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Presence of a Second Mechanism for the Posttranslational Regulation of Nitrogenase Activity in *Azospirillum brasilense* in Response to Ammonium

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Although ADP-ribosylation of dinitrogenase reductase plays a significant role in the regulation of nitrogenase activity in *Azospirillum brasilense*, it is not the only mechanism of that regulation. The replacement of an arginine residue at position 101 in the dinitrogenase reductase eliminated this ADP-ribosylation and revealed another regulatory system. While the constructed mutants had a low nitrogenase activity, NH_4^+ still partially inhibited their nitrogenase activity, independent of the dinitrogenase reductase ADP-ribosyltransferase/dinitrogenase reductase activating glycohydrolase (DRAT/DRAG) system. These mutated dinitrogenase reductases also were expressed in a *Rhodospirillum rubrum* strain that lacked its endogenous dinitrogenase reductase, and they supported high nitrogenase activity. These strains neither lost nitrogenase activity nor modified dinitrogenase reductase in response to darkness and NH_4^+, suggesting that the ADP-ribosylation of dinitrogenase reductase is probably the only mechanism for posttranslational regulation of nitrogenase activity in *R. rubrum* under these conditions.

Biological nitrogen fixation is catalyzed by the nitrogenase complex, which consists of dinitrogenase and dinitrogenase reductase. Dinitrogenase reductase (also known as Fe protein, encoded by *nifH*) transfers electrons from a low-potential electron donor to dinitrogenase (also known as MoFe protein, encoded by *nifDK*), and this protein then reduces dinitrogen to ammonia.

In some nitrogen-fixing bacteria, such as *Rhodospirillum rubrum*, *Azospirillum brasilense*, and *Rhodobacter capsulatus*, nitrogenase activity is subject to posttranslational regulation which involves reversible mono-ADP-ribosylation of dinitrogenase reductase (13, 17, 20, 31, 44). Two enzymes that perform this regulation have been found. Dinitrogenase reductase ADP-ribosyltransferase (DRAT, the gene product of *draT*) transfers ADP-ribose from NAD to one of the two identical subunits of the dinitrogenase reductase dimer and thus inactivates the enzyme. Dinitrogenase reductase activating glycohydrolase (DRAG, the gene product of *draG*) can remove the ADP-ribose from the covalently modified enzyme and restore its activity (28). This regulatory system responds to the addition of fixed nitrogen and energy depletion, such as darkness shifts in *R. rubrum* (20) and *R. capsulatus* (29, 31, 32) or anaerobic shifts in *A. brasilense* (16, 42). The *draT* and *draG* genes in these organisms have been cloned, mutagenized, and functionally characterized (11, 26, 29, 42, 44).

In *R. rubrum*, the site of ADP-ribosylation in dinitrogenase reductase has been determined to be a specific arginine residue by peptide analysis (33) and site-directed mutagenesis (25). When this residue was replaced by valine, the protein became inactive and could not be modified in response to darkness (25). This arginine residue is conserved in all examined dinitrogenase reductases regardless of whether the organism has the DRAT/DRAG regulatory system (6). Mutants with substitutions at this arginine residue of *Klebsiella pneumoniae* and *Azotobacter vinelandii*, neither of which has this regulatory ADP-ribosylation system, have been characterized. In *K. pneumoniae*, the mutant with a substitution of histidine for this arginine residue showed low nitrogenase activity during derepression in the presence of serine (4), and this low nitrogenase activity is probably due to the inefficient transfer of electrons to dinitrogenase by the altered dinitrogenase reductase (27). In *A. vinelandii*, some substitutions for this arginine residue such as tyrosine support diazotrophic growth, indicating that the arginine residue is optimal but not essential for protein function (39).

In *R. capsulatus*, this specific arginine residue has also been identified as the site for ADP-ribosylation of dinitrogenase reductase (19). Substitution of tyrosine or phenylalanine for this arginine residue supported high nitrogenase activity and diazotrophic growth, whereas some other substitutions, such as histidine and glutamine, showed a very low nitrogenase activity and failed to support diazotrophic growth (32). Interestingly, another posttranslational regulatory system for nitrogenase activity was revealed when the tyrosine and phenylalanine mutants still showed a rapid loss of nitrogenase activity in response to the addition of NH_4^+ but without ADP-ribosylation of dinitrogenase reductase (31). This second regulatory system regulates nitrogenase activity in response to NH_4^+ but does not respond to darkness (32). Unfortunately, the lack of appropriate constructs has prevented analysis of similar secondary systems in either *R. rubrum* or *A. brasilense*. Dinitrogenase reductase of *A. brasilense* also is regulated by the DRAT/DRAG regulatory system (13, 44), but neither the site nor the modified group on dinitrogenase reductase has been biochemically and genetically characterized.

