AmtB Is Necessary for NH_4^+ -Induced Nitrogenase Switch-Off and ADP-Ribosylation in *Rhodobacter capsulatus*‡

Alexander F. Yakunin† and Patrick C. Hallenbeck*

Département de Microbiologie et Immunologie, Université de Montréal, Succursale Centre-ville, Montréal, Québec H3C 3J7, Canada

Received 1 March 2002/Accepted 3 May 2002

***Rhodobacter capsulatus* possesses two genes potentially coding for ammonia transporters, *amtB* and *amtY*. In order to better understand their role in the physiology of this bacterium and their possible significance in nitrogen fixation, we created single-knockout mutants. Strains mutated in either *amtB* or *amtY* did not show a growth defect under any condition tested and were still capable of taking up ammonia at nearly wild-type rates, but an *amtB* mutant was no longer capable of transporting methylamine. The *amtB* strain but not the *amtY* strain was also totally defective in carrying out ADP-ribosylation of Fe-protein or the switch-off of in vivo nitrogenase activity in response to NH_4^+ addition. ADP-ribosylation in response to darkness was unaffected in *amtB* and *amtBY* strains, and glutamine synthetase activity was normally regulated in these strains in response to ammonium addition, suggesting that one role of AmtB is to function as an ammonia sensor for the processes that regulate nitrogenase activity.**

Nitrogenase is an enzymatic complex carrying out the biological reduction of dinitrogen to ammonia. Two protein components are necessary for nitrogenase activity; MoFe-protein (or dinitrogenase), which contains the active site for N_2 reduction, and Fe-protein (or dinitrogenase reductase), whose main function is to transfer electrons to MoFe-protein with the concomitant hydrolysis of MgATP. Nitrogen fixation represents a significant demand on cellular energy supplies (20 to 30 mol of MgATP for the reduction of 1 mol of N_2), and it is therefore not surprising that this process is tightly regulated at both the transcriptional and posttranslational levels. In all diazotrophs, the products of NH_4^+ assimilation inhibit transcription of *nif* genes (29). Additionally, externally added NH_4^+ induces a posttranslational regulation of nitrogenase in several different nitrogen-fixing microorganisms (25).

Shortly after the demonstration of nitrogen fixation in photosynthetic bacteria, the addition of ammonium to cultures of these organisms was reported to cause a rapid decrease in nitrogenase activity (12). Various studies have subsequently shown that ammonium addition usually causes complete inhibition of nitrogenase activity within 2 to 3 min. Full recovery of the initial rate of nitrogenase activity is usually observed within a relatively short period of time, which depends on the amount of ammonium added (42, 57). This type of regulation, termed the nitrogenase switch-off/switch-on effect, has been observed in many photosynthetic and chemotrophic bacteria (6, 14, 17, 18, 22, 54, 57).

There appear to be at least two different mechanisms underlying this effect. In some cases, nitrogenase switch-off by NH_4^+ is uniquely mediated by a two-enzyme system, dinitro-

genase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase activating glycohydrolase (DraG), which causes a covalent modification/demodification of the Fe-protein via ADP-ribosylation. The involvement of these proteins in the nitrogenase switch-off response to NH_4^+ addition has been amply demonstrated for the photosynthetic bacterium *Rhodospirillum rubrum* (25). In this organism, it appears that DraT and DraG are both necessary and sufficient for Fe-protein ADP-ribosylation and nitrogenase switch-off. In addition to NH_4^+ , Fe-protein ADP-ribosylation can also be induced by darkness (in some photosynthetic bacteria) or anaerobiosis (in *Azospirillum*) (25). On the other hand, other organisms seem to be capable of the metabolic modulation of nitrogenase activity in response to NH_4^+ addition without resorting to covalent modification (6, 48).

