

ADP-ribosylation of dinitrogenase reductase in *Azospirillum brasilense* is regulated by AmtB-dependent membrane sequestration of DraG

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Summary

Nitrogen fixation in some diazotrophic bacteria is regulated by mono-ADP-ribosylation of dinitrogenase reductase (NifH) that occurs in response to addition of ammonium to the extracellular medium. This process is mediated by dinitrogenase reductase ADP-ribosyltransferase (DraT) and reversed by dinitrogenase reductase glycohydrolase (DraG), but the means by which the activities of these enzymes are regulated are unknown. We have investigated the role of the P_{II} proteins (GlnB and GlnZ), the ammonia channel protein AmtB and the cellular localization of DraG in the regulation of the NifH-modification process in *Azospirillum brasilense*. GlnB, GlnZ and DraG were all membrane-associated after an ammonium shock, and both this membrane sequestration and ADP-ribosylation of NifH were defective in an *amtB* mutant. We now propose a model in which membrane association of DraG after an ammonium shock creates a physical separation from its cytoplasmic substrate NifH thereby inhibiting ADP-ribosyl-removal. Our observations identify a novel role for an ammonia channel (Amt) protein in the regulation of bacterial nitrogen metabolism by mediating membrane sequestration of a protein other than a P_{II} family member. They also suggest a model for control of ADP-ribosylation that is likely to be applicable to all diazotrophs that exhibit such post-translational regulation of nitrogenase.

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Introduction

Biological nitrogen fixation is catalysed by the nitrogenase enzyme, which consists of two proteins: dinitrogenase (NifDK) and dinitrogenase reductase (NifH). Dinitrogenase reductase is responsible for electron transfer to dinitrogenase, which contains the site of N₂ reduction. This process is very energy-demanding and is thus tightly regulated at the transcriptional level and in some organisms also at the post-translational level. Reversible post-translational modification of nitrogenase, also known as nitrogenase switch-off/switch-on, has been best characterized in the alpha-proteobacteria *Rhodobacter capsulatus*, *Rhodospirillum rubrum* and *Azospirillum brasilense*. There is, however, evidence to suggest that the process is also present in members of gamma, beta and delta proteobacteria (Nordlund, 2000; Pallen *et al.*, 2001).

In response to conditions unfavourable for nitrogenase activity, such as addition of ammonium to the medium, an ADP-ribose group from NAD⁺ is attached to an arginine residue in one subunit of the homodimeric NifH protein resulting in NifH inactivation (switch-off). This process is catalysed by dinitrogenase ADP-ribosyltransferase (DraT). When the added ammonium is exhausted by cellular metabolism, the ADP-ribose group is removed by dinitrogenase reductase activating glycohydrolase (DraG) leading to NifH activation (switch-on). NifH can also be modified in photosynthetic bacteria in response to darkness and in *A. brasilense* when cells are subjected to anaerobiosis (Zhang *et al.*, 1997; Nordlund, 2000).

Several lines of evidence indicate that the activities of both DraT and DraG are regulated *in vivo* but the regulatory factors involved remain largely unknown. The current model suggests that when nitrogen fixation is ongoing DraT is inactive and DraG is active. Upon energy depletion or ammonium addition, DraG is inactivated and DraT is transiently activated leading to modification and inactivation of NifH. DraG is reactivated as the negative stimulus is removed, causing NifH activation (Zhang *et al.*, 1997; Nordlund, 2000). As the signal for nitrogenase ADP-ribosylation is related to nitrogen metabolism (ammonium addition) or the energy/redox status (darkness/anaerobiosis), it seems likely that the initial parts of the pathways are different but it is not known if or where they merge

before affecting DraT/DraG activities (Nordlund, 2000). Despite the fact that the factors controlling DraT/DraG are not defined, it is very likely that they are similar in different organisms. This hypothesis is supported by data showing that heterologous expression of *A. brasilense draTG* in an *R. rubrum* mutant lacking DraT and/or DraG, and *vice versa*, restores switch-off control in the mutant (Zhang *et al.*, 1995).

Both DraT and DraG are active in cell extracts or when purified, leading to the hypothesis that these enzymes may be controlled by binding of labile or loosely bound negative regulators (Zhang *et al.*, 1997). Likewise over-expression of *R. rubrum* DraG and *A. brasilense* DraT and DraG leads to constitutively active enzymes suggesting titration of negative regulators (Grunwald *et al.*, 1995; Huergo *et al.*, 2005). The DraG proteins from *A. brasilense* and *R. rubrum* have been found to be membrane-associated (Nordlund *et al.*, 1977; Ljungstrom *et al.*, 1989). Moreover, some *R. rubrum* DraG variants with an altered regulation also show altered membrane affinity, suggesting that membrane association may be a possible mechanism of DraG regulation (Kim *et al.*, 2004).