As reported here, we substituted tyrosine and phenylalanine for the arginine residue at position 101 of dinitrogenase reductase of *A. brasilense* to investigate whether this organism has a second regulatory system. We also transferred these altered *A. brasilense nifHDK* genes into an *R. rubrum* mutant lacking nitrogenase to address the same issue for that organism.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
A. brasilense strains		
UB1 (Sp7)	Wild type	17
UB9	Δ*nifH1*::*kan* mutant	This report
UB10	Δ*draTG5*::*kan* mutant	43
UB40	Transconjugant of UB1 with pYPZ132	This report
UB41	Transconjugant of UB1 with pYPZ133	This report
UB42	Transconjugant of UB1 with pYPZ134	This report
UB43	Transconjugant of UB9 with pYPZ132	This report
UB44	Transconjugant of UB9 with pYPZ133	This report
UB45	Transconjugant of UB9 with pYPZ134	This report
UB46	*A. brasilense* wild-type (Arg-101) *nifHDK* genes were integrated into chromosome of UB9	This report
UB47	*A. brasilense* Tyr-101 *nifHDK* genes were integrated into chromosome of UB9	This report
UB50	Δ*nifH1*::*kan draT6*::*tet* mutant	This report
UB52	*A. brasilense* wild-type (Arg-101) *nifHDK* genes were integrated into chromosome of UB50	This report
UB53	*A. brasilense* Tyr-101 *nifHDK* genes were integrated into chromosome of UB50	This report
Plasmids		
pYPZ132	*A. brasilense* wild-type (Arg-101) *nifHDK* on pRK404	43
pYPZ133	*A. brasilense* Tyr-101 *nifHDK* on pRK404	This report
pYPZ134	*A. brasilense* Phe-101 *nifHDK* on pRK404	This report

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. Throughout this report, Arg (Arg-101, which is the wild type), Tyr (the Tyr-101 substitution), and Phe (the Phe-101 substitution) refer to the specific amino acids at position 101 of NifH of *A. brasilense*. Growth conditions and concentrations of antibiotics used in *Escherichia coli*, *A. brasilense*, and *R. rubrum* cells were the same as were used before (26, 44), except as noted.

Whole-cell nitrogenase activity assay. Nitrogenase activity in *A. brasilense* strains was measured in cells grown in semisolid medium (5, 35); this medium is very useful in studying this microaerobic nitrogen-fixing bacterium. A low level of NH_4Cl (2 mM) was added to the N-free semisolid medium to increase the growth rate, but this level had no effect on nitrogenase activity. *A. brasilense* strains were derepressed for nitrogenase in NfbHP-glutamate liquid medium, as described before (30, 44). *R. rubrum* strains were derepressed in MG medium as described previously (24). Nitrogenase activity of whole cells was measured by an acetylene reduction assay (2). For NH_4^+ treatment, a small volume of concentrated NH_4Cl (1 M) was added into derepressed *A. brasilense* or *R. rubrum* cultures. For darkness treatment, vials containing derepressed *R. rubrum* cells were wrapped with aluminum foil.

