ* Δ *amtB*₁, *draG* and Δ *glnB* Δ *glnJ* Δ *amtB*₁, *draG* mutants. A 1.7 kb *Bam*HI–*Hind*III fragment of *R. rubrum* *draTG* was cloned into pSUP202, yielding pUX343. *aacC1* from pUCGM was inserted into *draG* (same orientation) at *Eco*RV sites, yielding pUX347. This plasmid was conjugated into *R. rubrum* wild-type (UR2), Δ *glnJ-amtB*₁ (UR1147) and Δ *glnB* Δ *glnJ-amtB*₁ (UR1149) to create three *draG* mutants: *draG11*::*aacC1* (UR832), Δ *glnJ-amtB*₁2::*kan draG11*::*aacC1* (UR1406) and Δ *glnB3*

Δ *glnJ-amtB*₁2::*kan draG11*::*aacC1* (UR1410). As above, the orientation of *aacC1* was not important and only results from the first set are presented.

To restore nitrogenase activity, pCK3 carrying *K. pneumoniae nifA* (Kennedy & Drummond, 1985) was transferred into *R. rubrum glnB* mutants by the tri-parental mating method described previously (Grunwald *et al.*, 1995).

Construction of *amtB*₁ expression plasmids and strains. A 6 kb *Eco*RI–*Hind*III fragment containing *glnJ-amtB*₁ from pUX282 (which is similar to pUX281, but with different orientation of insert) was cloned into pRK404 (Ditta *et al.*, 1985), yielding pUX1109 (Fig. 1). pUX1109 was digested with *Bam*HI and religated, deleting a 231 bp fragment internal to *glnJ*, yielding pUX1139. This in-frame deletion of *glnJ* allows *amtB*₁ to be expressed from both the *glnJ* promoter and its own promoter. A 2.4 kb *Bgl*II fragment containing *glnJ'* *amtB*₁ from pUX282 was cloned into pRK404, yielding pUX1138. These plasmids were transformed into *E. coli* DH5 α and then conjugated into *R. rubrum glnB glnK* (UR757) and *glnJ glnK* (UR810) mutants. Sm^r Gm^r (Km^r) Tc^r *R. rubrum* colonies were selected. New strains were designated UR1183, UR1184, UR1185, UR1186, UR1203 and UR1204.

Membrane fractionation. *R. rubrum* was grown in SMN medium aerobically at 30 °C for 2 days, then diluted 100-fold into 200 ml MG medium and incubated anaerobically at 30 °C in the light for 1–2 days. When the cultures reached an OD₆₀₀ of 0.6–0.9, the bottles were quickly cooled to 4 °C in a dry-ice ethanol bath (2–3 min) while remaining in the light. Cells were concentrated 100-fold by centrifugation at 2000 g for 4 min, resuspended in 50 mM NaPO₄ (pH 6.8) buffer and immediately broken by three rounds of sonication for 30 s. Whole-cell extract was clarified by two rounds of centrifugation at 20 000 g for 1 min to remove unbroken cells and cell debris. Whole-cell extracts were subjected to three rounds of ultracentrifugation (200 000 g) for 30 min at 4 °C, with resuspension in 1 ml 50 mM NaPO₄ (pH 6.8) buffer after each round, with the final pellet used as the membrane fraction. The supernatant after the first round of ultracentrifugation was used as the cytoplasmic fraction.

Immunoblotting of GlnJ and GS. Trichloroacetic acid precipitation was used to extract and concentrate protein from whole-cell, cytoplasmic and membrane fractions (Zhang *et al.*, 1993). A Tricine SDS-PAGE gel was used (Schägger & von Jagow, 1987), but with a low-cross-linker acrylamide/bisacrylamide mix (40% T and 0.23% C) in the resolving gel to increase separation of the modified and unmodified subunits of P_{II} proteins. Proteins were electrophoretically transferred to a nitrocellulose membrane, probed with polyclonal antibody against *R. rubrum* GlnJ and visualized with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences).

GS immunoblotting was detected with horseradish peroxidase colour detection reagents (Bio-Rad) as described previously (Zhang *et al.*, 1993, 2000).

RESULTS

The absence of AmtB₁ dramatically perturbs the post-translational regulation of nitrogenase activity in response to NH₄⁺, but little effect is seen in a mutant lacking AmtB₂

