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# Posttranslational Regulation of Nitrogenase Activity in *Azospirillum brasilense ntrBC* Mutants: Ammonium and Anaerobic Switch-Off Occurs through Independent Signal Transduction Pathways

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**Nitrogenase activity is regulated by reversible ADP-ribosylation in response to $NH_4^+$ and anaerobic conditions in *Azospirillum brasilense*. The effect of mutations in *ntrBC* on this regulation was examined. While $NH_4^+$ addition to *ntrBC* mutants caused a partial loss of nitrogenase activity, the effect was substantially smaller than that seen in *ntr*$^+$ strains. In contrast, nitrogenase activity in these mutants was normally regulated in response to anaerobic conditions. The analysis of mutants lacking both the *ntrBC* gene products and dinitrogenase reductase activating glycohydrolase (DRAG) suggested that the primary effect of the *ntrBC* mutations was to alter the regulation of DRAG activity. Although *nif* expression in the *ntr* mutants appeared normal, as judged by activity, glutamine synthetase activity was significantly lower in *ntrBC* mutants than in the wild type. We hypothesize that this lower glutamine synthetase activity may delay the transduction of the $NH_4^+$ signal necessary for the inactivation of DRAG, resulting in a reduced response of nitrogenase activity to $NH_4^+$. Finally, data presented here suggest that different environmental stimuli use independent signal pathways to affect this reversible ADP-ribosylation system.**

Biological nitrogen fixation, the reduction of molecular dinitrogen to ammonium, is catalyzed by the nitrogenase complex. This complex consists of two enzymes: dinitrogenase (MoFe protein), which contains the active site of dinitrogen reduction, and dinitrogenase reductase (Fe protein), which donates electrons to dinitrogenase (3). Because nitrogen fixation is a very energy-demanding process, it is not surprising that the nitrogenase system is elaborately regulated at both the transcriptional and posttranslational levels.

Transcriptional regulation of *nif* gene expression appears to be exceedingly complicated, with both global and *nif*-specific regulators (29). In some diazotrophs, such as *Klebsiella pneumoniae* and *Rhodobacter capsulatus*, the global regulation in response to fixed nitrogen is due to the *ntr* system, in which the products of *ntrB* and *ntrC* (NTRB and NTRC, respectively) are typically required for *nif* transcription (7, 16, 36). In some other nitrogen-fixing bacteria, such as *Azotobacter vinelandii* and *Azospirillum brasilense*, this does not appear to be true, because mutations in *ntrB* or *ntrC* have no obvious effect on *nif* gene expression (19, 34).

Posttranslational regulation of nitrogenase activity, which also has been termed switch-off (39), has been found in several diverse nitrogen-fixing bacteria (21). This regulation has been described best for the photosynthetic bacterium *Rhodospirillum rubrum* and involves reversible mono-ADP-ribosylation of dinitrogenase reductase. Two enzymes that perform this regulation have been found. Dinitrogenase reductase ADP-ribosyl transferase (DRAT, the gene product of *draT*) catalyzes the transfer of ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase dimer of *R. rubrum* and thus inactivates the enzyme. Dinitrogenase reductase activating glycohydrolase (DRAG, the gene product of *draG*) can remove the ADP-ribose from the covalently modified dinitrogenase reductase and restore its activity (18, 20, 21, 30).

*Azospirillum* spp. are nonphotosynthetic, microaerobic, nitrogen-fixing bacteria associated with roots of many grasses and other economically important crops (6, 33). Posttranslational regulation of nitrogenase activity in *A. brasilense* has been studied extensively, and it also involves reversible modification of dinitrogenase reductase as seen in *R. rubrum* (10, 12, 13). The *draTG* genes of *A. brasilense* have been cloned, sequenced, and mutagenized (38). In this organism, the DRAT/DRAG system negatively regulates nitrogenase activity in response to exogenous $NH_4^+$ or anaerobic conditions (37, 38).

Through biochemical and genetic analysis, it has become clear that the activities of DRAT and DRAG are themselves subject to posttranslational regulation. DRAG is active and DRAT is inactive under *nif*-derepression conditions in *A. brasilense* and *R. rubrum* (18, 37, 38). When *A. brasilense* cells are treated with $NH_4^+$ or shifted from a microaerobic to an anaerobic environment, DRAG becomes inactive and DRAT is activated, resulting in modification of dinitrogenase reductase. Surprisingly, DRAT activity is observed only transiently under anaerobic conditions in vivo, while the duration of its activity in response to high concentrations of $NH_4^+$ is unclear. After $NH_4^+$ exhaustion or the return to microaerobic conditions, DRAG becomes active, whereupon nitrogenase activity is recovered (37). The actual mechanisms for the regulation of DRAT and DRAG activities in either *A. brasilense* or *R. rubrum* are unknown.

Recently Liang et al. (19) reported functional characterization of *ntrBC* genes from *A. brasilense*. The *ntrB* and *ntrC* mutants of *A. brasilense* have Nif$^+$ phenotypes, but the regulation of nitrogenase activity by $NH_4^+$ was shown to be reduced in these mutants. In this paper, we report further characterization of the regulation of nitrogenase activity and the modification of dinitrogenase reductase in these *ntrBC* mutants, in response to $NH_4^+$ and anaerobic conditions.

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## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *A. brasilense* Sp7 (ATCC 29145), also termed UB1, is the wild type obtained from the American Type Culture Collection, Rockville, Md. Two *A. brasilense* strains have been constructed previously by Liang et al. (19): one strain, Sp7 *ntrC*-148, contains an *ntrC* mutation (lacking NTRC, the strain is termed UB32 in this paper) and the other strain, Sp7 *ntrB*-158, contains an *ntrB* mutation (lacking NTRB, the strain is termed UB33 in this paper). Both of these strains are normal in terms of growth on a variety of media, with the exception of their inability to utilize nitrate as a nitrogen source (19) (see below). Their nitrogenase activity is approximately 80% that of the wild type (19). Strain UB4 (*draG3*::*kan*, lacking DRAG) has been described previously (38).