In the photosynthetic bacterium *Rhodobacter capsulatus*, exogenous NH_4^+ induces three different nitrogenase responses: an ADP-ribosylation of Fe-protein (13, 14, 18), an ADP-ribosylation-independent switch-off effect (10, 33, 50), and an ADP-ribosylation-independent magnitude response, where the quantity of added NH_4^+ affects the magnitude of the inhibition (50). Recently we have shown that in this bacterium, in addition to fast (short-term) Fe-protein ADP-ribosylation, which can be induced by NH_4^+ addition or darkness, there is a long-term (steady-state) Fe-protein modification which correlates with intracellular N status and can be observed during growth with different nitrogen sources (51).

Previously it has been shown that *R. capsulatus* appears to possess two ammonium uptake systems; one that is constitutively synthesized and a second system, capable of transporting methylammonium, that seems to be under global nitrogen control (34). Since the presence of nitrogenase switch-off in *R. capsulatus* is correlated with an increased activity of the second NH_4^+ uptake system (50), we suggested that the nitrogenase switch-off system responds to a signal generated by this NH_4^+ transport system. As a follow-up, we have now identified two genes in the genome of *R. capsulatus* potentially coding for

* Corresponding author. Mailing address: Département de Microbiologie et Immunologie, Université de Montréal, CP 6128, Succursale Centre-ville, Montréal, Québec H3C 3J7, Canada. Phone: (514) 343-5701. E-mail: patrick.hallenbeck@umontreal.ca.

† Permanent address: Institute of Basic Biological Problems, Russian Academy of Sciences, Puschino, Russia.

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ammonium transporters, *amtB* and *amtY*, and created disruption mutations in these genes. A strain mutated in *amtY* was unaltered in nitrogenase regulation. However, strains mutated in *amtB* were defective in appropriately regulating nitrogenase in response to the addition of ammonium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains S17-1 (39) and S17-1/ λ pir (2) were used for conjugal transfer of plasmids to *R. capsulatus* by filter mating (27). *E. coli* strains were grown in Luria-Bertani (LB) medium (35) supplemented with the appropriate antibiotics. *R. capsulatus* strains, including the wild-type SB1003 (52), were routinely maintained in liquid YPS (yeast extract/peptone/salts) medium (46) supplemented with the appropriate antibiotics. For all experiments shown, cultures were inoculated and grown photosynthetically in modified (51) liquid RCV medium (46). Highly nitrogen limited cultures, obtained by growing cultures to early stationary phase on RCV medium without an added nitrogen source, were used for all experiments on fast effects on nitrogenase because these conditions have previously been shown to be optimal for the switch-off response (50). Under these conditions, growth is presumably supported by the dissolved N_2 . For examining effects on long-term ADP-ribosylation, cultures were grown to early stationary phase on RCV medium with the indicated initial concentrations of ammonia as previously described (51).

Construction of an *amtB*::Km^r gene disruption. The *R. capsulatus amtB* gene was cloned from genomic DNA by PCR with oligonucleotide primers *amtB*-upstream (5'-CCGTTCAAGCTCGAGGAGGTCGCG-3'), which contains an *XhoI* site, and *amtB*-downstream (5'-TCAGCGGGAATGATGAGCGGCGC-3'), located downstream of a *Clal* site). The PCR product (1,944 bp) was cloned as an *XhoI/Clal* fragment into pBluescript KS- (Stratagene), and a kanamycin resistance cassette (Km^r; 1.2-kb *SphI* fragment) from pBSL86 (1) was inserted into an *NspI* site in the coding region of *amtB*. Subsequently, an *amtB*::Km^r fragment was subcloned into the *EcoRI* site of the suicide vector pSUP202 (39), and the resulting plasmid was mobilized from *E. coli* S17-1 into *R. capsulatus* SB1003. Replacement of the wild-type *amtB* gene was confirmed by Southern hybridization.

Construction of an *amtY*::Gm^r gene disruption. The *R. capsulatus amtY* gene was subcloned as a 1.9-kb *BamHI/EcoRI* fragment from pRCN102 (3) into pBluescript KS(-), and a gentamicin resistance cassette (Gm^r; 0.9-kb *BamHI* fragment) from pUCGM (37) was inserted into the *BclI* site of *amtY*. *amtY*::Gm^r was subcloned as a *BamHI/EcoRI* fragment into the mobilizable vector pK18mobsacB (36), and the resulting plasmid was mobilized from *E. coli* S17-1 into *R. capsulatus* SB1003. Double recombinants were confirmed by Southern hybridization.