Previous studies showed that both GlnB and GlnJ are involved in the regulation of the DRAT-DRAG system in response to darkness and NH₄⁺ stimuli (Zhang *et al.*, 2001a).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and description	Source or reference
<i>R. rubrum</i>		
UR2	Wild-type; Sm ^r	Fitzmaurice <i>et al.</i> (1989)
UR717	$\Delta glnB3$ (in-frame deletion) mutant; Sm ^r	Zhang <i>et al.</i> (2000)
UR757	$\Delta glnB3 \Delta glnK1::aacC1$ mutant; Sm ^r Gm ^r	Zhang <i>et al.</i> (2001a)
UR794	$\Delta amtB_1::aacC1$ mutant; Sm ^r Gm ^r	This study
UR795	$\Delta glnB3 \Delta amtB_1::aacC1$ double mutant; Sm ^r Gm ^r	This study
UR810	$\Delta glnK1::aacC1 \Delta glnJ1::kan$ double mutant; Sm ^r Gm ^r Km ^r	Zhang <i>et al.</i> (2001a)
UR832	$\Delta draG11::aacC1$ mutant; Sm ^r Gm ^r	This study
UR1115	Transconjugant of UR794 with pCK3; Sm ^r Gm ^r Tc ^r	This study
UR1117	Transconjugant of UR795 with pCK3; Sm ^r Gm ^r Tc ^r	This study
UR1147	$\Delta glnJ\text{-}amtB_1::kan$ mutant (same orientation); Sm ^r Km ^r	This study
UR1149	$\Delta glnB3 \Delta glnJ\text{-}amtB_1::kan$ mutant (same orientation); Sm ^r Km ^r	This study
UR1153	Transconjugant of UR1149 with pCK3; Sm ^r Km ^r Tc ^r	This study
UR1183	Transconjugant of UR757 with pUX1109; Sm ^r Km ^r Tc ^r	This study
UR1184	Transconjugant of UR757 with pUX1139; Sm ^r Km ^r Tc ^r	This study
UR1185	Transconjugant of UR810 with pUX1109; Sm ^r Km ^r Tc ^r	This study
UR1186	Transconjugant of UR810 with pUX1139; Sm ^r Km ^r Tc ^r	This study
UR1203	Transconjugant of UR757 with pUX1138; Sm ^r Km ^r Tc ^r	This study
UR1204	Transconjugant of UR810 with pUX1138; Sm ^r Km ^r Tc ^r	This study
UR1240	$\Delta glnB3 \Delta glnK1::aacC1 \Delta glnJ\text{-}amtB_1::kan$ mutant; Sm ^r Km ^r Gm ^r	This study
UR1406	$\Delta glnJ\text{-}amtB_1::kan \Delta draG11::aacC1$ mutant; Sm ^r Km ^r Gm ^r	This study
UR1410	$\Delta glnB3 \Delta glnJ\text{-}amtB_1::kan \Delta draG11::aacC1$ mutant; Sm ^r Km ^r Gm ^r	This study
UR1436	Transconjugant of UR1410 with pCK3; Sm ^r Km ^r Gm ^r Tc ^r	This study
UR1635	$\Delta amtB_1::kan$ mutant; Sm ^r Km ^r	This study
UR1790	$\Delta amtB_1::aacC1 \Delta amtB_1::kan$ mutant; Sm ^r Gm ^r Km ^r	This study
Plasmids		
pUX19	A suicide vector for <i>R. rubrum</i> ; Km ^r	Lies (1994)
pUCGM	pUC19 derivative containing <i>aacC1</i> (Gm ^r gene); Ap ^r Gm ^r	Schweizer (1993)
pUC4K	<i>kan</i> from Tn903 in pBR322; Ap ^r Km ^r	Tanigawa <i>et al.</i> (2002)
pSUP202	A suicide vector for <i>R. rubrum</i> ; Ap ^r Cm ^r Tc ^r	Simon <i>et al.</i> (1983)
pRK404	Broad-host-range plasmid for <i>R. rubrum</i> ; Tc ^r	Ditta <i>et al.</i> (1985)
pCK3	pRK290 derivative containing <i>K. pneumoniae nifA</i> ; Tc ^r	Kennedy & Drummond (1985)
pUX277	A 5 kb <i>EcoRI</i> fragment of <i>R. rubrum glnJ' amtB_1</i> cloned into pUX19; Km ^r	This study
pUX281	An 8 kb <i>EcoRI</i> fragment of <i>R. rubrum glnJ amtB_1</i> cloned into pBSKS(-); Ap ^r	This study
pUX282	Similar to pUX281, but <i>R. rubrum glnJ amtB_1</i> cloned into pBSKS(-) with different orientation; Ap ^r	This study
pUX283	<i>aacC1</i> from pUCGM inserted into <i>amtB_1</i> at <i>XcmI</i> sites of pUX277, with about 1.3 kb deletion of <i>amtB_1</i> and downstream region; Km ^r Gm ^r	This study
pUX343	A 1.7 kb <i>BamHI-HindIII</i> fragment of <i>R. rubrum draTG'</i> cloned into pSUP202; Ap ^r Cm ^r	This study
pUX347	<i>aacC1</i> from pUCGM inserted into <i>draG</i> at <i>EcoRV</i> sites of pUX343 (same orientation); Ap ^r Cm ^r Gm ^r	This study
pUX952	<i>kan</i> from pUC-4K inserted into the <i>BglII</i> sites in pUX281 in the same orientation, with a 2.4 kb deletion of <i>glnJ amtB_1</i> ; Ap ^r Km ^r	This study
pUX954	A 7 kb <i>EcoRV</i> fragment of $\Delta glnJ\text{-}amtB_1::kan$ from pUX952 cloned into pSUP202; Ap ^r Cm ^r Km ^r	This study
pUX1109	<i>HindIII-EcoRI</i> fragment of <i>glnJ amtB_1</i> from pUX282 cloned into pRK404; Tc ^r	This study
pUX1138	<i>P_{amtB_1}-amtB_1</i> ; <i>BglII</i> fragment of <i>glnJ' amtB_1</i> from pUX282 cloned into pRK404; Tc ^r	This study
pUX1139	<i>P_{glnJ}</i> and <i>P_{amtB_1}-amtB_1</i> ; pUX1109 derivative with a <i>BamHI</i> fragment which produces an in-frame deletion in <i>glnJ</i> ; Tc ^r	This study
pUX1664	A 3 kb <i>BglII-HindIII</i> fragment of <i>R. rubrum glnK amtB_2</i> cloned into pSUP202; Ap ^r Cm ^r	This study
pUX1974	<i>kan</i> from pUC4K was inserted into <i>amtB_2</i> at <i>PstI</i> sites of pUX1664; Ap ^r Cm ^r Km ^r	This study

Fig. 1. Physical map of plasmids for *amtB₁* expression. In pUX1109, *amtB₁* is expressed from both its own promoter and (with *glnJ*) from the *glnJ* promoter. In pUX1138, *amtB₁* is expressed only from its own promoter, since the *glnJ* promoter was deleted. In pUX1139, *amtB₁* is expressed from both its own promoter and that of *glnJ*, though *glnJ* itself has been deleted.

To test the hypothesis that the absence of AmtB₁ might affect P_{II} function and then perturb the regulation of DRAT and DRAG activities, we constructed *amtB₁* and *amtB₂* mutants and determined the roles of these AmtB homologues in the regulation of nitrogenase activity. MN⁻-grown cultures were used to study the NH₄⁺ response, since they show a more drastic NH₄⁺ response than do MG-grown cultures (Zhang *et al.*, 2001a). As shown in Table 2, UR2 (wild-type) had moderate nitrogenase activity in MN⁻ medium before the addition of NH₄⁺, and this activity decreased 95 % after NH₄⁺ addition. The *amtB₁* single mutant (UR794) showed slightly lower initial nitrogenase activity, but it lost only about 15 % of nitrogenase activity after NH₄⁺ addition. However, the *amtB₂* single mutant (UR1635) showed rapid loss of nitrogenase activity after NH₄⁺ addition, similar to that seen in wild-type (UR2). The *amtB₁ amtB₂* double mutant (UR1190) also showed an altered NH₄⁺ response, similar to that seen in the *amtB₁* single mutant (UR794). These results indicate that the regulation of the DRAT-DRAG system is dramatically perturbed in mutants lacking AmtB₁. In contrast, AmtB₂ appears to play a minor role in the regulation of the DRAT-DRAG system in response to NH₄⁺. An obvious possibility is that NH₃ transport is impaired in the *amtB₁* mutant, preventing the NH₄⁺ signal to the DRAT-DRAG regulatory system. However, as shown below, the effect of AmtB₁ on DRAT-DRAG is more complicated, and it is more likely that AmtB₁ is sequestering P_{II}, rather than directly regulating DRAT-DRAG. The fact that the *amtB₁ amtB₂* double mutants had substantial initial nitrogenase activity indicates that these AmtB homologues are not necessary for the uridylylation of GlnB, which is absolutely necessary for activating NifA for *nif* expression (Zhang *et al.*, 2000).