*A. brasilense* strains were grown in LD medium, and nitrogenase was derepressed in NfbHP-glutamate medium under microaerobic conditions as described previously (26, 37, 38). Nitrogenase activity of whole cells was determined by the acetylene reduction rate for short periods in a stoppered 9-ml vial under microaerobic conditions (37, 38). The ethylene produced was measured by gas chromatography (2). Anaerobic and $NH_4^+$ treatments of the whole cells were performed as described previously (37).

**Construction of *A. brasilense ntrB draG* and *ntrC draG* double mutants.** Double mutants were constructed by introducing *draG* mutations into the previously constructed *ntr* mutants. First, the *draTG* region, containing the $Km^r$ cassette gene from pUC4K inserted in the *draG* gene (38), was subcloned into pSUP202. Because the *ntrBC* mutants also contain $Km^r$ insertions, a different selectable marker was necessary, so the $Km^r$ in *draG* was replaced with $Tc^r$ by the following method. The pSUP202 derivative containing *draT*$^+$ *draG3*::*kan* was partially digested with *Hin*dIII (there are two *Hin*dIII sites on this plasmid; one is the $Km^r$ gene, and the other is in the replicon) and then ligated with a 2.1-kb *Hin*dIII fragment of the $Tc^r$ gene from pHP45Ω-$Tc^r$ (8). Desired clones were identified by selecting $Tc^r$ $Cm^r$ colonies and screening for $Km^s$, and the new allele was named *draG4*::*tet*. The pSUP202 derivative containing *draT*$^+$*draG4*::*tet* was then transformed into *E. coli* S17-1 and transferred into *A. brasilense ntrC* and *ntrB* mutants by conjugation as described previously (38). $Nx^r$ $Km^r$ $Tc^r$ *A. brasilense* colonies were selected and replica printed to identify $Cm^s$ colonies resulting from a double-crossover event. The *draG* mutations in these colonies were confirmed by Southern analysis. The *A. brasilense ntrB158 draG4*::*tet* mutant was named UB35, and the *ntrC148 draG4*::*tet* mutant was named UB36. Antibiotics were used at the following concentrations in *A. brasilense*: 10 mg of kanamycin per liter, 3 mg of nalidixic acid per liter, 7.5 mg of chloramphenicol per liter, and 5 to 7 mg of tetracycline per liter.

**Protein extraction, SDS-PAGE, and immunoblotting of dinitrogenase reductase.** To analyze the modification of dinitrogenase reductase under different conditions and minimize any artifactual change in the modification status during sample manipulation, we used the trichloroacetic acid precipitation method to extract protein quickly from *A. brasilense*, as described previously (37). Low-cross-linker sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein separation to obtain better resolution of the dinitrogenase reductase subunits; ADP-ribosylation slows migration of the modified subunit (14). Proteins from SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane, immunoblotted with polyclonal antibody against *Azotobacter vinelandii* dinitrogenase reductase, and visualized by color development with horseradish peroxidase, as described previously (13, 35, 37).

**GS activity assay.** Glutamine synthetase (GS) activity was measured by the γ-glutamyltransferase assay in the absence of $Mg^{2+}$ (31), and protein was measured in crude extracts by the method of Peterson (27).

## RESULTS

**$NH_4^+$ switch-off of nitrogenase activity in *ntrB* (UB33) and *ntrC* (UB32) mutants.** In wild-type *A. brasilense* UB1, exogenous $NH_4^+$ causes rapid and complete loss of nitrogenase activity, which can be recovered after $NH_4^+$ exhaustion; these effects correlate with the ADP-ribosylation of dinitrogenase reductase (37, 38). More recently, Liang et al. found that mutations in *ntrB* or *ntrC* reduced the extent of this regulatory response, but this effect was not further characterized (19). We have reexamined the effects of mutations in the *ntrBC* locus on this regulation of nitrogenase activity, and the results are shown in Fig. 1. In both *ntrC* and *ntrB* mutants, $NH_4^+$ caused rapid but only partial switch-off of nitrogenase activity with approximately 60% residual activity. Treatment with different $NH_4^+$ concentrations caused a similar level of residual activity but differed in the time of its exhaustion and the subsequent reactivation of nitrogenase. The residual activity seen with high concentrations of $NH_4^+$ remains at a constant level. While it is possible that this reflects competing but equivalent levels of DRAG and DRAT activities, we believe that both activities are absent under these conditions. It is not known why none of these strains recover full nitrogenase activity upon $NH_4^+$ exhaustion, but we have observed this result consistently with $NH_4^+$ concentrations of 0.5 mM and above (38).

We also examined this response at the level of the ADP-ribosylation of dinitrogenase reductase (Fig. 2). Modified (inactive) dinitrogenase reductase shows two protein bands on immunoblots of SDS-PAGE gels, because ADP-ribosylation slows the migration of the modified subunit while unmodified (active) dinitrogenase reductase has two identical subunits that migrate as a single band (14). As shown in Fig. 2, in *ntrB* and *ntrC* mutants less than 20% of the dinitrogenase reductase, as determined by scanning densitometry (37), was modified after $NH_4^+$ addition, which is substantially less than that seen in the wild type (Fig. 2A). Both the elevated residual nitrogenase activity and the reduced amount of ADP-ribosylation of dinitrogenase reductase seen in these *ntrBC* mutants indicate that *ntrBC* genes play some roles in the DRAG/DRAT regulatory system.

It should be noted, however, that the two assay systems gave different residual levels of nitrogenase activity in the *ntr* mutant following $NH_4^+$ addition: the activity measurements gave a residual level of 60%, while the SDS-PAGE assay indicated that more than 80% of the dinitrogenase reductase was unmodified. This discrepancy may reflect a second regulatory mechanism that responds to $NH_4^+$ but does not involve ADP-ribosylation of dinitrogenase reductase. A response reminiscent of this has been seen in *Rhodobacter capsulatus* (28).

