The nitrogenase activity of several diazotrophs is reversibly inhibited in vivo by addition of ammonium ions. The activity is fully recovered as the NH₄⁺ is consumed. This phenomenon is called nitrogenase switch-off/switch-on (27). In *Azospirillum brasilense*, *Azospirillum lipofermum* (9), *Rhodospirillum rubrum* (13, 14), and *Rhodobacter capsulatus* (12), nitrogenase switch-off upon addition of NH₄⁺ is due to the inactivation of the homodimeric iron protein (14, 25) by the transfer of an ADP-ribosyl residue from NAD⁺ to Arg-101 of one of the subunits, catalyzed by dinitrogenase reductase ADP-ribosyl transferase (DRAT) (14, 26). The activity of the iron protein is recovered by the removal of the ADP ribosyl moiety by dinitrogenase reductase-activating glycohydrolase (DRAG). In addition to NH₄⁺, the DRAT-DRAG system responds to fluctuations in cellular energy (26). The iron protein from *A. brasilense* is ADP-ribosylated under anaerobic conditions, and that of *R. capsulatus* and *R. rubrum* is ADP-ribosylated upon transfer to the dark (10, 13, 14). ADP-ribosylation has also been described in *Azotobacter chroococcum* (16) and accounts for a fraction of nitrogenase switch-off in this organism by addition of ammonium.

The enzymes DRAT and DRAG are coded by the *draTG* genes, which are constitutively expressed in *A. brasilense* (23, 26). The activities of DRAT and DRAG, on the other hand, are controlled by the presence of external stimuli, but the signaling pathways have not yet been clearly defined. Guanosine is capable of provoking nitrogenase switch-off (6, 10). Addition of L-methione-DL-sulfoximine (MSX), a specific glutamine synthetase inhibitor, to derepressed cells prevented nitrogenase switch-off by ammonium, suggesting that glutamine synthetase or glutamine may be part of the signal pathway to DRAT and DRAG (4, 10, 13, 26). Moreover, *ntrC* mutants of *A. brasilense* and *R. rubrum* are incapable of ADP-ribosylation (17, 20, 25), suggesting that NtrC may participate in a signaling circuit including DRAT/DRAG, possibly to regulate expression of a signal protein. The PII protein and its parologue, GlnZ in *A. brasilense*, are candidates for such signal proteins.

The glnZ mutant of *Azospirillum brasilense* (strain 7611) showed only partial recovery (20 to 40%) after 80 min of ammonia-induced nitrogenase switch-off, whereas the wild type recovered totally within 10 min. In contrast, the two strains showed identical anoxic-induced switch-on/switch-off, indicating no cross talk between the two reactivation mechanisms.

The glnZ gene of *Azospirillum brasilense* (2) but not that of *glnB* (3). Further evidence suggested that the expression of *glnB* is dependent on the ammonium levels, which points to a novel regulatory system responsive to NH₄⁺ (3). The data suggest a probable link between NtrC and *A. brasilense* nitrogenase switch-off through the activity of GlnZ. Here we analyze the role of a mutation in the *glnZ* gene in the control of nitrogenase activity in *A. brasilense*.

The *A. brasilense* strains FP2 (wild type) (17), 7611 (*glnZ::Ω*), (2), 7611 pJ11 (*glnZ::Ω* carrying the plasmid-borne wild-type *A. brasilense* *glnZ* gene *Te*') were grown in liquid NFBHPN medium (15) at 30°C at 120 rpm. Plasmid pJ11 was constructed by cloning a 0.37-kbp *SacII*-HindIII DNA fragment containing the *A. brasilense* glnZ gene into the vector pLAFR3.18. Plasmid pJ11 carried the glnZ gene from the lacZ promoter in all *A. brasilense* strains. For nitrogenase switch-off/switch-on, *A. brasilense* cells grown in NFBHPN medium were harvested by centrifugation at 5,000 × g for 5 min, resuspended in N-free NFBHP medium (optical density at 600 nm of 1.2; 8 ml), and derepressed for nitrogenase activity in 25-ml flasks for 4 h at 30°C. After this period nitrogenase activity was determined by the acetylene reduction method (18). Protein was determined as described previously (1).

Addition of ammonium chloride (0.2 mmol/liter) to derepressed *A. brasilense* FP2 (wild-type) cultures caused almost complete inhibition of nitrogenase activity (Fig. 1A). The activity was fully recovered after approximately 10 min, following exhaustion of ammonium ions from the medium (Fig. 1A). These effects correlate with ADP-ribosylation of dinitrogenase reductase (10, 24, 27). As in the wild-type strain, the *A. brasilense* strain 7611 (*glnZ* mutant) was fully derepressed for nitrogenase activity, and the addition of ammonium ions caused a similar level of inhibition (Fig. 1B). However, recovery of the initial nitrogenase activity was partial (20 to 40%) even 80 min after ammonium addition. Addition of the protein synthesis inhibitors chloramphenicol or tetracycline prior to the addition of ammonium had no effect on the control of nitrogenase activity (not shown). Ammonium ions added were completely taken up after 10 min both in the wild type and in *glnZ* mutant strains (Fig. 1D). *A. brasilense* has two ammonium uptake systems: a high-affinity uptake for transporting ammonium and methylammonium and a low-affinity uptake specific...
The results presented here showed that the overall ammonium uptake rates were very similar in the glnZ mutant and in the wild-type strains under our conditions and were not affected by the glnZ mutation. Apparently the low-affinity ammonium uptake system can efficiently uptake ammonium under the conditions employed in this work.