NH₄⁺ response in *amtB₁* mutants that also lack GlnB, GlnJ or both

To test the notion that the effect of the *amtB₁* mutation on DRAT-DRAG was exerted through P_{II}, we then analysed the impact of the absence of one or more P_{II} proteins in the *amtB₁* mutant background. All *glnB* mutants lack nitrogenase activity, since GlnB-UMP is required for the activation of NifA. We circumvented the GlnB requirement by introducing a plasmid expressing NifA from *K. pneumoniae* (Kennedy & Drummond, 1985), as done previously (Zhang *et al.*, 2000). The *K. pneumoniae* NifA does not itself perturb DRAT-DRAG regulation, as shown by a comparison of strains UR794 and UR1115 (Table 2) and other previous data (Zhang *et al.*, 2000).

As controls, strains lacking *glnB*, *glnJ* or both were also examined. As shown in Table 2 and reported previously (Zhang *et al.*, 2001a), the *glnJ* mutation alone (UR806) does not show any significant effect on the DRAT-DRAG regulatory system. The *glnB* mutant with *K. pneumoniae nifA* (UR720) responded to NH₄⁺, but with slightly higher residual activity, indicating that ADP-ribosylation of dinitrogenase reductase was altered slightly in this mutant. As seen previously, a poor response to NH₄⁺ was seen in the *glnB glnJ* double mutant (UR824) (Zhang *et al.*, 2001a). All tested *amtB₁* strains, including *glnB amtB₁*, *glnJ amtB₁* and *glnB glnJ amtB₁* mutants, completely failed to respond to NH₄⁺ addition (Table 2). Strains lacking *amtB₁* and either *glnB* or *glnJ* appeared to be even more perturbed than strains lacking only *amtB₁*. All of these results show that the absence of AmtB₁ dramatically perturbs the regulation of the DRAT-DRAG system irrespective of the presence or absence of P_{II} homologues. This suggests that proper DRAT-DRAG regulation requires the presence of AmtB₁ and either GlnB or GlnJ, consistent with a functional interaction between AmtB₁ and the P_{II} homologues. The data also show that the *glnB glnJ amtB₁* mutant (UR1153) is even more perturbed than is the *glnB glnJ* strain (UR824). We believe this reflects the availability of GlnK, as discussed below.

The absence of AmtB₁ also perturbs the post-translational regulation of nitrogenase activity in response to darkness

We also examined the impact of AmtB₁ on the response of the DRAT-DRAG system to energy depletion. Unlike the slow response to NH₄⁺ addition to MG medium (Zhang *et al.*, 1995), wild-type (UR2) grown in MG medium showed a dramatic decline in nitrogenase activity upon removal of light and an equally dramatic recovery of activity upon its restoration (Table 3). The MG medium contains the poor nitrogen source glutamate, which supports higher nitrogenase activity than does the nitrogen-free medium used in Table 2. The *amtB₁* mutants, with or without *nifA* of *K. pneumoniae*, had slightly lower initial nitrogenase activities, but only a partial response to the removal of light, followed by a complete recovery of activities after the return of light

Table 2. Nitrogenase activity and NH_4^+ response in MN^- -grown *R. rubrum* wild-type, *amtB*₁, *amtB*₂ and other mutants

Strain	Chromosomal genotype	Gene(s) on plasmids*	Nitrogenase activity before and after addition of 10 mM NH_4Cl †		
			Initial activity (= 100 %)	10 min (%)	50 min (%)
UR2	Wild-type	None	420	5	4
UR794	<i>amtB</i> ₁	None	240	85	87
UR1635	<i>amtB</i> ₂	None	250	4	3
UR1790	<i>amtB</i> ₁ <i>amtB</i> ₂	None	200	78	80
UR1115	<i>amtB</i> ₁	<i>K.p. nifA</i>	260	84	80
UR720‡	<i>glnB</i>	<i>K.p. nifA</i>	400	20	5
UR806‡	<i>glnJ</i>	None	350	1	1
UR824‡	<i>glnB glnJ</i>	<i>K.p. nifA</i>	300	70	60
UR1117	<i>glnB amtB</i> ₁	<i>K.p. nifA</i>	220	102	90
UR1147	<i>glnJ amtB</i> ₁	None	250	98	96
UR1153	<i>glnB glnJ amtB</i> ₁	<i>K.p. nifA</i>	370	96	91

**K.p. nifA*, *K. pneumoniae nifA* carried on pCK3 (Kennedy & Drummond, 1985).

†Cultures were grown in MN^- medium for derepression of nitrogenase. Nitrogenase activity was assayed before NH_4^+ addition (initial activity), and 10 min and 50 min after cells were treated with 10 mM NH_4Cl . The percentage of initial nitrogenase activity remaining after NH_4^+ addition is indicated. Initial nitrogenase activity is expressed as nmol ethylene produced h^{-1} (ml cells at $\text{OD}_{600} = 1$)⁻¹. Each activity value is from at least 10 replicate assays from different individually grown cultures. The SD is between 5 and 15 %.

‡Data from UR720, UR806 and UR824 were published previously (Zhang *et al.*, 2001a).