Construction of a double *amtB amtY* mutant. To create an *R. capsulatus amtB amtY* double mutant, the 0.7-kb *BclI/HindIII* fragment of the *amtY* gene was subcloned into the broad-host-range mobilizable suicide vector pKNOCK-gentamicin (2) which had been digested with *BamHI/HindIII*, and the resulting plasmid was mobilized from *E. coli* S17-1/ λ pir into the *R. capsulatus amtB*::Km^r strain. Recombinants were selected for kanamycin and gentamicin resistance. As a result of homologous recombination (confirmed by Southern hybridization), two mutant copies of the *amtY* gene, separated by the inserted pKNOCK, were formed, with one lacking the 5' region (456 bp) and the other the 3' region (233 bp).

Complementation studies. The entire *glnK-amtB* operon as well as 390 bp of upstream sequence (2.4 kb total) was cloned by PCR and inserted into the polylinker of the broad-host-range vector pJB3Tc20 (4). The resulting plasmid, pAY99, was mobilized from *E. coli* S17-1 into *R. capsulatus amtB* or *amtB amtY* mutant strains.

Determination of in vivo nitrogenase activity and Fe-protein modification state. In vivo nitrogenase activity was measured by the acetylene reduction method (13, 14) with 5-ml culture samples. Stimuli were applied as follows: light was removed by wrapping the vials in aluminum foil, and ammonium was added as an anaerobic solution. Solutions were made anaerobic by sparging with argon which had been passed through a heated copper catalyst to remove any traces of oxygen. At the indicated times, 50- μ l aliquots of the gas phase and the culture were separately withdrawn from the vials for the analysis of ethylene by gas chromatography and Fe-protein modification state.

To monitor the modification state of Fe-protein, the entire culture samples without prior treatment were brought to a 1 $\times$ sodium dodecyl sulfate sample buffer concentration (23) and immediately incubated in a boiling-water bath for 5 min. Equal amounts of total protein (2 μ g/well) were analyzed by Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% total acryl-

amide) with low-crosslinker gels (20) and subsequent immunoblotting with chemiluminescence detection as described previously (49). In this system, *R. capsulatus* Fe-protein shows an apparent molecular mass of 34.9 kDa for the unmodified subunit and 38.7 kDa for the ADP-ribosylated subunit. (On high-resolution gels, the unmodified Fe-protein usually produces a double band due to the transient formation of incompletely denatured intermediates.) Since only one of the two Fe-protein subunits of the Fe-protein dimer is modified, 100% modification of Fe-protein dimers would correspond to two bands of equal intensity.

Ammonium and methylammonium uptake. For measurement of ammonium uptake, early-stationary-phase cells grown photosynthetically on RCV medium without an added nitrogen source were incubated for 5 min at room temperature under light and anaerobic conditions. The reaction was initiated by the addition of ammonium (0.2 mM), and ammonium concentrations in culture supernatants from samples taken at 2.5-min intervals were measured with the phenol-hypochlorite method (40). [¹⁴C]methylammonium uptake was measured as previously described (34).

Ammonium excretion and intracellular ammonium concentrations. To measure ammonium excretion, highly nitrogen limited cultures in early stationary phase were sparged with N_2 , and the appearance of ammonium in culture supernatants over time was measured with the phenol-hypochlorite method (40). Intracellular ammonium concentrations were also determined by the phenol-hypochlorite method after ice-cold methanol extraction of cell pellets. Intracellular concentrations were calculated based on an intracellular volume of 4 μ l/mg of protein (7).

Glutamine synthetase assays. Glutamine synthetase activity (γ -glutamyltransferase) of whole cells and the relative state of glutamine synthetase adenylation were measured as previously described (19). Protein concentrations were determined by the Bradford method (5) after 2 min of sonication.