Proteins of the nitrogen signal transduction P_{II} family and of the ammonia channel Amt family have been implicated in the regulation of DraT and DraG activities in several organisms. *R. rubrum* encodes three P_{II} proteins (GlnB, GlnJ and GlnK) and in the absence of GlnB and GlnJ there is no modification of *R. rubrum* NifH in response to ammonium or darkness (Zhang *et al.*, 2001). Similarly in *R. capsulatus*, a *glnB*, *glnK* double mutant does not modify NifH in response to ammonium, although it is not clear if this phenotype is due to the absence of GlnK or a polar effect on AmtB expression (Drepper *et al.*, 2003). In *R. capsulatus*, the presence of AmtB is also necessary for ammonium-induced but not for darkness-induced ADP-ribosylation (Yakunin and Hallenbeck, 2002). In *Azoarcus* sp., ammonium-induced NifH modification requires GlnB, GlnK and AmtB whilst the anaerobic response requires GlnB or GlnK (Martin and Reinhold-Hurek, 2002). In *A. brasilense*, GlnZ was shown to be involved in the reactivation of NifH in response to ammonium (Klassen *et al.*, 2001) and GlnB has been proposed as a candidate for triggering DraT activity in response to ammonium (L. F. Huergo *et al.*, unpublished; Klassen *et al.*, 2005). Interaction of *R. capsulatus* DraT with either GlnB or GlnK has also been shown using a yeast two-hybrid system (Pawlowski *et al.*, 2003). However, despite the P_{II} and Amt proteins being consistently implicated in regulation of NifH modification, there has so far been no conclusive demonstration of how this regulation takes place.

Using Western blot analysis we have studied ADP-ribosylation of NifH in *A. brasilense* in response to ammonium addition. Our results show that GlnB, GlnZ and DraG

reversibly associate with the membrane according to the cellular nitrogen status in an AmtB-dependent manner. These findings suggest a model for the regulation of DraG activity in which an ammonium shock causes DraG to associate with the membrane creating a physical separation from its cytoplasmic substrate ADP-ribosylated NifH and thereby inhibiting the ADP-ribosyl-removing process.

Results

NifH modification in response to ammonium addition is synchronized with GlnB/GlnZ deuridylylation and membrane association

In order to address the possible role of the P_{II} proteins GlnZ and GlnB in the ammonium-induced NifH ADP-ribosylation in *A. brasilense*, we followed the NifH, glutamine synthetase (GS) and P_{II} protein modification cycles in response to ammonium addition in wild-type and P_{II} mutant strains. The only way to assess DraT and DraG activities *in vivo* is indirect, by assessing the modification status of NifH and we therefore decided to examine whether the modification profile of NifH correlates with uridylylation/deuridylylation of GlnB and GlnZ in *A. brasilense* after an ammonium shock. *A. brasilense* cells derepressed for nitrogenase were subjected to a shock of 200 μ M NH₄Cl, samples were taken before and after ammonium addition and immediately frozen in liquid nitrogen. The samples were analysed for NifH and GS modification by SDS-PAGE and for GlnB and GlnZ modification by native PAGE (Fig. 1A–D).

In many bacteria, including *A. brasilense*, the dodecameric GS enzyme is also modified in response to extracellular ammonium addition, usually by adenylation of a tyrosine residue on each subunit. This leads to progressive inactivation of GS as the intracellular nitrogen status rises and consequently GS modification provides an independent indicator of the intracellular response to ammonium addition and should be directly related to the internal glutamine pool. GS modification in *A. brasilense* differs from that in *Escherichia coli* where GlnB plays a role in regulating the activity of adenylyltransferase (ATase). In *A. brasilense*, neither GlnB nor GlnZ appears to influence GS modification significantly (de Zamaroczy, 1998), suggesting that ATase may be regulated solely by the allosteric effects of glutamine binding which is also known to occur in *E. coli* (Jiang *et al.*, 1998).