Table 3. Nitrogenase activity and its response to darkness in MG-grown *R. rubrum* wild-type, *amtB*₁, *amtB*₂ and other mutants

Strain	Chromosomal genotype	Gene(s) on plasmids*	Nitrogenase activity before and after dark/light shifts†		
			Initial activity (= 100%)	60 min dark (%)	10 min light (%)
UR2	Wild-type	None	800	7	87
UR794	<i>amtB</i> ₁	None	660	53	94
UR1115	<i>amtB</i> ₁	<i>K.p. nifA</i>	650	40	98
UR1635	<i>amtB</i> ₂	None	700	20	99
UR1790	<i>amtB</i> ₁ <i>amtB</i> ₂	None	650	65	110
UR720‡	<i>glnB</i>	<i>K.p. nifA</i>	550	12	85
UR806‡	<i>glnJ</i>	None	820	7	97
UR824‡	<i>glnB glnJ</i>	<i>K.p. nifA</i>	700	103	101
UR1117	<i>glnB amtB</i> ₁	<i>K.p. nifA</i>	460	101	110
UR1147	<i>glnJ amtB</i> ₁	None	660§	47	86
UR1153	<i>glnB glnJ amtB</i> ₁	<i>K.p. nifA</i>	380§	110	105
UR832	<i>draG</i>	None	500	5	25
UR1550	<i>amtB</i> ₁ <i>draG</i>	None	120	NT	NT
UR1406	<i>glnJ amtB</i> ₁ <i>draG</i>	None	70	NT	NT
UR1436	<i>glnB glnJ amtB</i> ₁ <i>draG</i>	<i>K.p. nifA</i>	80	NT	NT

**K.p. nifA*, *K. pneumoniae nifA* carried on pCK3 (Kennedy & Drummond, 1985).

†Cultures were grown in MG medium for derepression of nitrogenase. Nitrogenase activity was assayed before darkness shifts (initial activity), 60 min after cells were shifted to dark and 10 min after cells were returned to light. The percentage of initial nitrogenase activity remaining after the shift to darkness or re-illumination is indicated. Initial nitrogenase activity is expressed as nmol ethylene produced h^{-1} (ml cells at $\text{OD}_{600} = 1$)⁻¹. Each activity value is from at least 10 replicate assays from different individually grown cultures. The SD is between 5 and 15 %. NT, Not tested.

‡Data from UR720, UR806 and UR824 were published previously (Zhang *et al.*, 2001a).

§More $\Delta\text{glnJ-amtB}_1$, $\Delta\text{glnB } \Delta\text{glnJ-amtB}_1$ mutants with Km^r or Gm^r inserted in the opposite orientation were constructed and analysed. All showed identical results to the similar mutants listed in this table.

(UR794 and UR1115 in Table 3). The *amtB₂* single mutant (UR1635) also showed a normal darkness response, with only slightly higher residual activity remaining under darkness conditions than that seen in wild-type (UR2), while the *amtB₁ amtB₂* double mutant (UR1790) showed a partial response to darkness shifts, similar to that seen in the *amtB₁* single mutant (UR794). This result indicates that AmtB₁, but not AmtB₂, is necessary for the proper response of DRAT-DRAG to energy depletion. This is important because such an energy response defect is very difficult to explain based on the known role of AmtB₁ in NH₃ transport. Because of our previous results implicating P_{II} proteins in this light/dark regulation of nitrogenase activity, it was an obvious possibility that the effect of the absence of AmtB₁ might be mediated through the P_{II} proteins.

We then examined the impact of *glnB* and *glnJ* mutations in the Δ *amtB₁* background on the darkness response. As controls, strains lacking *glnB*, *glnJ* or both were also examined. As shown in Table 3 and reported previously (Zhang *et al.*, 2001a), the *glnB* or *glnJ* mutations alone do not show any significant effect on the darkness response (UR720 and UR806), although the darkness response was abolished in the *glnB glnJ* double mutant (UR824). In a Δ *amtB₁* strain the presence or absence of *glnJ* was largely irrelevant (UR794 and UR1147 in Table 3), but the absence of *glnB* completely eliminated the response to darkness in the Δ *amtB₁* background (UR1117 and UR1153 in Table 3).

These results have two implications. First, the interaction of AmtB₁ and GlnB/J is important not only to the NH₄⁺ response, but also to the darkness response. We note, however, that mutants lacking AmtB₁ and/or GlnB/J showed differences in the response to these different stimuli, indicating that darkness and NH₄⁺ might have different mechanisms for regulation of DRAT-DRAG. Second, the result shows that GlnB and GlnJ are not equivalent in a Δ *amtB₁* background, which might reflect a differential interaction between AmtB₁ and these two P_{II} homologues. These two issues will be discussed below.

Finally, we were curious to know if the absence of AmtB₁ affected DRAT, DRAG or both, because the perturbed regulation seen in these *amtB₁* mutants might result from perturbation of the activation of DRAT or inactivation of DRAG. As mentioned in the Introduction, DRAT modifies dinitrogenase reductase by ADP-ribosylation under NH₄⁺-excess or energy-limiting conditions, resulting in inactivation of that enzyme. DRAG restores dinitrogenase reductase activity by removing the ADP-ribose group. We therefore examined strains lacking *draG*, so that we could focus only on the activity of DRAT without the complication of the competing DRAG activity. In an otherwise wild-type background, a *draG* mutant has fairly normal initial nitrogenase activity because DRAT is very tightly regulated until nitrogen addition or energy depletion (UR832 in Table 3), which is consistent with a previous report of other *draG* mutants (Liang *et al.*, 1991). However, all *draG* mutants lacking *amtB₁*, *glnJ amtB₁* or *glnB glnJ amtB₁* show low initial

nitrogenase activities (UR1550, UR1406 and UR1436 in Table 3), indicating that some DRAT had already escaped normal down-regulation and was able to modify dinitrogenase reductase in these *amtB₁* mutant backgrounds even before treatment. Therefore, the failure of the DRAT-DRAG system to turn off nitrogenase activity in response to NH₄⁺ or darkness in *amtB₁* mutant backgrounds cannot be attributed to the failure to activate DRAT activity, but must instead be the result of the mutant's inability to inactivate DRAG under these conditions.

Effects of darkness do not seem to be mediated through glutamine pools

The effects of darkness on GS activity have been reported for different organisms (Johansson & Gest, 1977; Margués *et al.*, 1992; Reyes & Florencio, 1995; Reyes *et al.*, 1995), including *R. rubrum* where GS activity decreased when cells were shifted to dark and recovered on re-illumination (Nordlund *et al.*, 1985). We tested the notion that changes in nitrogen regulation by a darkness shift might result from direct or indirect effects on pools of nitrogen metabolites, such as glutamine. However, this hypothesis seems not to be the case for the darkness effect in *R. rubrum* for several reasons. First, we examined the effect of both darkness and NH₄⁺ on the timing of DRAT activation. We reasoned that the response to NH₄⁺ should certainly be at least as rapid as to darkness if the latter treatment led indirectly to changes in the nitrogen status. We used a *draG* mutant (UR832) to avoid complications with DRAG. In fact, when UR832 was derepressed for nitrogenase in MG medium, the response to darkness was significantly faster than to NH₄⁺, implying that darkness is not signalled through an indirect effect on nitrogen status (Fig. 2). Consistent with the efficacy of NH₄⁺ treatment, GS was rapidly modified in response to NH₄⁺ addition, but showed only a very slow response to darkness (Fig. 3).