RESULTS

***R. capsulatus* contains two *amtB* homologues.** A BLAST search of the available genome sequence of *R. capsulatus* (<http://wit.mcs.anl.gov/WIT/CGI/>) revealed the presence of two genes potentially coding for ammonium transport proteins, which we call *amtB* and *amtY*. *amtB* is predicted to be part of a *glnK-amtB* operon, as in many other bacteria (43, 44), whereas the determined DNA sequence suggests that *amtY* is a monocistronic gene found a short distance (2.5 kb) from *ntrBC* and *nifR3*. The predicted AmtB protein (453 amino acids, 46.8 kDa) is highly similar to that of other bacterial and archaeal putative AmtBs, ranging from 59% identity to AmtB from *Rhizobium etli* to 30% for that from *Thermotoga maritima*. The predicted *R. capsulatus* AmtY protein (392 amino acids, 41.2 kDa) is less similar to putative AmtBs, with the highest similarity, 39% identity, to AmtB from *Azospirillum brasilense*. The *R. capsulatus* AmtB and AmtY proteins are only distantly related, 38% identity, and differ substantially in length, with the greatest difference being in the N-terminal region.

Disruption of *amtB* homologues in a number of bacteria has no effect on growth on various nitrogen sources, including ammonium, at neutral pH (28, 30, 31, 47). However, growth of an *A. brasilense amtB* mutant on low (0.1 mM) ammonium at pH 7.0 is affected (45), and an *E. coli amtB* mutant shows a growth defect on low concentrations of ammonium (≤ 1 mM) when growth is at pH 5 (41).

R. capsulatus amtB or *amtY* single mutants showed wild-type growth on rich organic medium (YPS) as well as on mineral medium (RCV) with NH_4^+ (30 mM), N_2 , or glutamate (7 mM) as a nitrogen source (data not shown). Upon transfer from YPS to RCV medium containing NH_4^+ as the nitrogen source, *amtB amtY* double mutants also grew at wild-type rates after a somewhat longer lag phase (data not shown). Thus, disruption

FIG. 1. Ammonium (A) and [^{14}C]methylammonium (B) uptake. Cultures were grown under photoheterotrophic anaerobic conditions on RCV medium which lacked an added nitrogen source to the early stationary phase ($0.07 \text{ mg of protein ml}^{-1}$), and uptake was measured after the addition of NH_4Cl to $200 \mu\text{M}$ for ammonium uptake assays or the addition of [^{14}C]CH $_3\text{NH}_3^+$ to $75 \mu\text{M}$ for methylammonium uptake assays. The values shown are the means of three independent replicates. Standard deviations are indicated as vertical bars.

of *amtB* and/or *amtY* does not result in an observable growth defect at neutral pH.

Ammonium and methylammonium uptake. The ability of the mutant strains to grow on ammonium strongly suggests that ammonium is entering these cells, and several lines of evidence clearly corroborate this, as presented below. Physiological studies have previously shown that *R. capsulatus* possesses two ammonium transport systems, a system that appears to be constitutively synthesized, and a second system that appears to be Ntr regulated (34, 38) because it is abolished in the mutant J61, which carries a defect in *ntrC* (3). In contrast to the constitutively expressed system, the second system is competent for the uptake of methylammonium (34).

Here we measured the ammonium and methylammonium uptake activities of *amt* mutants of *R. capsulatus*. To test their uptake abilities, cultures were grown under nitrogen-limited conditions, thus permitting the expression of the Ntr-regulated system as well as the constitutively expressed system. Consis-

tent with their ability to grow on ammonium, nitrogen-limited cultures of both the *amtB* and *amtY* mutants as well as the *amtB amtY* double mutant demonstrated very nearly wild-type rates of ammonium uptake (Fig. 1A). A mutation in *amtY* actually appeared to lead to a small increase in the apparent rate of ammonium uptake. This demonstrates that ammonium uptake and assimilation in *R. capsulatus* can proceed in the absence of AmtB and AmtY, which is consistent with the notion that, at least for the enteric bacteria *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, unmediated diffusion of NH_3 is sufficient for optimal growth at reasonable concentrations of ammonium (greater than $50 \mu\text{M}$) (41). Methylammonium uptake was largely unaffected in the *amtY* strain (in fact, levels somewhat higher than the wild-type level were seen), whereas the *amtB* mutant was no longer capable of transporting methylammonium (Fig. 1B). This suggests that AmtB corresponds to the Ntr-regulated system described previously (34). In support of this is the observation that the *glnK-amtB* operon is preceded by two DNA sequences 137 and 154 bp upstream of the translational start codon exhibiting strong similarity to NtrC binding sites (GC-N $_7$ -T-N $_3$ -GC) (11).