**$NH_4^+$ switch-off of nitrogenase activity in *ntrB draG* (UB35) or *ntrC draG* (UB36) double mutants.** Our previous studies have shown that both DRAG and DRAT activities are subject to posttranslational regulation in *A. brasilense* (37). Specifically, prior to a negative stimulus, DRAG is active and DRAT is inactive, but after such a stimulus, DRAT is activated and DRAG become inactive. Differences in residual nitrogenase activity between the wild type and these *ntrBC* mutants after a negative stimulus therefore could reflect effects on either the

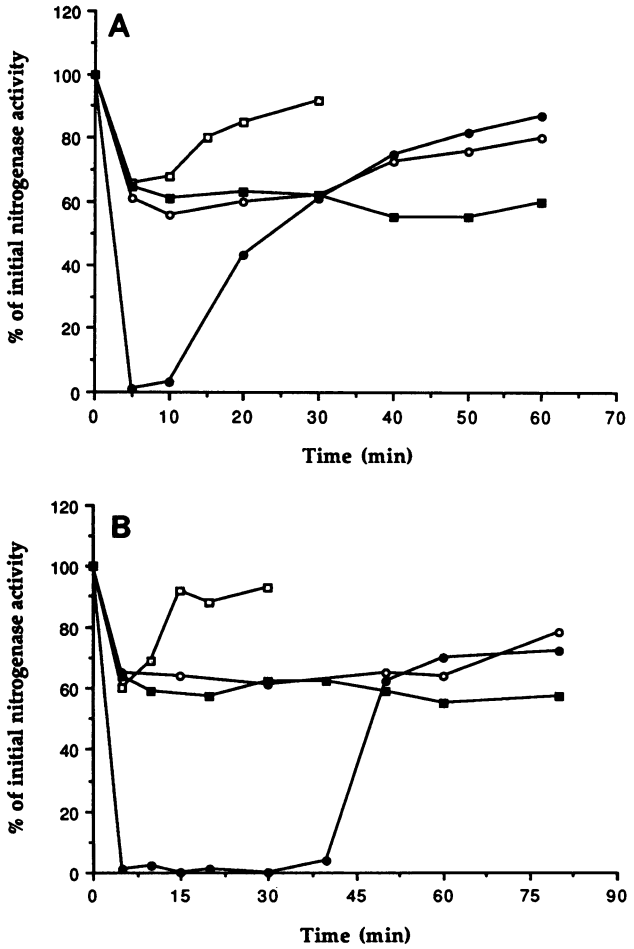


FIG. 1. Regulation of nitrogenase activity by $NH_4^+$ in *A. brasilense* UB1 (wild type), UB32 (*ntrC*), and UB33 (*ntrB*). (A) At $t = 0$, $NH_4Cl$ was added to derepressed cells of UB32 to a final concentration of 0.2 mM (□), 0.5 mM (○), and 20 mM (■), while $NH_4Cl$ was added to UB1 cells (●) to a final concentration of 0.5 mM as a positive control. (B) At $t = 0$, $NH_4Cl$ was added to derepressed cells of UB33 to a final concentration of 0.1 mM (□), 1 mM (○), and 2 mM (■), and $NH_4Cl$ was added to UB1 cells to a final concentration of 1 mM as a control (●). At the times indicated, 1-ml portions of cells were withdrawn and assayed for nitrogenase activity under microaerobic conditions for 2 min. Initial nitrogenase activities (100%) in UB1, UB32, and UB33 were, respectively, about 1,400, 1,100 and 1,100 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 of at least three replicate runs.

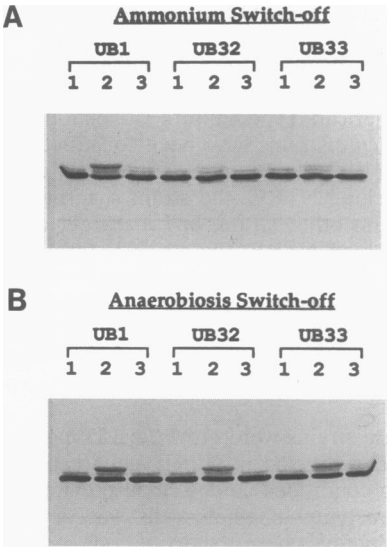


FIG. 2. ADP-ribosylation of dinitrogenase reductase in response to $NH_4^+$ and anaerobiosis. (A) Immunoblotting of dinitrogenase reductase of strains UB1 (wild type), UB32 (*ntrC*), and UB33 (*ntrB*), during $NH_4^+$ switch-off. Crude extracts were prepared from cells before $NH_4^+$ addition (lane 1), 5 min after $NH_4Cl$ addition (250 μM final concentration) (lane 2), and 15 min after $NH_4Cl$ addition (lane 3). (B) Immunoblotting of dinitrogenase reductase of strains UB1, UB32, and UB33, during anaerobic switch-off. Crude extracts were prepared from cells before anaerobiosis (lane 1), 10 min after anaerobiosis (lane 2), and 10 min after cells were returned to microaerobic conditions (lane 3).

accumulation of DRAT/DRAG proteins or the regulation of their activities. To characterize effects of *ntrBC* mutations on DRAT alone, we constructed double mutants of *ntrB draG* and *ntrC draG* and compared them with *ntr*$^+$ *draG* strains. Because these strains lack DRAG, any response differences would indicate an *ntr* effect on DRAT activity.