The modifications of both NifH and GS were synchronized after the ammonium shock (Fig. 1A and B). Modified forms of both proteins could be observed 2 min after ammonium addition and the modifying group was removed after 15 min, by which time the added ammonium will have been exhausted by cellular metabolism (Klassen *et al.*, 2001). Hence NifH modification appears to correlate with an increase in intracellular glutamine

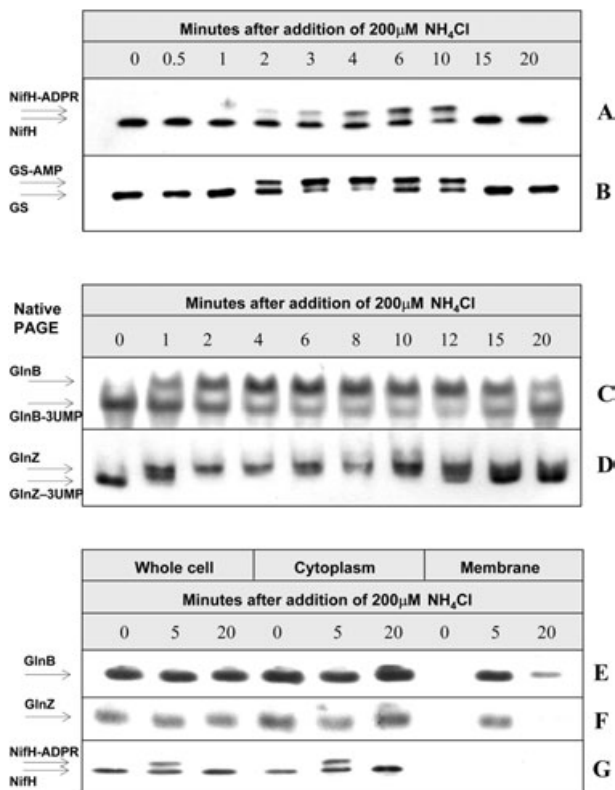


Fig. 1. Modification profile of NifH, GS, GlnB and GlnZ after an ammonium shock in wild-type *A. brasilense* cells. Wild-type cells (FP2) derepressed for nitrogenase were subjected to an ammonium shock (200 μ M NH_4Cl) at time zero. Samples were collected, processed and analysed by Western blotting at the time point indicated. Cell extracts were subjected to SDS-PAGE followed by Western blotting using anti-NifH antibody (A) or anti-GS antibody (B). Whole cell extracts were subjected to Native PAGE followed by Western blotting with anti-GlnB antibody (C) or anti-GlnZ antibody (D). Whole cell extracts, cytoplasmic and membrane fractions were subjected to SDS-PAGE followed by Western blotting with anti-GlnB antibody (E), anti-GlnZ antibody (F) or anti-NifH antibody (G).

levels in agreement with previous results (Hartmann and Burris, 1987).

Given the trimeric nature of P_{II} proteins, GlnB and GlnZ can exist in four different forms according to their uridylylation status (0, 1, 2 and 3 UMP) with the fully uridylylated form showing the highest mobility on native gels (Araujo *et al.*, 2004). Not all four forms of GlnB or GlnZ were resolved on Native PAGE gels but we consider that both proteins are most likely in the 3-UMP form before the ammonium shock and that the most slowly migrating band observed after ammonium addition represents the 0-UMP form (Fig. 1C and D). Indeed when a much higher ammonium shock was used (20 mM NH_4Cl for 20 min), the migration of GlnB and GlnZ was comparable with the upper bands in Fig. 1C and D respectively (data not shown). The profiles of GlnB and GlnZ after the ammonium shock were quite different (Fig. 1C and D). Deuridylylation of both proteins started within 1 min of ammonium

addition and was complete for GlnZ after 2 min, whereas for GlnB deuridylylation was still not complete after 12 min and some putative GlnB 3-UMP was present at all the time points. Re-uridylylation of GlnZ started after 12 min and was complete by 20 min whereas GlnB re-uridylylation started only after 15 min and there was still a detectable level of GlnB 0-UMP after 20 min.

In *E. coli* both P_{II} proteins, GlnB and GlnK, are sequestered to the membrane after an ammonium shock (Coutts *et al.*, 2002; Javelle *et al.*, 2004). In order to determine if this is also the case in *A. brasilense*, we monitored whole cell extracts, cytoplasmic and membrane fractions for the presence of GlnB and GlnZ before and after the ammonium shock with 200 μ M NH_4Cl (Fig. 1E and F). The purity of the membrane fractions was confirmed by Western blot with an antibody against NifH, which is only present in the cytoplasm (Fig. 1G). Neither GlnB nor GlnZ were membrane-associated before ammonium addition; however, 5 min after addition a proportion of both proteins was membrane-associated. After 20 min, when the internal glutamine pool is inferred to have fallen again as judged by the deadenylylated state of GS, only a small fraction of GlnB was still membrane-associated and no GlnZ signal was detected in the membrane fraction. In *E. coli* membrane association of GlnK reflects its uridylylation state and this also seems to be the case in *A. brasilense*. Comparison of the native profile and the membrane association status of GlnB and GlnZ (compare Fig. 1C with 1E and Fig. 1D with 1F) suggest that both proteins are membrane-associated only when they are in the 0-UMP form.