Ammonium retention. One hypothesis concerning the possible role of AmtB has been that it functions as an ammonium transporter, regulating the concentration of ammonium in the interior of the cell (21). Since the uncharged species, NH_3 , is in equilibrium with the charged species, NH_4^+ , $\text{NH}_3 + \text{H}^+ \rightleftharpoons \text{NH}_4^+$; $\text{pK}_a = 9.25$, there should be appreciable quantities of intracellular NH_3 at pHs close to neutral and NH_3 should pass rapidly across the cytoplasmic membrane by diffusion. However, the fact that free ammonium concentrations inside the cell have been determined to be much higher (in the millimolar range) than outside the cell (in the micromolar range) led to the postulation of active ammonium transporters (21).

According to this model, the NH_3 formed inside the cell must continuously diffuse out of the cell only to be taken back up as NH_4^+ through a transport process, i.e., a futile cycle is created. According to Kleiner (21), who postulated the existence of this futile cycle, "Proof for this hypothesis would be the demonstration of constant NH_3 excretion upon loss of NH_4^+ transport." We checked for leakage of ammonium into the extracellular milieu by actively nitrogen-fixing cultures of

FIG. 2. Ammonium retention by wild-type and mutant cells actively fixing dinitrogen. Cultures were grown as described in the legend to Fig. 1. At time zero, the cultures were sparged with oxygen-free dinitrogen, and extracellular ammonium was determined as described in Materials and Methods. The values shown are the means of duplicate assays. Standard deviations are indicated as vertical bars.

FIG. 3. Effect of ammonium addition on in vivo nitrogenase activity and Fe-protein modification state. Cultures were grown as described in the legend to Fig. 1, and 5-ml aliquots were transferred by syringe to 25-ml argon-filled vials for the simultaneous analysis of nitrogenase activity by acetylene reduction, presented as graphs, and Fe-protein modification state, presented as immunoblots below the graphs. At the times indicated by the arrows, NH_4Cl was added. On all immunoblots, the lower bands correspond to the monomer form of unmodified Fe-protein, and the upper bands correspond to the ADP-ribosylated monomer. Since only one of the two Fe-protein subunits of the Fe-protein dimer is modified, 100% modification of Fe-protein dimers would correspond to two equal-intensity bands. Lane Std (immunoblots C to F) contains an ADP-ribosylated Fe-protein standard.

wild-type and *amt* strains (Fig. 2). Under these conditions, measurements of intracellular ammonium concentrations showed that the concentration of intracellular ammonium was in the millimolar range (results not shown). The wild-type

strain showed little ammonium in the external milieu, showing that the newly fixed ammonium was retained within the cells. However, analysis of the *amtB* and *amtB amtY* mutant strains showed that appreciable quantities of ammonium accumulated

FIG. 4. Effect of ammonium addition on in vivo nitrogenase activity and Fe-protein modification state in *R. capsulatus* *amtB* (A) and *amtB amtY* double (B) mutants complemented with *amtB* in trans. The broad-host-range replicon-based plasmid pAY99, carrying *amtB* under the control of the natural *glnK* promoter, was introduced by conjugation into the *amtB* and *amtB amtY* mutant strains. Experimental conditions were as described in the legend to Fig. 2.

in the culture supernatants over time. Thus, AmtB is required for ammonium retention under these conditions.