Site-directed mutagenesis of the arginine residue of dinitrogenase reductase. The unique site elimination method of Deng and Nickoloff (7) was used with some modifications to generate mutations. A 0.8-kb *Xho*I-*Pst*I fragment from pYPZ103 (44), containing part of the *A. brasilense nifH* gene, was cloned into pBSKS(−) (Stratagene, La Jolla, Calif.), yielding pYPZ120. Plasmid pYPZ120 was used as a template DNA and was annealed with two primers: one is the unique site elimination primer that replaced the *Afl*III site with a *Bgl*II site in the vector (7), and the other is one of two synthetic oligonucleotides that replace the Arg-101 codon (CGC) with TAC (Tyr) or TTC (Phe). After phosphorylation, 2 pmol of each primer (the primer for the residue 101 substitutions and the unique site elimination primer) and 0.05 pmol of double-stranded template DNA were mixed with annealing buffer to a total volume of 20 μl. The mixture was boiled for 3 min, then put on ice for 5 min, and kept at room temperature for 30 min. After the extension reaction, the DNA was precipitated with 2 volumes of ethanol, resuspended in 18 μl of double-distilled water, and then digested with *Afl*III. Digested DNA (5 μl) was transformed into *E. coli* BMH 71-18 (*mutS*) (41). DNA was isolated en masse from transformants, redigested with *Afl*III, and run on a 0.8% agarose gel. Undigested DNA was excised and recovered from the gel with a GENECLEAN II Kit (Bio 101 Inc., La Jolla, Calif.) and transformed into *E. coli* DH5α. This step significantly increased the detection of mutant plasmids in the next step of screening. Plasmids were isolated from the transformants and digested with *Afl*III; those that were not cut by *Afl*III were digested with *Bgl*II to confirm unique site exchange. The frequency of plasmids with *Afl*III-*Bgl*II exchange was very high (more than 80%) by the last step. The plasmids with *Afl*III-*Bgl*II exchange were subjected to DNA sequencing to check the sequence encoding residue 101; desired substitutions were found at a frequency of 30 to 100%.

Construction of *nifH* and *nifH draT* mutants of *A. brasilense*. A *nifH* deletion mutant of *A. brasilense* was constructed by partially digesting plasmid pYPZ103 (44), containing *A. brasilense nifHD'* and *draTG*, with *Nae*I and ligating it with a 1.4-kb *Hin*cII fragment carrying the Km^r gene from pUC4K (38). In the construct chosen for further use, a 100-bp *Nae*I fragment within *nifH* was deleted and replaced with the Km^r gene. An 8.1-kb *Sal*I fragment containing the mutated *nifH* gene from this plasmid was subcloned into pSUP202 (34), transformed into *E. coli* S17-1 (34), and then conjugated into the *A. brasilense* wild type as described previously (44). Nx^r Km^r *A. brasilense* colonies were selected and replica printed to identify Cm^s colonies resulting from a double crossover event. The replacement of the wild-type *nifH* allele with the mutant was confirmed by Southern analysis. The mutation was designated Δ*nifH1*::*kan*, and the mutant was named UB9.

Construction of a *nifH draT* double mutant utilized the *nifH* mutant (UB9) described above and plasmids used for constructing a *draT* mutant (UB2) described previously (44). Because the *nifH* mutant also contains a Km^r gene insertion, a different selectable marker was necessary, so the Km^r gene inserted in *draT* was replaced with a Tc^r gene by the following method. A 2.0-kb *Hin*dIII fragment containing the Tc^r gene from pHP45Ω-Tc (10) was inserted into the Km^r gene located in the *draT* gene. After being subcloned into pSUP202, the mutated *draT* region was transferred into UB9. Desired clones were identified by selecting Nx^r Km^r Tc^r colonies and screening for Cm^s. This *draT6*::*tet nifH1*::*kan* double mutant was named UB50.

Mobilization of the cloned *nifHDK* region into *A. brasilense* and *R. rubrum* cells. The *nifHDK* genes from *A. brasilense* were previously cloned into the broad host-range plasmid, pRK404 (8), yielding pYPZ132 (43). A 0.8-kb *Xho*I-*Pst*I fragment from mutated plasmids, containing either a Tyr or Phe substitution in *nifH*, was inserted into pYPZ132, replacing the wild-type fragment and yielding pYPZ133 and pYPZ134, respectively. pYPZ132, pYPZ133, and pYPZ134 were mobilized from *E. coli* DH5α to *A. brasilense* or *R. rubrum* by using the methods described previously (14, 26, 44).

To integrate *A. brasilense nifHDK* genes into the *A. brasilense* chromosome, the 6.7-kb *Eco*RI fragment containing either the wild-type or Tyr-101 *nifHDK* was subcloned into pSUP202 and then conjugated into UB9 and UB50. In this case, a single crossover event was desired, so that Nx^r Km^r Tc^r colonies were selected. Although UB50 is resistant to 5 to 7 μg of tetracycline per ml, strains with the integrated Tc^r gene from pSUP202 can grow at a 12.5-μg/ml level. The constructed strains were named UB46, UB47, UB52, and UB53 (Table 1).