$NH_4^+$ switch-off of nitrogenase activity in these double mutants was studied, and the results are shown in Fig. 3. After $NH_4^+$ addition, UB35 (*ntrB draG*) and UB36 (*ntrC draG*) showed a rapid complete loss of nitrogenase activity, very similar to that seen in UB4 (*draG*). Because DRAG is absent in all these strains, nitrogenase activity is not recovered after $NH_4^+$ exhaustion. The modification of dinitrogenase reductase in UB36 was examined directly (Fig. 4). Following $NH_4^+$ addition, modification of dinitrogenase reductase in UB36 was similar to that seen in the wild type. No demodification of dinitrogenase reductase occurred after $NH_4^+$ exhaustion. Similar results were seen with UB35 (*ntrB draG*) (data not shown). These results indicate that DRAT is approximately normal in its ability to modify dinitrogenase reductase in *ntrBC* mutants.

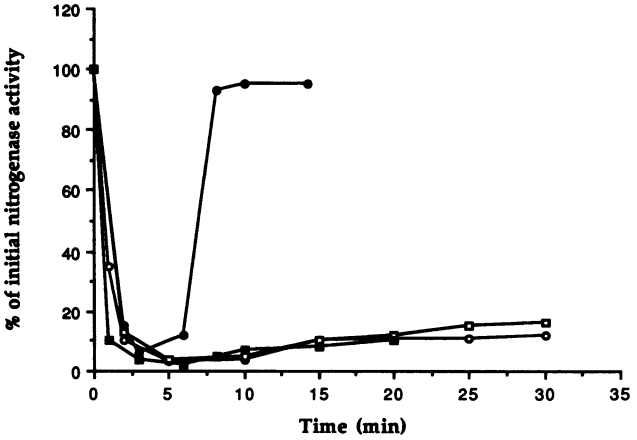


FIG. 3. Regulation of nitrogenase activity by $NH_4Cl$ in UB35 (*ntrB draG*) (○) and UB36 (*ntrC draG*) (□). At $t = 0$, $NH_4Cl$ was added to a final concentration of 0.2 mM. Samples of the cells (1 ml) were withdrawn and assayed for nitrogenase activity for 2 min at the times indicated. UB1 (wild type) (●) and UB4 (*draG*) (■) were treated with the same concentration of $NH_4Cl$ as the control. Initial activities of UB35 and UB36 were about 500 nmol of ethylene produced per h per ml of cells at an optical density at 600 nm of 5.0, while initial activities of UB1 and UB4 were about 1,400 and 800 nmol/h/ml, respectively.

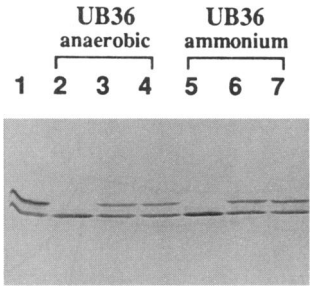


FIG. 4. ADP-ribosylation of dinitrogenase reductase in response to anaerobiosis and $NH_4^+$ in UB36 (*ntrC draG*). Crude extracts were prepared from cells before anaerobiosis (lane 2), 20 min after anaerobiosis (lane 3), and 10 min after the cells were returned to microaerobic conditions (lane 4). Crude extracts were prepared from cells before $NH_4^+$ addition (lane 5), 5 min after $NH_4Cl$ addition (200 μM final concentration) (lane 6), and 15 min after $NH_4Cl$ addition (lane 7). Modified dinitrogenase reductase from UB1 (wild type) crude extract was used as a control (lane 1).

Before $NH_4^+$ treatment, there was little modification of dinitrogenase reductase in either UB36 (*ntrC draG*) or UB4 (*draG*), but UB36 has a lower initial nitrogenase activity (about 500 nmol of ethylene per h per ml) than UB4 does (about 800 nmol of ethylene per h per ml) (see the legend to Fig. 3). The reason for this difference is unknown, but it does not appear to involve the ADP-ribosylation of dinitrogenase reductase.

Another indication of DRAT activity is its rate of modification of dinitrogenase reductase in response to negative stimuli. The results in Fig. 5A show that the loss of nitrogenase activity after $NH_4^+$ addition in UB36 (*ntrC draG*) is extremely fast, again suggesting approximately normal functionality of DRAT. It is significantly faster than that in UB1 (wild type) but a little slower than that in UB4 (*draG*). In UB4, a low level of $NH_4^+$, such as 30 and 50 μM $NH_4Cl$, causes only partial loss of nitrogenase activity, because DRAT activity ceases after $NH_4^+$ is metabolized (37) (Fig. 5B). Similarly, UB36 also showed only a partial loss of nitrogenase activity when treated with a low concentration of $NH_4^+$ (Fig. 5B), but UB36 has a slightly lower residual nitrogenase activity than UB4 does at the same low level of $NH_4^+$.

These results show that UB36 (*ntrC draG*) has a response to $NH_4^+$ similar to that of UB4 (*draG*), suggesting that the DRAT in *ntrBC* mutants is normal in its response to $NH_4^+$ addition. The very similar responses to low levels of $NH_4^+$ in the double mutants also suggest that $NH_4^+$ transport is not dramatically altered by the presence of *ntrBC* mutations. The small differences between UB4 and UB36 in the rates of loss of nitrogenase activity and the residual activity after treatment with low levels of $NH_4^+$ may suggest that the mutations in *ntrBC* have a slight effect on DRAT in the time of its activation and the duration of its activity. However, this small effect on DRAT regulation cannot explain the dramatically altered $NH_4^+$ switch-off in *ntrBC* mutants. A difference in the initial nitrogenase activities in UB36 and UB4 also may contribute to these slight effects. Therefore, we favor the hypothesis that the elevated residual nitrogenase activity seen in *ntrBC* mutants after $NH_4^+$ addition results mainly from the altered regulation of DRAG rather than DRAT.