The results described so far clearly show that the process of NifH modification, and thus changes in DraT and DraG activities, are synchronized with the modification status of the P_{II} proteins GlnB and GlnZ as well with their changes in cellular location.

Effects of a glnB mutation on the NifH, GS and GlnZ modification cycles

In the absence of GlnB, NifH is not expressed because the *nif* transcriptional activator protein NifA is not active (de Zamaroczy *et al.*, 1993). However, it is possible to study the NifH modification process in a *glnB* mutant by introducing a plasmid (pEMS136) that expresses a constitutively active, N-truncated, form of NifA from *Herbaspirillum seropedicae* (Souza *et al.*, 1999). Using this approach the *A. brasilense* GlnB protein has been shown to be necessary for nitrogenase inactivation after ammonium addition because the *glnB::km* strain (7628) showed only partial nitrogenase switch-off after ammonium addition (Klassen *et al.*, 2005). In *A. brasilense*, the GS structural gene, *glnA*, is immediately downstream of *glnB* and under conditions of nitrogen limitation *glnA*

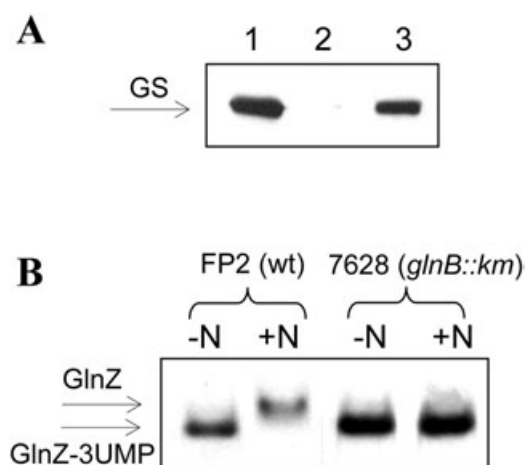


Fig. 2. Glutamine synthetase (GS) expression and GlnZ deuridylylation in *glnB* mutant strains.

A. Whole cell extracts prepared from nitrogen limited cells were subjected to SDS-PAGE followed by Western blotting with anti-GS antibody. Lane 1, wild-type strain FP2. Lane 2, strain 7628 (*glnB::km*). Lane 3, strain LFH3 (*glnB* non-polar mutant).

B. Whole cell extracts prepared from nitrogen-limited cells collected before (-N) or 20 min after (+N) the addition of 20 mM NH_4Cl . Samples were subjected to Native PAGE followed by Western blotting with anti-GlnZ antibody.

expression occurs mainly from the *glnB* promoter (de Zamaroczy *et al.*, 1990; 1993; Huergo *et al.*, 2003) and consequently an insertion mutation in *glnB* is expected to be polar on *glnA*. Indeed the GS activity of strain 7628 grown under nitrogen limitation was reported to be five times less than that observed in the wild-type (de Zamaroczy *et al.*, 1996), and we found that, by Western blotting, GS was almost undetectable in extracts from nitrogen limited cells of the *glnB::km* mutant (Fig. 2A). We therefore examined whether this reduction in GS expression affected deuridylylation of GlnZ in response to a high ammonium shock (20 mM NH_4Cl for 20 min). In the wild-type GlnZ became deuridylylated after the ammonium addition as judged by the slower migration rate under native conditions (Fig. 2B) but in the *glnB::km* mutant the GlnZ migration rate did not change after ammonium addition indicating that GlnZ deuridylylation is severely compromised in this strain (Fig. 2B). Our initial interpretation of this result was that the reduced GS levels in the *glnB::km* mutant are such that the intracellular glutamine pool does not increase even in the presence of high external ammonium concentrations, thus preventing GlnZ deuridylylation.

In order to avoid polar effects on *glnA* expression, a *glnB* non-polar deletion (LFH3) was constructed. Expression levels of GS in this mutant were very close to wild-type levels as judged by Western blotting using cell extracts from nitrogen-limited cells (Fig. 2A) and this mutant showed a wild-type GS adenylylation/deadenylation

response to ammonium addition (data not shown). However surprisingly, strain LFH3 also failed to deuridylylate GlnZ in response to a high ammonium shock (20 mM NH_4Cl for 20 min) (data not shown). The reason for this is presently unknown but it would appear that in *A. brasilense* GlnB is required for deuridylylation of GlnZ.