Next, we re-examined the effect of methionine sulfoximine (MSX), an inhibitor of GS activity, on the regulation of nitrogenase activity after NH₄⁺ or dark treatments in *R. rubrum*. Previous reports showed that MSX completely blocked the effect of NH₄⁺ on nitrogenase activity, while the effect of MSX on the darkness response has been controversial in *R. rubrum* (Kanemoto & Ludden, 1984; Li *et al.*, 1987; Sweet & Burris, 1981; Yoch & Gotto, 1982). We again used a *draG* mutant (UR832) to obtain more interpretable results. After pre-treatment with MSX, UR832 (*draG*) showed a diminished rate of loss of nitrogenase activity after addition of NH₄⁺, with about 60 % activity remaining after 60 min of treatment, similar to that seen in cells treated with MSX alone (data not shown). In contrast, after pre-treatment with MSX, UR832 still showed a rapid loss of nitrogenase activity in response to darkness shifts. Similarly, MSX treatment had no significant effect on the darkness response in wild-type (data not shown). These results indicate that glutamine is not the signal for the darkness response. Consistent with this, a rapid and drastic increase in the glutamine pool has been seen after addition of NH₄⁺, but only a very small change was seen after the darkness

Fig. 2. Regulation of nitrogenase activity by NH_4^+ ($\square$) and darkness ($\circ$) in an *R. rubrum draG* mutant (UR832). At time zero, NH_4Cl was added to derepressed cultures of UR832 to a final concentration of 10 mM or cultures were shifted to darkness and then returned to light at 60 min. At the times indicated, 1 ml portions of cells were withdrawn and assayed for nitrogenase activity anaerobically under illumination conditions for 2 min. Initial nitrogenase activities (100 %) in UR832 were about 600 nmol ethylene produced h^{-1} (ml cells at $\text{OD}_{600}=1$) $^{-1}$. Each point represents a mean of at least three replicate assays.

shift in *R. rubrum* (Kanemoto & Ludden, 1987; Li *et al.*, 1987). These data suggest that the modification of GS and the regulation of nitrogenase activity are two independent events, and both are regulated by P_{II} in response to NH_4^+ and darkness stimuli.

AmtB₁ is required to sequester unmodified GlnJ to the membrane in *R. rubrum*

A number of reports have been published demonstrating that AmtB homologues can sequester P_{II} protein to the cell membrane (Coutts *et al.*, 2002; Detsch & Stülke, 2003; Javelle *et al.*, 2004; Strösser *et al.*, 2004). If the P_{II} proteins sequestered by AmtB₁ were altered in their ability to regulate receptor proteins, this would be an obvious mechanism for explaining the physiological roles of AmtB₁ in response to NH_4^+ and darkness. There are, however, particular technical challenges in testing this with a photosynthetically grown organism: abundant intracytoplasmic membranes make separation of membrane and cytoplasm challenging, and the very act of harvesting the cells by centrifugation allows for a darkness response. We therefore grew wild-type (UR2) and *amtB*₁ (UR794) strains in MG medium to early exponential phase and then treated them with NH_4Cl or darkness shifts, or left them untreated. Cultures were chilled before harvesting, as described in Methods, to reduce the rate of response to darkness. The extracts were analysed by Western blotting developed with antibody to GlnJ. Whole-cell,

Fig. 3. The modification of GS in an *R. rubrum draG* mutant (UR832) after NH_4^+ (a) and darkness (b) treatments. Derepressed cultures of UR832 were shifted to darkness at time zero. At the times indicated, 1 ml portions of the same culture were rapidly extracted by the trichloroacetic acid precipitation method. Protein samples were loaded on low cross-linker 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.

cytoplasmic and membrane extracts were loaded such that both protein and P_{II} levels in the cytoplasmic and membrane lanes added up to the levels in the original whole-cell extract. This GlnJ antibody is significantly more responsive to GlnJ than to GlnB, and GlnJ is the most abundant P_{II} homologue in *R. rubrum* under these conditions (Zhang *et al.*, 2005). We therefore interpret the results below in terms of GlnJ location, but cannot exclude the additional presence of lesser amounts of other P_{II} proteins.

As shown in Fig. 4, in the wild-type grown in the light under nitrogen-deficient conditions, GlnJ is mostly uridylylated and primarily found in the cytoplasm. There is some GlnJ associated with the membrane under these conditions, but this may reflect the difficulty of stopping the physiological response to darkness during cell harvest. It is notable that the majority of cytoplasmic GlnJ is uridylylated while membrane-associated GlnJ is deuridylylated, suggesting that unmodified GlnJ preferentially binds to AmtB₁, consistent with previous reports in *E. coli* (Coutts *et al.*, 2002; Javelle *et al.*, 2004). In response to darkness or the addition of 10 mM NH_4Cl , GlnJ is completely deuridylylated and the ratio of GlnJ found in the membrane to that found in the cytoplasm is reversed from the initial sample, consistent with a significant increase in membrane association. The samples from the *amtB*₁ strain are roughly similar to those of the wild-type in terms of the uridylylation state of GlnJ, but the striking difference is the apparently undetectable levels of GlnJ in the membrane fraction under any conditions tested. Thus, AmtB₁ does not appear to be necessary for the approximately normal regulation of GlnJ modification by GlnD under the tested conditions, but is necessary for GlnJ membrane association.

Fig. 4. AmtB₁-dependent sequestration of GlnJ to the membrane in *R. rubrum*. Cells of wild-type (UR2) and an *amtB*₁ mutant (UR794) were collected and separated into cytoplasmic and membrane fractions, as described in Methods, after 60 min of darkness treatment, 30 min after addition of NH₄Cl to a final concentration of 10 mM, or with no treatment. Samples were resuspended in identical final volumes of loading buffer and equal volumes of whole-cell (WC), cytoplasmic (C) and membrane (M) samples were loaded on low cross-linker Tricine SDS-PAGE gels and immunoblotted with antibody against *R. rubrum* GlnJ. M indicates the position of modified GlnJ and U indicates the position of unmodified GlnJ.