Nitrogenase switch-off and short-term Fe-protein ADP-ribosylation. Addition of ammonium to actively nitrogen-fixing cells of *R. capsulatus* causes quick, short-term-reversible responses. Recently, we have shown that these responses, nitrogenase switch-off and short-term Fe-protein ADP-ribosylation, are modulated by the cellular nitrogen status (50). Since methyllummonium uptake activity was similarly affected by the cellular nitrogen status, we hypothesized that the nitrogenase

switch-off effect observed upon addition of ammonium to actively nitrogen-fixing cells of *R. capsulatus* is mediated by the Ntr-regulated ammonium permease system (50). Here we examined the ability of *amt* strains to modulate nitrogenase activity and to ADP-ribosylate the Fe-protein of nitrogenase. Both the wild-type and *amtY* strains demonstrated a classical in vivo nitrogenase switch-off response to the addition of ammonium (200 μ M final concentration) (Fig. 3). Almost complete inhibition of acetylene reduction was evident within 5 min of ammonium addition, and full recovery of the initial rate of nitrogenase activity was obtained within a relatively short period of time. In both strains, the addition of ammonium also induced fast, short-term ADP-ribosylation of Fe-protein (Fig. 3).

In striking contrast, the addition of the same amount of ammonium or more (50 mM final concentration) had no effect on the in vivo nitrogenase activity and Fe-protein modification state in either the *amtB* mutant or the *amtB amtY* double mutant (Fig. 3). To directly demonstrate that this effect was due to the introduced mutation, we studied the effects of complementation with pAY99, a plasmid carrying the cloned *glnK-amtB* operon in which expression of *amtB* is under the control of the native promoter upstream of *glnK*. Analysis of the effects of ammonium addition on nitrogenase activity and Fe-protein modification showed that the wild-type response had been restored in the plasmid-carrying strains (Fig. 4).

Thus, disruption of *amtB* completely abolished the fast response to external ammonium addition. We wished to show that the effect occurred upstream of the regulatory processes and that therefore the nitrogenase modification system itself was intact. Apart from ammonium addition, short-term reversible ADP-ribosylation of Fe-protein is modulated by dark-light cycles (25). However, the timing and extent of Fe-protein ADP-ribosylation in response to darkness was unaltered in *amtB* and *amtY* mutants (Fig. 5). These results show that *amtB* mutants seem to be specifically affected in communicating the presence of extracellular ammonium to the nitrogenase switch-off and modification systems.

Nitrogenase synthesis and long-term Fe-protein ADP-ribosylation. We wished to determine if cellular processes regulated with respect to the intracellular concentration of fixed nitrogen, such as nitrogenase expression (29) and long-term Fe-protein ADP-ribosylation (51), were affected. The sensitivity of nitrogenase synthesis to the presence of ammonium in *amt* strains was checked by varying the initial concentration of ammonium (0 to 25 mM) in the growth medium of batch cultures. Studies have previously shown that this produces cultures with different severities of nitrogen limitation, as assessed by the measurement of internal glutamine pools (51).

Within the range of ammonium concentrations used, the wild-type and *amt* strains demonstrated essentially the same relationship between the initial ammonium concentration in the medium and the final culture density as well as the presence of nitrogenase proteins. We assessed effects on synthesis by checking levels of nitrogenase protein by immunoblotting. The synthesis of nitrogenase in all strains was abolished with 21 to 22 mM ammonium (Fig. 6), although a slightly higher amount of initial ammonium appeared to be required to achieve complete inhibition of nitrogenase synthesis in the *amtY* strain. Thus, disruption of *amtB* or *amtY* does not appear

FIG. 5. Effect of light switch-off/switch-on on the modification state of Fe-protein. Cultures were grown as described in the legend to Fig. 1. Growth tubes were incubated for 15 min at room temperature under light, and 100- μ l culture aliquots were withdrawn for the analysis of Fe-protein modification state (samples 1). After that, the tubes were incubated in darkness (sample 2, 5 min; sample 3, 10 min; sample 4, 20 min; and sample 5, 30 min). Sample 6, after 5 min of reincubation in the light.

to greatly affect the level of internal fixed nitrogen needed to suppress *nif* expression.