The results suggest that the GlnZ protein is involved in the mechanism of reactivation of the ADP-ribosylated iron protein but is not essential for nitrogenase switch-off. Furthermore, complementation of the nitrogenase activity control by ammonium in the glnZ mutant by constitutively expressed A. brasilense glnZ gene strongly supports this role for the GlnZ protein (Fig. 1C).

Nitrogenase switch-off also occurs in response to anaerobic conditions in A. brasilense (9). Nitrogenase activity of derepressed cultures of the FP2 (wild-type) and the 7611 (glnZ mutant) strains was inactivated when maintained static for 10 min to deplete oxygen, but nitrogenase activity was recovered immediately upon shaking to restore the rate of oxygenation.

This result indicates that GlnZ is involved neither in the mechanism of anaerobic inactivation nor in the aerobic reactivation of nitrogenase (Fig. 2).

In A. brasilense under nitrogen-fixing conditions (low ammonium and microaerobiosis), DRAT is inactive and DRAG is active (26). Upon the stimulus (increase of NH₄⁺ or anaerobiosis), DRAT is temporarily activated and DRAG is inactivated, resulting in a rapid increase in the ADP-ribosylated iron protein. Ten to 15 minutes after the stimulus, the transferase is inactivated, and the glycosidase is reactivated either after consumption of ammonium or on return to microaerobiosis, followed by recovery of nitrogenase activity (26, 25). The mechanisms through which the activities of DRAG and DRAT are controlled are not yet clear. Both enzymes are fully active in cell extracts or when purified, suggesting that the potential effectors are probably loosely bound and lost during in vitro manipulation (23). Effectors such as energy charge, pyridine nucleotides, and amino acids have been suggested, but neither a small molecule nor a protein has been identified. Here we show that the GlnZ protein is required for nitrogenase
switch-on in *A. brasilense*. Since DRAT inactivation seems to occur even in the presence of a persistent stimulus (26), it is likely that GlnZ is required for DRAG reactivation in *A. brasilense*. The PII protein, a paralogue of GlnZ, has been reported to be involved in the reversible inactivation of nitrogenase. Hallenbeck (8) showed that a nitrogen fixation-constitutive glnB mutant of *R. capsulatus* was deficient in nitrogenase switch-off by ammonium. Johansson and Nordlund (11) noted a correlation between the uridylylation state of PII in *R. rubrum* and nitrogenase switch-off by NH₄⁺/H₂. A Y51F mutant in the glnB gene of *R. rubrum* partially abolished the negative regulation of DRAT (22). In addition, in the *Klebsiella pneumoniae* heterologous background, lack of glnB and glnK alters the activity of the DRAT and DRAG from *R. rubrum*, reinforcing the role of PII-like proteins in the control of both enzymes (21), although a glnK mutant of *R. rubrum* was not affected in switch-off/switch-on (7). It is possible, therefore, that the PII protein is also involved in the signal pathway leading to the reversible ADP-ribosylation of the iron protein in *A. brasilense*.

*ntrBC* mutants of *A. brasilense* (17, 24) and *R. rubrum* (20) failed to inactivate the iron protein by ADP-ribosylation upon addition of NH₄⁺ but did switch off under anaerobiosis and in darkness, respectively. These results suggested that the ammonium- and energy-triggered pathways for nitrogenase activity control are different. In agreement with this suggestion, activation of DRAT by NH₄⁺ required active glutamine synthetase and led to an increase in the levels of glutamine; anaerobiosis, in contrast, failed to alter glutamine concentrations (5, 26). Likewise, there seem to be two separate signal pathways to restore the activity of the iron protein since the pattern of the switch-off/switch-on of the glnZ mutant under anaerobiosis was the same as that of the wild-type strain (Fig. 2). To determine if reactivation of the nitrogenase iron protein by microaeration could override persistent ammonium switch-off in the glnZ mutant, derepressed cultures were inactivated by anaerobiosis followed by the addition 0.2 mmol of NH₄Cl per liter. No increase in the extent of reactivation was observed (not shown), suggesting the absence of cross talk between the signal pathways.

The only clear phenotype so far ascribed to glnZ mutants of *A. brasilense* was an increase in methylammonium uptake rate, whereas overexpression of glnZ depressed it (2). Here we show that the GlnZ protein is required for reactivation of nitrogenase following ammonium switch-off and also that the signal pathways for ADP-ribosylation and removal of ADP-ribosylation are different.

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