AmtB₁ might sequester GlnK and prevent it from functioning in some P_{II} roles in the cell

As reported previously, *glnB glnJ* double mutants grow poorly on all examined media (Zhang *et al.*, 2001a). The additional elimination of the third P_{II} homologue, *glnK*, has little effect, which we assumed was because its level of expression is very low under all conditions examined. However, in the course of constructing some of the mutants described above, we found that the elimination of *amtB*₁ allows normal growth in a *glnB glnJ* background, but not in a *glnB glnJ glnK* background. To further examine this, we measured the growth rates of a set of different mutants. UR1149 ($\Delta glnB \Delta glnJ$ -*amtB*₁) had a wild-type growth rate, while UR1240 ($\Delta glnB \Delta glnK \Delta glnJ$ -*amtB*₁) grew slowly in SMN, similar to the growth of *glnBJ* (UR808) and *glnBJK* (UR812) mutants (Table 4). This result has two implications. First, it shows that GlnK apparently has the biochemical properties necessary to support normal growth. As we will show elsewhere, these properties probably reflect the ability of GlnK to properly regulate NtrB and GlnE to modulate GS activity levels to a satisfactory degree (Y. Zhang & others, unpublished results). Second, the result implies that the presence of AmtB₁ interferes with that ability. We

attempted to test this hypothesis directly, but we were unable to reliably detect GlnK in our analyses (data not shown). GlnK is the least abundant P_{II} protein in *R. rubrum* (Zhang *et al.*, 2005), and *glnK::lacZ* expression studies showed that *glnK* is expressed at background levels in otherwise wild-type backgrounds (D. M. Wolfe & others, unpublished data).

There is an apparent specificity of interaction between AmtB₁ and GlnJ

Some of the results described above are most easily rationalized by a model of direct interactions between AmtB₁ and P_{II} homologues, but there are suggestions that the effect of AmtB₁ is not equivalent for all P_{II} homologues. For example, a *glnJ amtB*₁ mutant (UR1147) has approximately the same defect in the darkness response as does the *amtB*₁ single mutant (UR794), but the *glnB amtB*₁ strain (UR1117) completely lacks a response to darkness (Table 3). The simplest interpretation is that both GlnB and GlnJ are involved in the darkness response, but that AmtB₁ is only important for GlnJ function. In this case, the absence of *glnJ* in a $\Delta amtB$ ₁ background has little effect because GlnJ is already rendered non-functional by the absence of AmtB₁. In contrast, GlnB apparently has a substantial effect even in the absence of AmtB₁, since its removal from a $\Delta amtB$ ₁ strain has a striking effect. This suggests a greater specificity of interaction of AmtB₁ with GlnJ than with other P_{II} proteins.

To test this hypothesis more directly, we created expression systems that overexpressed AmtB₁ at two different levels and examined the effects with or without the simultaneous overexpression of GlnJ. We took advantage of our observation that there appear to be both NtrC- and RpoN-binding sites 5' of *glnJ* and *amtB*₁ (as well as *glnB* and a number of other genes). Based on the DNA sequence alignment in Fig. 5, we believe that *amtB*₁ can be expressed from both the *glnJ* promoter and its own, and the following results are also consistent with this hypothesis.

Table 4. Growth of *R. rubrum* wild-type, *glnB glnJ*, *glnB glnK glnJ*, *glnB glnJ*-*amtB*₁ and *glnB glnK glnJ*-*amtB*₁ mutants in SMN medium

Strains	Chromosomal genotype	Generation time (h) and stability in SMN medium
UR2	Wild-type	4.2; large stable colonies
UR808	$\Delta glnB3 \Delta glnJ1$	10.4; small unstable colonies
UR812	$\Delta glnB3 \Delta glnK1 \Delta glnJ1$	11.1; small unstable colonies
UR1149	$\Delta glnB3 \Delta glnJ$ - <i>amtB</i> ₁ 2	4.4; large stable colonies
UR1240	$\Delta glnB3 \Delta glnK1 \Delta glnJ$ - <i>amtB</i> ₁ 2	10.3; small unstable colonies

RpoN-binding site (σ^{54} -dependent promoter)			
NTGGCACGNNNNNTT	GCA		Consensus
TTGGCACGCTGGGT	GCA	71	<i>nifH</i>
TTGGCATCATCCTT	GCT	52	<i>nifB</i>
GTGGCACGAGGCGT	GAT	64	<i>glnB</i>
GCGGCACGCGTCTT	GAA	121	<i>glnJ</i>
GTGGCACGGTTCTT	GTT	65	<i>amtB₁</i>
NtrC-binding site			
TGCACNNNNNTGGT	GCA		Consensus
TGCCTATATTTTCGG	GCA	178	<i>glnB</i>
TGCCCAAGGCTTAG	GCA	201	<i>glnJ</i>
TGCCCGCTTTACGG	GCA	144	<i>amtB₁</i>

Fig. 5. Sequence alignment of RpoN- and NtrC-binding sites from the promoter regions of *R. rubrum* *nifH*, *nifB*, *glnB*, *glnJ* and *amtB₁*. Consensus bases, thought to be essential for these binding sites, are shown in bold. Highly conserved bases are in bold type, and less conserved or non-conserved bases are labelled as N. The number indicates the distance in nucleotides between the binding site and the start codon of translation.