In addition to being sensitive to ammonium addition and light-dark cycles, the ADP-ribosylation status of the Fe-protein is regulated over the long term at a steady-state level by the internal pool of fixed nitrogen (measured as glutamine) (51). Immunoblot analysis also demonstrated that this long-term Fe-protein ADP-ribosylation was largely unaffected in the *amtB* mutant strain, where, as in the wild-type strain SB1003, constitutive Fe-protein ADP-ribosylation was observed in cells grown at moderate and higher initial ammonium concentrations (Fig. 6). This response appeared to be somewhat affected in the *amtY* mutants and the *amtB amtY* double mutant, where ADP-ribosylation was seen at slightly lower initial ammonium concentrations (13 and 14 mM). Thus, disruption of *amtB* or *amtY* does not appear to greatly affect the level of internal fixed nitrogen needed to repress nitrogenase expression or to induce long-term modification.

Regulation of glutamine synthetase activity. We wondered if the effect of the *amtB* gene disruption was specific to short-term nitrogenase regulation or if it extended to other enzymes whose activity is known to be regulated in the short term in response to exogenously added ammonium, such as glutamine synthetase. In *R. capsulatus*, as in many other bacteria, the addition of ammonium to cells results in a fast (within 5 to 10 min) decrease in glutamine synthetase specific activity as well as in the inactivation of this enzyme by adenylation (9, 16, 32, 53). Therefore, we checked if the regulation of glutamine syn-

FIG. 6. Immunoblot analysis of nitrogenase Fe-protein in *R. capsulatus* strains grown with different initial concentrations of ammonium (indicated below the blots, in millimolar units). RCV medium containing the indicated initial concentrations of ammonium was inoculated with cells grown on RCV-ammonium excess (30 mM NH_4^+) medium. On all immunoblots, the lower band represents unmodified Fe-protein monomer, while the upper, slower migrating band is an ADP-ribosylated subunit.

thetase activity was affected in *R. capsulatus amtB* or *amtY* strains. Interestingly, based on the total activity as measured here, somewhat higher amounts of glutamine synthetase appeared to be present in the *amtB* and *amtB amtY* strains.

If total activity is a true reflection of the amount of protein present, this would suggest that AmtB might affect the synthesis of glutamine synthetase. Nevertheless, the addition of ammonium to cultures of the *amt* mutants caused a fast decrease in glutamine synthetase activity (Fig. 7) and an increase in adenylation state (not shown), similar to what has been observed for wild-type cultures. Thus, a different mechanism is involved in the regulation of glutamine synthetase activity by ammonium.

DISCUSSION

The survival and competitiveness of microorganisms depend on their ability to sense and respond to the external milieu. This is particularly evident with compounds that are metabolized to form the basic building blocks of the cell. Nutrient-based regulation has been extensively studied with respect to transcriptional control, yet little is known about the direct sensing of nutrient availability. Ammonium (we use this term to designate both NH_4^+ and NH_3) is almost invariably the preferred source of nitrogen for the growth of microorganisms, and therefore, the availability of ammonium inhibits the synthesis and activity of proteins involved in the uptake and assimilation of other nitrogen sources. Although considerable information on the genetic and biochemical aspects of global

nitrogen control is available, the networks described are intracellular, and it has been uncertain if sensing of external nitrogen compounds can contribute to metabolic regulation. It has recently been proposed that the yeast high-affinity ammonium permease Mep2p, an AmtB homologue, functions as an ammonium sensor, generating a signal which activates signal transduction cascades that regulate filamentous growth in response to ammonium starvation (24). In addition, there are mammalian homologues belonging to this family, such as the human rhesus-associated RhAG protein and the kidney-specific RhGK protein (26).

The AmtBs (ammonium transport B; TC 2.A.49.3.2) are ubiquitous membrane proteins found in all three domains of life (24, 26, 43). The function of bacterial AmtBs has been unclear, and until now, gene disruption has usually resulted in little or no discernible phenotype at neutral pH, although growth at low pH is affected, leading to the proposal that AmtB/Mep proteins facilitate the equilibration of NH_3 across the cytoplasmic membrane (41). Alternatively, other studies have suggested that AmtB/Mep proteins may be true ammonium transporters (15).