Preparation of protein samples, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting of dinitrogenase reductase. Trichloroacetic acid precipitation was used to extract proteins quickly from *A. brasilense* and *R. rubrum* cells as described before (42). Low cross-linker SDS gels were used to obtain better separation of the dinitrogenase reductase subunits; ADP-ribosylation slows migration of the modified subunit (20). Protein was transferred to a nitrocellulose membrane, which was incubated with a polyclonal antibody against *A. vinelandii* dinitrogenase reductase, and then visualized with horseradish peroxidase as described previously (17, 37).

RESULTS AND DISCUSSION

Nitrogenase activity of arginine substitution mutants of *A. brasilense* in semisolid medium. To study the effects of substitutions at Arg-101, a *nifH* deletion mutant of *A. brasilense* was constructed as described in Materials and Methods. This mutant (UB9) showed no nitrogenase activity when grown in

either semisolid medium or NfbHP-glutamate liquid medium and failed to accumulate dinitrogenase reductase (data not shown). Plasmids pYPZ132, pYPZ133, and pYPZ134, which contain wild-type (Arg) or Tyr- or Phe-substituted *nifHDK*, respectively, were transferred into UB1 (wild type) and UB9 (*nifH*). Cultures were grown in semisolid medium containing 2 mM NH_4Cl, and the whole-cell nitrogenase activity was measured. In the wild-type (UB1) background, the Tyr and Phe mutants (UB41 and UB42) had nitrogenase activities of approximately 68 nmol of ethylene per h per tube; this is similar to that of the control strain UB40, which contains multiple copies of wild-type *nifHDK*. However, nitrogenase activity in all these strains is only one-third of that in wild-type UB1, presumably because the multiple copies of *nifHDK* affect the expression of the other *nif* operons. All introduced genes were present on derivatives of plasmid pRK404, of which there are approximately 10 copies per cell (36).

In the *nifH* background (UB9), the following nitrogenase activities (nanomoles of acetylene produced per hour per tube) were seen: UB43 (Arg), 42; UB44 (Tyr), 10; and UB45 (Phe), 5. The higher nitrogenase activity of the mutants in the UB1 background, compared with that in the UB9 background, suggests that the detected activity mainly reflected expression of the wild-type *nifHDK* in the chromosome of UB1.

It is rather surprising that these altered low-activity dinitrogenase reductases have no significant effect on the activity of the wild-type dinitrogenase reductase (UB41 and UB42). As will be shown below, these same plasmids support a very significant level of dinitrogenase reductase activities in an *R. rubrum* background, so that mutated proteins must certainly be expressed and accumulated in the homologous background. One possibility is that these mutant dinitrogenase reductases cannot interact with their physiological electron donor in vivo and therefore cannot be reduced and compete with the wild-type protein for interaction with dinitrogenase.

Taken together, these results demonstrate significantly reduced accumulation of nitrogenase activity from the plasmid-encoded genes. We were concerned that the low activity in the above-mentioned strains would hinder interpretation of the posttranslational response to NH_4^+, so we undertook the alternate approach described below to try to increase nitrogenase activity.

Effect of NH_4^+ on nitrogenase activity in Tyr mutant of *A. brasilense*. Because the multiple copies of *nifHDK* decreased nitrogenase activity in *A. brasilense*, we decided to introduce the cloned region in a single copy as described in Materials and Methods. Single-copy integrants of pSUP202 carrying either the Arg-101 (wild-type) or Tyr-101 *nifHDK* into the UB9 (*nifH*) background, UB46 and UB47, respectively, were obtained. To study the effect of NH_4^+ on nitrogenase activity in these strains, NfbHP-glutamate liquid medium was used because such an experiment cannot be done in semisolid medium. Upon derepression, nitrogenase activity in UB46 was 1,000 nmol of ethylene h^{-1} ml^{-1}, threefold higher than that in UB43, which contains multicopies of wild-type *nifHDK*. However, nitrogenase activity in UB47 still was low (about 40 nmol of ethylene h^{-1} ml^{-1}), similar to that in UB44, indicating that copy number was not the source of the low activity in the Tyr mutant.