**Anaerobic switch-off of nitrogenase activity in *ntrB* (UB33), *ntrC* (UB32), and *ntrB/C draG* (UB35 and UB36) mutants.** In addition to $NH_4^+$ switch-off, the nitrogenase activity in *A. brasilense* UB1 (wild type) is regulated negatively in response to anaerobiosis, and this response is also known to be catalyzed

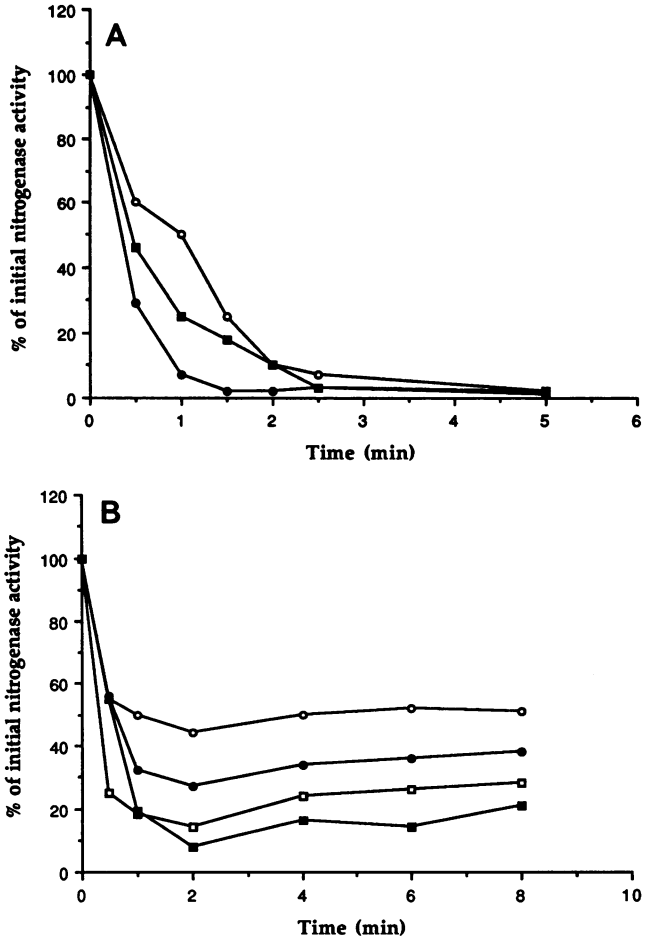


FIG. 5. Comparison of $NH_4^+$ switch-off in UB1 (wild type), UB4 (*draG*), and UB36 (*ntrC draG*) strains. (A) Rate of decrease of nitrogenase activity during $NH_4^+$ switch-off in UB1 (○), UB4 (●), and UB36 (■). At $t = 0$, $NH_4Cl$ was added to a final concentration of 0.5 mM. Samples of cells (1 ml) were withdrawn for nitrogenase assay at the times indicated. (B) Rate of loss of nitrogenase activity after treatment with low concentrations of $NH_4^+$ in UB4 and UB36. At $t = 0$, $NH_4Cl$ was added to a final concentrations of 30 μM into UB4 (○) and UB36 (●) and 50 μM into UB4 (□) and UB36 (■). Nitrogenase activity was assayed as described above, with the initial activities of UB1, UB4, and UB36 being the same as in the experiments in Fig. 1 and 3. Similar results with UB1 and UB4 have been published in our previous paper (37).

by the DRAT/DRAG regulatory system (37). We examined whether these *ntrBC* mutations have dramatic effects on anaerobic switch-off of nitrogenase activity.

Derepressed cells of *ntrBC* mutants were shifted from microaerobic conditions to anaerobic conditions and the changes in nitrogenase activity were monitored. Because the assay for nitrogenase activity of *A. brasilense* must be done under microaerobic conditions which also support the reactivation of dinitrogenase reductase, a very short assay time (0.5 min) was used here, as described previously (37). As shown in Fig. 6A, in response to anaerobic conditions, UB33 (*ntrB*) and UB32 (*ntrC*) mutants showed reductions of nitrogenase activity similar to that of UB1 (wild type); usually a slightly higher residual nitrogenase activity was observed in the *ntr* mutants under anaerobic conditions. In all strains, nitrogenase activity was recovered when cells were returned to microaerobic

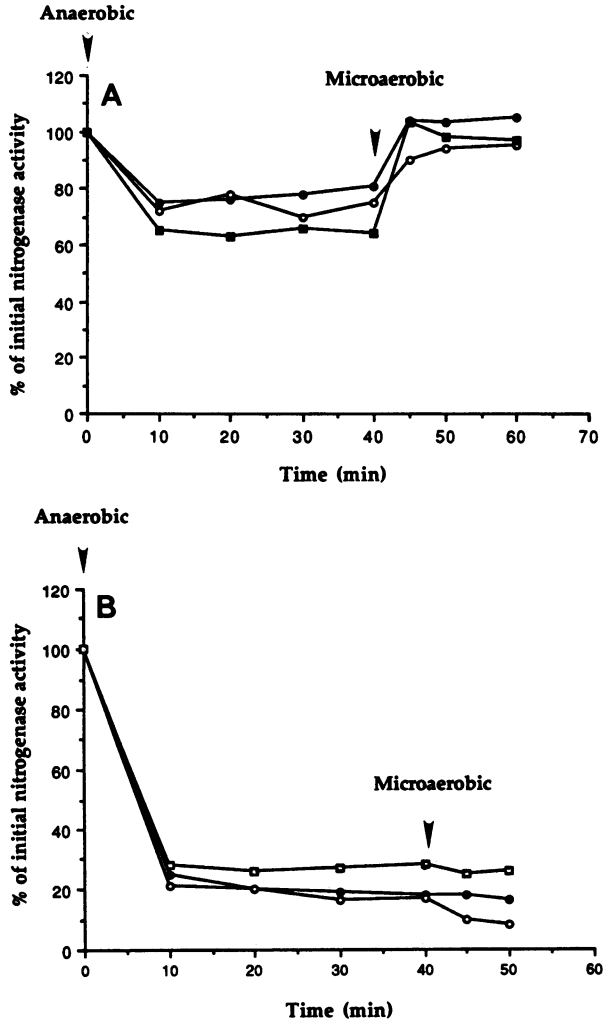


FIG. 6. Regulation of nitrogenase activity by anaerobiosis. (A) Anaerobic switch-off of nitrogenase activity in UB1 (wild type) (■), UB32 (*ntrC*) (●), and UB33 (*ntrB*) (○). At time zero, cells were made anaerobic as described in Materials and Methods, and at 40 min, cells were returned to microaerobic conditions. At the times indicated, 1-ml aliquots of the cells were withdrawn anaerobically and assayed for nitrogenase activity under microaerobic conditions for 0.5 min. (B) Anaerobic switch-off of nitrogenase activity in UB4 (*draG*) (□), UB35 (*ntrB draG*) (○), and UB36 (*ntrC draG*) (●). The nitrogenase activity was assayed under microaerobic conditions as described above, but the assay time was extended from 0.5 to 2 min. Initial activity in these strains was the same as in the experiments in Fig. 1 and 3. Similar results with UB1 and UB4 have been published in our previous paper (37).