Here we have shown that AmtB and AmtY are not essential for ammonium uptake. Presumably, in their absence, uncharged ammonia diffuses into the cell and is trapped through conversion into glutamine by the action of glutamine synthetase. This bulk process appears to be sufficient to support growth on ammonium at neutral pH. Do AmtB and AmtY play a physiological role in terms of ammonium uptake? It is unclear from the data presently in hand what the role of AmtY may be. It clearly is incapable of methylammonium uptake and presumably therefore is not a high-affinity ammonium uptake system. (Ammonium permeases that can be probed with methylammonium are usually high-affinity ammonium transporters.) Further work is needed to clarify this point. However, AmtB is clearly responsible for methylammonium uptake, and it appears to play a role in ammonium retention under conditions in which active nitrogen fixation is taking place (Fig. 2). Presumably, under these conditions AmtB functions to recycle the ammonium lost to the cell through passive diffusion.

Taken together, these results suggest that AmtB functions in the uptake of ammonium from low-ammonium-concentration solutions. The most striking metabolic effect observed with the *amtB* mutant was the inability to carry out the normal fast nitrogenase responses (switch-off and short-term Fe-protein ADP-ribosylation) to ammonium addition. Further investigation along this line may enable a molecular understanding of the phenomenon of nitrogenase switch-off by NH_4^+ first observed 50 years ago. Since ammonium uptake is largely unaffected in an *amtB* strain, as shown by measurement of gross ammonium uptake and by the response of glutamine synthetase to ammonium addition, our results suggest that AmtB responds to external ammonium.

How AmtB might transmit the presence of ammonium is unclear. Either binding or transport of ammonium might trigger conformational changes that are then communicated to intracellular proteins, for example, GlnK. In this regard, the very recent report of a reversible association of GlnK with the membrane that is AmtB and ammonium dependent is intriguing (8). The GlnK of *Klebsiella pneumoniae* has been shown to be involved in the posttranslational regulation of *Rhodospirillum rubrum* DraT and DraG when they are expressed in *K. pneumoniae* (55). While subsequent studies with *R. rubrum* have shown that these results cannot be strictly extrapolated to *R. rubrum*, it is clear that several of the *R. rubrum* P_{II} proteins, namely, GlnB and GlnJ, are somehow involved in the posttranslational regulation of DraT and DraG in that organism (56).

FIG. 7. Effect of ammonium addition on glutamine synthetase (GS) activity. Cultures were grown as described in the legend to Fig. 1. Growth tubes were incubated for 15 min at room temperature under light, and 1-ml aliquots were withdrawn for the analysis of glutamine synthetase transferase activity ($-\text{NH}_4^+$). A second set of samples ($+\text{NH}_4^+$) were withdrawn 5 min after the addition of NH_4Cl (1 mM final concentration). Each bar represents an average of the results from at least three independent determinations, with standard deviations indicated by error bars.

R. rubrum DraT and DraG when they are expressed in *K. pneumoniae* (55). While subsequent studies with *R. rubrum* have shown that these results cannot be strictly extrapolated to *R. rubrum*, it is clear that several of the *R. rubrum* P_{II} proteins, namely, GlnB and GlnJ, are somehow involved in the posttranslational regulation of DraT and DraG in that organism (56).

R. capsulatus contains two P_{II} proteins, GlnK and GlnB, and it is therefore likely that one or both are involved in the regulation of *R. capsulatus* DraT and DraG activity. AmtB could play a role in signaling the presence of ammonium to either GlnK, GlnB, or both. In addition to mono-ADP-ribosylation, the nitrogenase activity of *R. capsulatus* is also regulated in an unknown manner by a non-ADP-ribosylation pathway. From the results presented here, AmtB appears to play a decisive role in this process as well, and this regulatory pathway could also involve GlnK and/or GlnB. Indeed, we have found that mutations in *glnK* and *glnB* drastically alter the response of nitrogenase activity to ammonium additions (T. Drepper, S. Gross, A. F. Yakunin, P. C. Hallenbeck, B. Masepohl, and W. Klipp, unpublished observations). Regardless of the mechanism, it is clear from the results presented here that perception of ammonium by AmtB is necessary for the proper regulation of nitrogenase.

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