A set of vectors with different expression levels of AmtB₁ was constructed as described in Methods (Fig. 1). When *glnJ* and *amtB₁* were both overexpressed from their normal promoters on a multi-copy plasmid (pUX1109) in a background with a wild-type copy of *glnJ* (but lacking both *glnB* and *glnK*), growth appeared normal (UR1183 in Table 5). However, when only *amtB₁* was overexpressed from its normal promoter on a multi-copy plasmid (pUX1138), it caused slow growth in UR1203. This suggests that the excess AmtB₁ is sequestering the normal levels of GlnJ and creating a cell that is phenotypically GlnB[−] GlnJ[−], which grows poorly as we have already shown (Zhang *et al.*,

2001a). We then used an in-frame deletion within *glnJ* to create a plasmid that expressed *amtB₁* from both the *glnJ* and normal *amtB₁* promoters (pUX1139). This is expected to provide even higher levels of AmtB₁, and indeed caused an even more severe growth defect in the *glnBK* mutant background (UR1184). The poorly growing colonies produced fast-growing suppressor-containing strains, as we have seen with *glnB glnJ* mutants (Zhang *et al.*, 2001a). In contrast, the same expression plasmids in a strain with normal levels of GlnB, but lacking GlnJ and GlnK, resulted in perturbed growth only at the highest levels of AmtB₁ expression, and then only slightly (UR1186). The same background strains overexpressing *glnJ-amtB₁* (UR1185) or *amtB₁* from its normal promoter (UR1204) showed wild-type growth. We interpret this to mean that AmtB₁ is much more effective at sequestering GlnJ than GlnB, since the presence of either is sufficient to promote good growth. Because we lack pure AmtB₁ protein and antibody against it, we are unable to quantify the exact level of AmtB₁ and P_{II} in these different strains.

DISCUSSION

AmtB is a gas channel that conducts uncharged NH₃ and it has been proposed that one function of P_{II} is to regulate AmtB channel function by direct interaction (Andrade *et al.*, 2005; Coutts *et al.*, 2002; Javelle *et al.*, 2004). While this is reasonable, it does not appear to explain the results reported here. Indeed, if regulation of AmtB activity is so important, it is odd that elimination of AmtB has such a modest effect on growth in most organisms under most tested conditions. The exception is the reduced growth at low NH₃ concentration seen in several organisms lacking *amtB* homologues (Soupene *et al.*, 1998; Van Dommelen *et al.*, 1998). The other mutant phenotype reported for *amtB* mutants is the failure to transport methylammonium (Meletzus *et al.*,

Table 5. Growth of *R. rubrum* *glnBK* or *glnJK* mutants carrying multiple-copy plasmids expressing either *glnJ amtB₁* or *amtB₁* alone

Strain	Background strain	Plasmid (relevant genes)	Generation time (h) and stability in SMN medium
UR1183	UR757 (<i>glnBK</i>)	pUX1109 (<i>glnJ amtB₁</i>)*	4·7; large stable colonies
UR1203	UR757 (<i>glnBK</i>)	pUX1138 (P _{<i>amtB₁</i>} - <i>amtB₁</i>)†	11·0; small unstable colonies‡
UR1184	UR757 (<i>glnBK</i>)	pUX1139 (P _{<i>glnJ</i>} /P _{<i>amtB₁</i>} - <i>amtB₁</i>)§	> 15; tiny unstable colonies
UR1185	UR810 (<i>glnJK</i>)	pUX1109 (<i>glnJ amtB₁</i>)	4·7; large stable colonies
UR1204	UR810 (<i>glnJK</i>)	pUX1138 (P _{<i>amtB₁</i>} - <i>amtB₁</i>)	4·5; large stable colonies
UR1186	UR810 (<i>glnJK</i>)	pUX1139 (P _{<i>glnJ</i>} /P _{<i>amtB₁</i>} - <i>amtB₁</i>)	8·8; small unstable colonies

**glnJ* and *amtB₁* are expressed together from pUX1109.
†*amtB₁* is only expressed from its own promoter from pUX1138.
‡Overall, UR1203 is more stable on SMN plates than UR1184, but a few faster-growing colonies were occasionally seen during the purification of this strain.
§*amtB₁* is expressed from both the *glnJ* promoter and its own promoter from pUX1139.
||UR1184 grew so poorly in SMN and with such a high frequency of suppressor mutations that it was impossible to get a meaningful generation time. It grew slower than UR1203 on SMN plates.

1998; Michel-Reydellet *et al.*, 1998; Soupene *et al.*, 1998; Van Dommelen *et al.*, 1998; Yakunin & Hallenbeck, 2002). Rather, it appears that the model of a direct interaction between AmtB and P_{II} to affect P_{II} activities (Blauwkamp & Ninfa, 2003) is more consistent with our data. We therefore favour the notion that interaction between AmtB_I and P_{II} is a regulated phenomenon with the effect of removing P_{II} from the cytoplasm and decreasing its effective concentration for some targets. Because P_{II} apparently acts by protein–protein interactions with various target proteins, a decrease in P_{II} concentration would potentially affect all of these interactions. Indeed, the model is easiest to understand in the enteric bacteria where the gene for the ‘extra’ P_{II} (termed GlnK, but analogous to GlnJ and GlnK of *R. rubrum*) is only occasionally expressed and is also closely linked to *amtB*. In this case, growth conditions that demand higher levels of P_{II} lead to GlnK synthesis, but that decision might be rapidly reversed by sequestration of GlnK by AmtB, restoring P_{II} levels to those seen prior to *glnK* expression. This close linkage between *amtB* and the gene for the extra P_{II} protein is found in most prokaryotic genomes, suggesting that interactions between the protein products of the two genes might be a common theme (Thomas *et al.*, 2000a). The co-transcription of a *glnK-amtB* operon could prevent titration of GlnB when production of AmtB is elevated under nitrogen-deficient conditions (Blauwkamp & Ninfa, 2003).

We do not know the basis of the darkness effect, but speculate that ATP or α -KG might be the direct signal. It is recognized that α -KG and ATP bind synergistically to P_{II} homologues of both the enterics and the cyanobacteria, though there are differences in the specific details (Forchhammer, 2004; Ninfa & Jiang, 2005). Because α -KG is thought to have effects on P_{II} function *in vivo*, it is clear that ATP binding could at least have *indirect* effects through α -KG. Given the clear role of ATP on P_{II} function *in vitro* (Jiang *et al.*, 1998b; Kamberov *et al.*, 1995), it is somewhat surprising that an *in vivo* role for ATP in the process has not been proposed. Indeed, there are a number of observations that might be explained by fluctuations in ATP pools. In the cyanobacteria there is a clear effect of redox state on P_{II} function (Hisbergues *et al.*, 1999), which has been interpreted as an effect on the α -KG pool, though without direct support (Forchhammer, 2004). It was also shown many years ago by the Magasanik group (Bender *et al.*, 1977) that the method of breaking *Klebsiella aerogenes* cells affected the GS adenylation state and again this has been assumed to reflect a direct perturbation of pools of nitrogen, though this was not demonstrated and perturbation of ATP pools is a possibility. The notion that ATP levels can fluctuate and have important biological effects has been clearly demonstrated in the regulation of rRNA expression in *E. coli* (Paul *et al.*, 2004; Schneider *et al.*, 2002). However, the adenylate and pyridine nucleotide pools have been analysed in *R. rubrum* in response to darkness and NH_4^+ treatments (Nordlund & Höglund, 1986; Paul & Ludden, 1984) and only a slight transient decrease of the ATP pool was found after a shift to darkness (Paul & Ludden, 1984). Because of