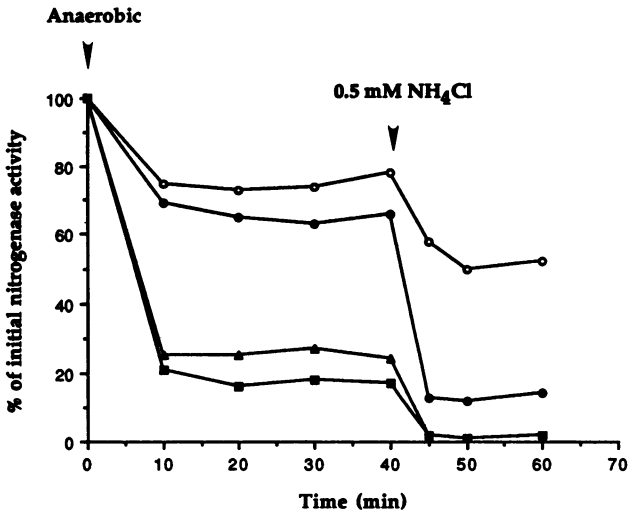


FIG. 7. Effect of a combination of stimuli on nitrogenase activity in UB1 (wild type) (●), UB4 (*draG*) (▲), UB32 (*ntrC*) (○), and UB36 (*ntrC draG*) (■). $N_2$-fixing cells were shifted to anaerobic conditions at time zero, and at 40 min $NH_4Cl$ was added anaerobically to a final concentration of 0.5 mM. At the times indicated, 1-ml portions of the cells were withdrawn and assayed for nitrogenase activity under microaerobic conditions for 0.5 min (UB1 and UB32) and for 2 min (UB4 and UB36). Initial activity was the same as in the experiments in Fig. 1 and 3. Similar results with UB1 and UB4 have been published in our previous paper (37).

conditions. A similar pattern was seen when ADP-ribosylation of dinitrogenase reductase was examined directly in these strains (Fig. 2B); only slightly less modified dinitrogenase reductase was detected in UB32 and UB33 under these conditions. These results show that anaerobic switch-off is approximately normal in these *ntrBC* mutants.

Anaerobic switch-off of nitrogenase activity in UB35 (*ntrB draG*) and UB36 (*ntrC draG*) was examined and compared with that in UB4 (*draG*) (Fig. 6B). The general pattern of response was very similar, although the residual nitrogenase activity is slightly lower in these mutants than in UB4 (*ntr*$^+$ *draG*) under anaerobic conditions. All mutants lacking DRAG failed to recover nitrogenase activity after a return to microaerobic conditions. The modification of dinitrogenase reductase in UB36 was correlated with the loss of nitrogenase activity (Fig. 4). These results indicate that the regulatory response to anaerobiosis is relatively unaffected in *ntrBC* mutants and that the altered response to $NH_4Cl$ does not result from differential accumulation of DRAG and DRAT in *ntr* strains but, rather, from altered regulation of their activity in response to $NH_4^+$ stimuli.

**$NH_4^+$ and anaerobicity have competing effects on DRAG and therefore do not share the same signal transduction pathway.** In a previous study (37), the effect of successive treatments with anaerobicity and $NH_4^+$ on nitrogenase activity in UB1 (wild type) was demonstrated, and we show a similar result in Fig. 7. The further decrease in nitrogenase activity caused by $NH_4^+$ (following anaerobicity) shows that DRAT activity only has been transiently stimulated by the anaerobic shift. The earlier paper did not comment on the fact that a significant level of residual nitrogenase activity was still detectable in UB1 after the second stimulus, even though $NH_4^+$ by itself (under microaerobic conditions) causes a complete loss of nitrogenase activity (Fig. 1 and 2). This leaves the paradox that a portion of the dinitrogenase reductase must be activated during the (microaerobic) nitrogenase assay itself, even though DRAG should have been inactivated by the preceding anaerobic treatment and should be further inactivated in response to $NH_4^+$.

We explain this paradox by hypothesizing that, because the cells must be shifted from anaerobic to microaerobic conditions for the nitrogenase assay, DRAG is being activated by this shift and is then activating nitrogenase during the actual nitrogenase assay. This residual nitrogenase activity was not detected in $NH_4^+$-treated microaerobic cells under the same assay conditions (see, e.g., Fig. 1 and 2), presumably because

the cells were already microaerobic and therefore no microaerobic shift was necessary to support the nitrogenase assay. The implication is therefore that DRAG is being activated by a return to microaerobicity, even though the cells are still in the presence of excess $NH_4^+$, suggesting a competition between the two signals. Appropriately, this residual activity is absent in the isogenic *draG* mutant (UB4) (Fig. 7).

A similar but even more striking difference is seen with UB32 (*ntrC*) and its isogenic *draG* derivative, UB36 (Fig. 7). As above, the significant level of residual nitrogenase activity reflects the ability of DRAG to become active and demodify dinitrogenase reductase even in the presence of $NH_4^+$.