The effect of the addition of NH_4^+ on nitrogenase activity in UB46 and UB47 was studied, and the results are shown in Fig. 1. UB46 showed the loss of nitrogenase activity in response to the addition of NH_4^+, and the activity was recovered after NH_4^+ exhaustion, as seen previously with the wild type (44). The modification of dinitrogenase reductase was also monitored, and the result is shown in Fig. 2. After the addition of

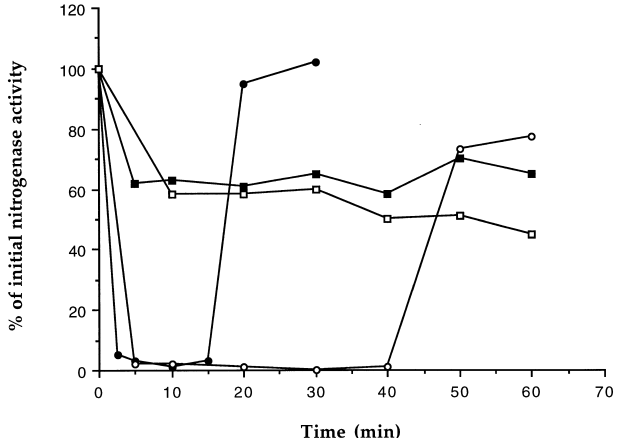

FIG. 1. Regulation of nitrogenase activity by NH_4^+ in *A. brasilense* UB46 (Δ*nifH*, integrated with wild-type *nifHDK*) and UB47 (Δ*nifH*, integrated with Tyr-101 *nifHDK*). *A. brasilense* cells were derepressed in NfbHP-glutamate liquid medium. At time zero, NH_4Cl was added to different cultures to the following concentrations: UB46, 0.4 mM (●); UB46, 1 mM (○); UB47, 0.5 mM (■); and UB47, 2 mM (□). At the times indicated, 1-ml aliquots were withdrawn and assayed for nitrogenase activity under microaerobic conditions for 2 min for UB46 and 10 min for UB47. Initial nitrogenase activities (100%) in UB46 and UB47 were, respectively, 1,000 and 40 nmol of ethylene produced per h per ml of cells at an optical density of 5.0 at 600 nm. Each point represents an average for at least three replicate assays.

NH_4^+, dinitrogenase reductase showed two bands on the SDS-PAGE Western blot (immunoblot), since ADP-ribosylation slows migration of the modified subunit. In the Tyr mutant (UB47), the addition of NH_4^+ partially inhibited nitrogenase activity (Fig. 1), but there was no modification of dinitrogenase reductase (a single band) after the addition of NH_4^+ (Fig. 2). The Tyr-101 dinitrogenase reductase migrates slightly more slowly than the wild-type protein; this result is similar to that obtained with the tyrosine-substituted dinitrogenase reductase in *R. capsulatus* (31). The loss of activity without apparent modification indicates that ADP-ribosylation of dinitrogenase reductase is not the only mechanism by which *A. brasilense*

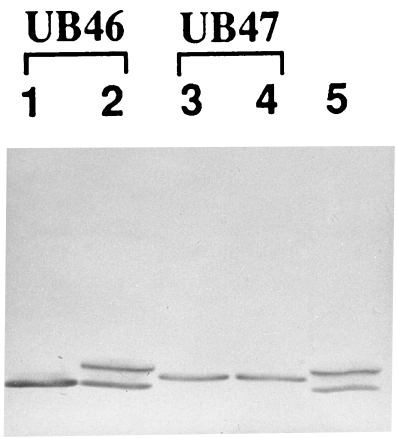

FIG. 2. ADP ribosylation of dinitrogenase reductase in response to NH_4^+ in *A. brasilense* UB46 (Δ*nifH*, integrated with wild-type *nifHDK*) and UB47 (Δ*nifH*, integrated with Tyr-101 *nifHDK*). Crude extracts were prepared from derepressed cells before the addition of NH_4^+ (lanes 1 and 3) and 10 min after the addition of NH_4^+ to a final concentration of 2 mM (lanes 2 and 4). Modified dinitrogenase reductase from NH_4^+-treated crude extract of *A. brasilense* wild type was used as a control (lane 5). In lanes 2 and 5, the upper band is the modified subunit of dinitrogenase reductase.