the obvious role of ATP in modulating P_{II} function *in vitro*, we favour the general model that energy deprivation (darkness in our system) creates an ATP-deficient form of P_{II} that behaves sufficiently like unmodified P_{II} , irrespective of the actual presence of UMP, at least in terms of regulation of nitrogenase activity. It is possible that the modest ATP effects previously detected in *R. rubrum* are sufficient to affect P_{II} function.

We still lack some critical information in sorting out exactly how GlnB and GlnJ effect DRAT and DRAG regulation, but the following model is consistent with the above argument as well as previous and recent data on the system (Huergo *et al.*, 2005; Wang *et al.*, 2005; Zhang *et al.*, 2005, 2003, 2001b; Zhu *et al.*, 2006). P_{II} binds DRAT and probably activates its activity under nitrogen-excess conditions. However, P_{II} -UMP binds and inhibits DRAT and also activates DRAG under nitrogen-deficient conditions. The addition of NH_4^+ causes P_{II} to be deuridylylated, eliminating DRAT inhibition and inactivating DRAG. The inhibition of DRAG activity is actually the result of the sequestration of DRAG by membrane-associated AmtB_I, binding the P_{II} -DRAG complex.

This model is consistent with data from two recent papers on the membrane sequestration of DRAG in mutants lacking P_{II} or AmtB (Huergo *et al.*, 2006; Wang *et al.*, 2005). Wang *et al.* (2005) reported the different location of DRAG in *R. rubrum* mutants lacking P_{II} proteins. Substantial amounts of DRAG were found in the cytosolic fraction in *glnB glnJ* and *glnB glnJ glnK* mutants, but very little DRAG was seen in the cytosolic fraction in a *glnB* mutant and much less in a *glnJ* mutant. Similar to our results, they found that an *R. rubrum amtB_I* mutant showed an altered response to NH_4^+ treatment in regulating nitrogenase activity and the modification of dinitrogenase reductase. Interestingly, DRAG was found predominantly in the cytosol in this *amtB_I* mutant (Wang *et al.*, 2005). Similarly, Huergo *et al.* (2006) reported that, in *Azospirillum brasilense*, P_{II} proteins (GlnB and GlnZ) and DRAG were also found to be associated with the membrane after NH_4^+ treatments, and this membrane-sequestration was AmtB-dependent. In an *amtB* mutant, the inability of DRAG to be associated with the membrane altered the regulation of its activity, resulting in a poor response to NH_4^+ (Huergo *et al.*, 2006). These results indicated that the inhibition of DRAG activity is actually the result of the sequestration of DRAG by membrane-associated AmtB_I, probably binding the P_{II} -DRAG complex. Thus the elimination of either GlnB/GlnJ or AmtB_I abolishes the turn-off of nitrogenase activity in response to NH_4^+ or darkness because DRAG cannot be properly regulated by sequestration in the absence of either P_{II} protein. Darkness might create an ATP-deficient form of P_{II} that is in the nitrogen-sufficient state. This model is also consistent with the previous data on the system: (i) DRAG has been detected in association with the membrane fraction of *R. rubrum* cells in which DRAG activity is down-regulated (Norén & Nordlund, 1997); (ii) in *A. brasilense*,

the reactivation of DRAG activity was altered in a mutant lacking GlnZ, a GlnK-like protein (Klassen *et al.*, 2001); (iii) in *R. capsulatus*, DRAT interacts with both GlnB and GlnK in the yeast two-hybrid system (Pawlowski *et al.*, 2003) and we have detected similar interactions between DRAT and all three P_{II} homologues of *R. rubrum* (Zhu *et al.*, 2006). Thus, although we specifically propose this scheme for the *R. rubrum* system, it is consistent with results seen with *Azoarcus*, *R. capsulatus* and *Azospirillum brasilense* as well, in which the DRAT-DRAG regulation is altered in mutants lacking P_{II} or AmtB (Drepper *et al.*, 2003; Klassen *et al.*, 2001; Martin & Reinhold-Hurek, 2002; Yakunin & Hallenbeck, 2002). We note that rather different results have been seen in the case of *R. capsulatus*, where mutants lacking two AmtB homologues (AmtB and AmtY) affected the DRAT-DRAG response to NH₄⁺, but not to darkness; the basis for this difference is unclear (Yakunin & Hallenbeck, 2002).

The results presented here also imply some degree of specificity between AmtB₁ and the different P_{II} species in *R. rubrum*. Such specificity has not been well documented previously. In *E. coli*, AmtB can sequester both GlnB and GlnK, although not identically (Cou tts *et al.*, 2002). GlnB and GlnK antagonize AmtB similarly (Blauwkamp & Ninfa, 2003). However, Javelle *et al.* (2004) suggested that GlnK and GlnB have quite distinct functions in *E. coli*, GlnK being concerned with AmtB regulation and GlnB with control of NtrB. The situation in *R. rubrum* is apparently different. In response to energy deprivation, for example, a *glnB amtB*₁ mutant is significantly more perturbed than a *glnJ amtB*₁ mutant. The overexpression experiments (Table 5) also show an apparent preference of AmtB₁ for GlnJ. In contrast, the differential growth rates of *glnBJ*, *glnBJ amtB*₁ and *glnBJK amtB*₁ mutants strongly imply a significant degree of affinity of AmtB₁ for GlnK as well. Such specificity in P_{II} interactions is clearly of great potential importance, since it affects the differential P_{II} effects on the various receptor proteins.

In summary, these results show that AmtB₁ is critical for the response of the DRAT-DRAG system to both NH₄⁺ addition and energy deprivation. The latter role is difficult to attribute to the role of AmtB₁ as either an NH₃ channel or an NH₃ sensor. Lastly, the results strongly imply selectivity in the interaction of AmtB₁ with specific P_{II} proteins.

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