The difference seen between the responses of UB1 (wild type) and UB32 (*ntrC*) to the double stimuli (Fig. 7) are consistent with the above hypothesis and with the view that the predominant effect of the *ntr* mutations is on signal transduction to DRAG. The difference in detected activity cannot reflect differences in DRAG or DRAT response to microaerobicity, since the data in Fig. 2 and 6A show that the two strains respond similarly. Differential DRAT activity is probably also not the cause of the difference, again because the *ntrC* mutation appears to have little effect on DRAT in response to both $NH_4^+$ (Fig. 3 and 4) and anaerobic conditions (Fig. 4 and 6B). Instead, the difference apparently lies in the ability of the existing $NH_4^+$ signal to counteract the effect of microaerobicity on DRAG; the *ntrC* mutation appears to alter this $NH_4^+$ signal in some way, making it less effective at keeping DRAG inactive. We suggest, then, that while the DRAG in both strains is similarly activated to some extent by the microaerobic shift, the continued presence of $NH_4^+$ either rapidly reduces or partially reverses this activation in the wild type. In the *ntr* mutant, the impaired ability to process the "$NH_4^+$ signal" caused a slower inactivation of DRAG activity by $NH_4^+$, resulting in a higher nitrogenase residual activity.

This model is consistent with the results obtained with the direct assay of the ADP-ribosylation of dinitrogenase reductase by using Western immunoblot analysis. Unlike the nitrogenase assay, this assay freezes the ADP-ribosylation state of dinitrogenase reductase, avoiding demodification during the assay. In this assay, all these strains showed a complete modification of dinitrogenase reductase after anaerobic and $NH_4^+$ treatment (data not shown), indicating that the residual activity results from reactivation during the assay.

**Other effects of *ntrBC* mutations.** The GS activity in both the wild type (UB1) and *ntrBC* mutants was measured as described in Materials and Methods. The GS activities (micromoles of glutamyl hydroxamate per minute per milligram of protein) were as follows: UR32 (*ntrC*), 1.2; UB33 (*ntrB*), 1.1; and UB1 (wild type), 2.9.

It was reported previously that L-methionine-D-sulfoximine (MSX), an inhibitor of GS, could significantly block $NH_4^+$ switch-off of nitrogenase activity in both *A. brasilense* (13) and *R. rubrum* (14). The effect of MSX under our experimental conditions is shown in Fig. 8. After treatment with 1 mM MSX, UB1 (wild type) showed only partial loss of nitrogenase activity after $NH_4^+$ addition, reminiscent of the response of the *ntrBC* mutants to $NH_4^+$ addition. UB1 treated with a low level of MSX, such as 0.1 or 0.5 mM, showed lower residual activity (data not shown). Addition of 0.5 mM glutamine to cultures of all three strains, derepressed on NfbHP-glutamate medium, had no significant effect on nitrogenase activity (data not shown).

As reported previously, *A. brasilense* wild type (UB1) can use nitrate to support nitrogenase activity under anaerobic conditions, resulting in the demodification of dinitrogenase reductase after nitrate addition (1, 23, 37). We also have found that

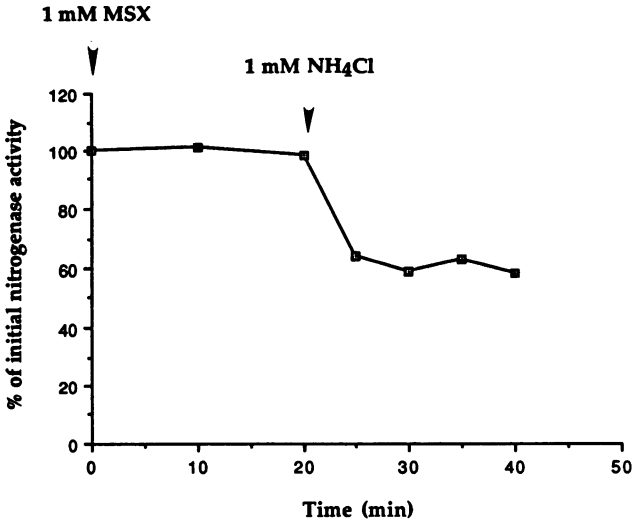


FIG. 8. Effect of MSX on $NH_4^+$ switch-off of nitrogenase activity in UB1 (wild type). At $t = 0$, MSX was added to a final concentration of 1 mM, and at 20 min, $NH_4Cl$ was added to a final concentration of 1 mM. Aliquots of the cultures (1 ml) were withdrawn and assayed for nitrogenase activity for 2 min at the times indicated. The initial nitrogenase activity of UB1 was the same as in the experiment in Fig. 1.

*A. brasilense ntrBC* mutants can use nitrate as an electron acceptor to support nitrogenase activity under anaerobic conditions. UB32 (*ntrC*) and UB33 (*ntrB*) were derepressed for nitrogenase under microaerobic conditions, the cultures were made anaerobic, and 20 mM $NaNO_3$ was added. Nitrogenase activity recovered in these mutants just as in the wild type (data not shown). These results suggest that dissimilatory nitrate reductase is functional in these mutants and confirm the previous report (19).

## DISCUSSION

**Role of *ntrBC* in nitrogenase regulation.** Although the general function of DRAT and DRAG in the regulation of nitrogenase activity in *A. brasilense* has been characterized, the mechanisms for the regulation of DRAT and DRAG activities are still unknown. Several regulatory *A. brasilense* mutants, which showed high nitrogenase activity in the presence of $NH_4^+$, were isolated. Some of these mutants lacked GS activity (11), and some had effects on histidine transport (9) or $NH_4^+$ uptake and excretion (22). Unfortunately, neither the nature of these mutations nor their effects on the DRAT/DRAG system were determined.

Recently, Liang et al. (19) reported the isolation of the *ntrBC* region of *A. brasilense* and characterized the effects of Tn*5* insertions in each of these genes. Surprisingly, the transcriptional regulation of the *nif* system seemed to be normal, indicating that these particular *ntrBC* homologs are not critical for *nif* derepression as seen in other organisms, such as in *K. pneumoniae* and *Rhodobacter capsulatus* (7, 16, 36). In contrast, the regulation of nitrogenase activity by $NH_4^+$ was altered in these mutants, with $NH_4^+$ addition showing less of an effect than in the wild type.