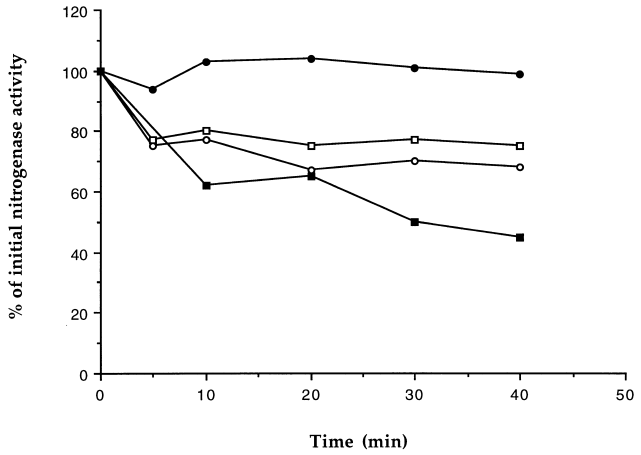

FIG. 3. Effects of the addition of NH_4^+ and Na^+ on nitrogenase activity in *A. brasilense* UB52 (Δ*nifH draT*, integrated with wild-type *nifHDK*) and UB53 (Δ*nifH draT*, integrated with Tyr-101 *nifHDK*). At $t = 0$, NH_4Cl (final concentration, 2 mM) was added to UB52 (□) and UB53 (■), while ammonium acetate (final concentration, 2 mM) (○) or 2 mM NaCl (●) was added to UB52. At the times indicated, 1-ml aliquots were withdrawn and assayed for nitrogenase activity under microaerobic conditions for 2 min for UB52 and 10 min for UB53. Initial nitrogenase activities (100%) in UB52 and UB53 were, respectively, 300 and 30 nmol of ethylene produced per h per ml of cells at an optical density of 5.0 at 600 nm. Each point represents an average for at least three replicate assays.

regulates nitrogenase activity. The failure to detect an electrophoretic shift of dinitrogenase reductase in the Tyr mutant also demonstrates that the Arg-101 residue is necessary for this modification, strongly suggesting that it is the site for ADP-ribosylation in *A. brasilense.*

We note two curiosities in Fig. 1. The first is that the activity of UB47 (Tyr) shows little or no recovery of activity in the time frame of this experiment in numerous repeats, although the NH_4^+ has certainly been exhausted, suggesting that the second mechanism was not reversible. In light of this result, it is therefore surprising that the activity of UB46 (Arg) recovers completely, as the second mechanism should be operating here also. We believe that the explanation for both of these results is that the nitrogenase activity in the mutant is unstable and there is simply less total nitrogenase activity present when the second mechanism ceases to affect activity. In the case of the wild type, the nitrogenase activity remains stable and both ADP ribosylation and the second mechanism cease to affect nitrogenase activity upon NH_4^+ exhaustion.

The newly identified regulatory system is DRAT/DRAG independent. Whereas ADP-ribosylation of dinitrogenase reductase is not responsible for the loss of nitrogenase activity in the Tyr mutant, it remained a formal possibility that the effect of the addition of NH_4^+ resulted from DRAT modifying some other protein necessary for nitrogenase activity. To test this possibility, a *draT nifH* double mutant (UB50) was constructed as described in Materials and Methods. Either the wild-type or the Tyr-101 *nifHDK* genes were integrated into the chromosome of UB50 in a single copy, yielding UB52 and UB53, respectively.

UB52 (*draT*/Arg) has a lower nitrogenase activity than that of the wild type or *draT* mutant (about 300 nmol of ethylene h^{-1} ml^{-1}). Figure 3 shows that the addition of NH_4^+ causes a 20 to 30% inhibition of nitrogenase activity in UB52, which is significantly greater than that seen previously with *draT* mutants (43, 44). UB53 (*draT*/Tyr) had a low initial nitrogenase activity, and the addition of NH_4^+ partially inhibited this nitrogenase activity (Fig. 3); this was similar to the response in UB47 (Fig. 1). The effect is attributed to NH_4^+, as ammonium acetate caused a similar inhibition, but NaCl had no effect (Fig. 3). Neither UB52 nor UB53 showed detectable modification of dinitrogenase reductase in response to NH_4^+ (data not shown). These results indicate that the mechanism of this partial inhibition of nitrogenase activity by NH_4^+ in *A. brasilense* is independent of the DRAT/DRAG system.