In this paper, we have shown that the effect of *ntrBC* mutations on the response to $NH_4^+$ addition is due to changes in the DRAT/DRAG regulatory system. The normal response of *ntrBC* mutants to anaerobic conditions suggests that the

levels of DRAG and DRAT proteins are approximately normal, and therefore the effect of the *ntr* mutations on $NH_4^+$ regulation reflects an alteration in the regulation of DRAG and/or DRAT activities. However, we cannot rule out small effects of the *ntr* mutations on DRAG and DRAT levels because of the difficulty in accurate quantitation of these very scarce proteins. It also indicates that this *ntrBC* locus is involved mainly in the response of the cell to fixed nitrogen.

The comparison of the response to $NH_4^+$ of *ntrB/C draG* mutants and a *draG* mutant allows us to focus only on *ntr* effects on DRAT, since these strains lack DRAG. Their similar response to $NH_4^+$ indicates that DRAT is functioning similarly to regulate nitrogenase activity in these strains and therefore that its regulation is not substantially affected by the *ntr* mutations. The large effect of *ntrBC* mutations on the response of *dra*$^+$ strains to $NH_4^+$ is therefore apparently due to changes in the regulation of DRAG activity.

We cannot rule out some effect of the *ntr* mutations on $NH_4^+$ transport, but we believe that this is not likely to be the primary mechanism of the *ntr* effects. We base this on the normal response of the *ntr draG* double mutants to $NH_4^+$, since these strains should have the same $NH_4^+$ transport as the *ntr* strain but without the complication of DRAG. A strong effect of the degree of aerobicity on $NH_4^+$ uptake is also unlikely, because we have previously shown that $NH_4^+$ addition also causes switch-off of nitrogenase activity in anaerobic cultures (37).

**Candidates for signal molecules for the DRAT/DRAG regulatory system.** Although several attempts have been made to correlate the modification of dinitrogenase reductase with changes in the pools of small molecules (15, 17, 24, 25, 32), the signals for the regulation of the DRAT/DRAG system are still unknown.

The ability of MSX, an inhibitor of GS, to block $NH_4^+$ switch-off of nitrogenase activity suggests that glutamine or a metabolite, rather than $NH_4^+$ itself, is the direct signal for the DRAT/DRAG system. However, while the intracellular glutamine concentration in *R. rubrum* does rise rapidly after $NH_4^+$ treatment, there is little change in response to other DRAG/DRAT stimuli such as darkness (15, 17). Hartmann and Burris also reported that the glutamine pool changed during $NH_4^+$ switch-off but not during anaerobic treatments in *A. brasilense* (12). All these data suggest that, while glutamine or a metabolite may play a role in $NH_4^+$ switch-off, darkness (in *R. rubrum*) or anaerobic switch-off (in *A. brasilense*) must have a different signal.

The effect of mutations in *ntrBC* on $NH_4^+$ switch-off in *A. brasilense* can be rationalized through an effect on GS. The GS activity in both *ntrB* and *ntrC* mutants is significantly lower than that in the wild type, and this might result in a delay in processing $NH_4^+$ to the actual signal molecule. In turn, this delay could explain the reduced effect of $NH_4^+$ addition on DRAG in these mutants.

The reason for the *ntr* effect on GS levels is unclear. Although one promoter of the *glnB glnA* operon of *A. brasilense* seems to be $\sigma^{54}$ dependent (4) and therefore mutations in *ntrBC* could reduce its transcription, direct *lacZ* fusion studies showed no significant effect of *ntrBC* mutations on *glnA* expression (5). The basis for the effect of *ntrBC* mutations on GS activity must be investigated further.

The result that mutations in *ntrBC* of *A. brasilense* strongly affect $NH_4^+$ switch-off of nitrogenase activity but only slightly affect anaerobic switch-off is consistent with the model that suggests independent (or branched) pathways for the different signals. Whether or not the two stimuli use different signal pathways, we explain the different degrees of effect in response to these stimuli by hypothesizing that a high concentration of $NH_4^+$ sends a stronger or more lasting signal than does anaerobicity and that this allows DRAT to cause virtually complete modification of dinitrogenase reductase.

While mutations in *ntrBC* show significant effects on the regulation of DRAG, they show rather less effect on the regulation of DRAT. This suggests that activities of DRAG and DRAT are not regulated in a completely coordinated but inverse manner; this is consistent with previous observations (37).

Another candidate for an effector that might mediate both $NH_4^+$ and anaerobic switch-off could be the NAD level in the cell. Because NAD, and not NADH, is the substrate for DRAT and the source of the ADP-ribose for the modification of dinitrogenase reductase, a change in that pool might be the basis for the observed changes in DRAT activity in vivo. The NAD would be expected to be reduced to NADH under anaerobic conditions. The partial relief of that reduction upon $NH_4^+$ addition might be due to the synthesis of glutamate from glutamine and α-ketoglutarate by glutamate synthase, producing NAD from NADH. It has also been reported that exogenous NAD can partially inhibit nitrogenase activity in *R. rubrum* (32). However, the direct assay of intracellular NAD and NADH pools during $NH_4^+$ switch-off shows little change and therefore does not appear to support this hypothesis (24).

**Effect of competing signals on the DRAT/DRAG system.** Perhaps the most surprising result of this work is the response of strains to "activating" signals such as microaerobicity when still in the presence of a negative stimulus such as $NH_4^+$. This response suggests that the cells are not integrating these signals, because logically the presence of $NH_4^+$ should cause a complete loss of nitrogenase activity. Rather, it suggests that these different environmental signals use independent pathways to affect the DRAG/DRAT system and that the cell is affected predominantly by the most recent signal.

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