NH_4^+ inhibition of nitrogenase activity without modification of dinitrogenase reductase has been reported for some nitrogen-fixing bacteria, such as *A. vinelandii* (21, 22), *Azospirillum amazonense* (17), *Acetobacter diazotrophicus* (1, 3), *Herbaspirillum seropedicae* (12), *Anabaena variabilis* (9), and *Rhodobacter sphaeroides* (15, 18, 40). These bacteria appear to lack the DRAT/DRAG system, and the mechanism for this inhibition of nitrogenase activity by NH_4^+ is unknown; it has been suggested that the regulation in *A. vinelandii* might involve the reallocation of electron flow to nitrogenase (22). In *R. capsulatus*, the second regulatory system is very rapid and completely inhibits nitrogenase activity (31). Furthermore, this second system might involve DRAT/DRAG, because a *draT* mutant of *R. capsulatus* was unable to respond to the addition of NH_4^+ (29); the relationship between these two regulatory systems in *R. capsulatus* needs further investigation.

The second regulatory system in *A. brasilense* shows some differences from the system in *R. capsulatus*. It is DRAT/DRAG independent, and its effects on nitrogenase activity are less dramatic. A variable degree (10 to 40%) of this secondary regulatory effect was seen with different strains, and it probably reflects their different initial nitrogenase activities. Strains with a lower initial nitrogenase activity consistently show a greater degree of inhibition by NH_4^+.

The nitrogenase activity of *A. brasilense* also is regulated in response to anaerobic shifts (42). However, because of the low initial nitrogenase activity in the Tyr-101 mutant (see above) and the rapid reactivation of anaerobic-treated dinitrogenase reductase during nitrogenase assay under microaerobic conditions in *A. brasilense* (42), we were unable to determine whether this second regulatory system also can respond to anaerobic shifts.

Absence of a secondary regulatory system in *R. rubrum*. Our previous experiments showed that wild-type *nifHDK* genes from *A. brasilense* could be expressed well in an *R. rubrum* mutant lacking nitrogenase (43). Therefore we transferred pYPZ133 and pYPZ134, containing the Tyr-101 and Phe-101 *nifHDK* genes of *A. brasilense*, respectively, into *R. rubrum* UR206 (*nifH*::*kan*) (23). Surprisingly, in contrast to the results with the *A. brasilense* background, these plasmids support high nitrogenase activity (about 600 nmol of ethylene h^{-1} ml^{-1}), which is similar to that seen with a control with the wild-type *nifHDK* from *A. brasilense* (43). With the wild-type plasmid, NH_4^+ or darkness caused a decrease in nitrogenase activity, concomitant with ADP-ribosylation of dinitrogenase reductase (data not shown). In contrast, these stimuli had no detectable effect on nitrogenase activity or ADP-ribosylation with the mutants (data not shown). These results strongly suggest that there is no second regulatory system controlling nitrogenase activity in *R. rubrum*.

In vitro nitrogenase activity in *A. brasilense* mutants. The high activity of these mutant proteins in *R. rubrum* suggested that their low activity in *A. brasilense* might reflect poor interaction of these proteins with their endogenous electron donor. Were this the case, we would have expected to see substantial nitrogenase activity in vitro, when an exogenous electron donor (dithionite) was supplied. To avoid any modification of dinitrogenase reductase during sample manipulation, we used strains lacking DRAT. While crude extracts of UB53 (*draTG*

nifH/Tyr) displayed undetectable nitrogenase activity in vitro, extracts of UB10 (*draTG*) and UB52 (*draTG nifH*/Arg) also had surprisingly low nitrogenase activities, indicating that the problem was not a result of the Tyr substitution.

The addition of purified dinitrogenase and dinitrogenase reductase from *A. vinelandii* to the extracts revealed substantial dinitrogenase activity in UB10 and UB52 but very little in UB53. All the extracts lacked significant dinitrogenase reductase activity, although Western blot analysis showed that both nitrogenase proteins were comparably accumulated (data not shown). The causes of the low dinitrogenase reductase activity in all strains and of the low dinitrogenase activity in UB53 are unknown.

Conclusions. The ability of NH_4^+ to inhibit nitrogenase activity partially in *A. brasilense* strains lacking both DRAT and the probable site of ADP-ribosylation demonstrates the presence of another mechanism for posttranslational regulation of nitrogenase activity in this organism. Overall, this second regulatory system has a less dramatic effect on nitrogenase activity in response to NH_4^+ than does the DRAT/DRAG regulatory system. In contrast, in *R. rubrum*, alteration of the target residue for ADP-ribosylation eliminated its posttranslational response to darkness and NH_4^+; this strongly suggests that DRAT/DRAG is the sole mechanism for the regulation of nitrogenase activity in response to the addition of NH_4^+ and darkness shifts in this organism.

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