To further characterize the effects of the *glnB* mutation we followed NifH and GS modification in response to ammonium addition in strain LFH3 (pEMS136). This strain did not show any NifH modification after ammonium addition (Fig. 3A) whereas GS modification was still responsive to ammonium shock although it had a different profile from that observed in wild-type cells (compare Figs 1B and 3B). No deuridylylation (data not shown) or membrane sequestration of GlnZ was observed after ammonium addition in this strain (Fig. 3C). Introduction of the pJC1 plasmid, expressing *A. brasilense glnB*, into strain LFH3 restored a wild-type NifH and GS response (data

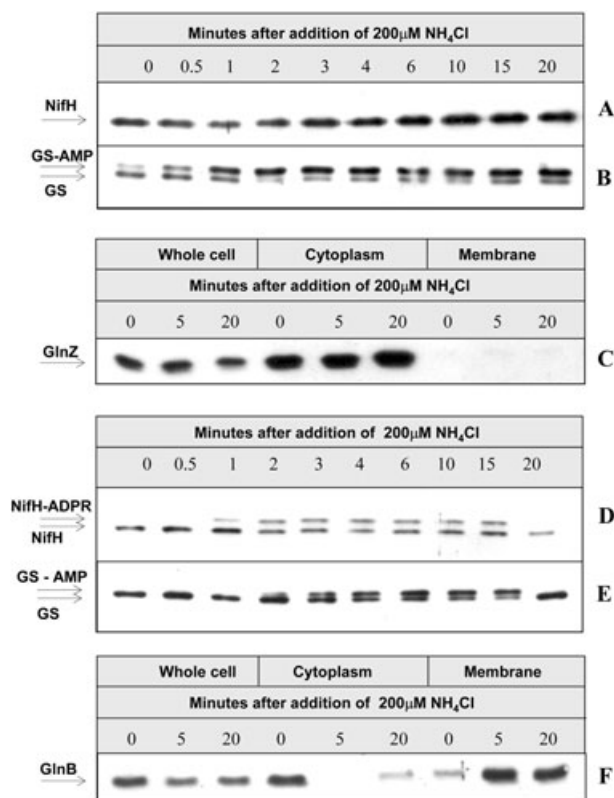


Fig. 3. Modification profile of NifH and GS in *A. brasilense*, *glnB* and *glnZ* mutant strains. Non-polar *glnB* mutant strain LFH3 (pems136) (panels A to C) or *glnZ* mutant strain 7611 (panels D to F), derepressed for nitrogenase were subjected to an ammonium shock (200 μM NH_4Cl) at time zero. Samples were collected, processed and analysed by Western blotting at the time point indicated. Cell extracts were subjected to SDS-PAGE followed by Western blotting using anti-NifH antibody (A and D) or anti-GS antibody (B and E). Whole cell extracts, cytoplasmic and membrane fractions were subjected to SDS-PAGE followed by Western blotting with anti-GlnZ antibody (C) or anti-GlnB antibody (F).

not shown) and the wild-type strain carrying pEMS136 showed normal NifH modification after ammonium addition (data not shown). The data reported here corroborate the observation (Klassen *et al.*, 2005) that GlnB is required for NifH modification after ammonium addition, but given the pleiotropic nature of the *glnB* mutation it is difficult to address whether this effect is direct or indirect.

No differences in the anaerobic induction of NifH modification were observed in the *glnB* mutant compared with the wild-type (data not shown).

Effects of a *glnZ* mutation on the NifH, GS and GlnB modification cycles

A previous report has shown that GlnZ is necessary for nitrogenase reactivation after an ammonium shock (Klassen *et al.*, 2001). To further characterize this effect we analysed the NifH, GS and GlnB modification profile in a *glnZ* mutant using the same set of conditions described for the wild-type strain.

The NifH modification rate was faster and lasted for longer in a *glnZ* mutant compared with the wild-type (compare Figs 1A and 3D). Modification of GS was also altered in the absence of GlnZ in that both adenylation and deadenylation occurred more slowly in the mutant (compare Figs 1B and 3E). This alteration in GS adenylation would be expected to affect the intracellular glutamine pool and could explain the changed rate of NifH ADP-ribosylation. In the *glnZ* mutant, GlnB was deuridylylated/re-uridylylated in a similar manner to the wild-type strain (data not shown) but the membrane association of GlnB was dramatically changed in comparison with the wild-type (compare Figs 1E and 3F). In the absence of GlnZ, GlnB was found in the membrane fraction even before ammonium addition; by 5 min there was no GlnB in the cytoplasmic fraction; and a large fraction of the GlnB pool was still membrane-associated after 20 min. Hence it would appear that GlnB has a significantly greater affinity for the membrane when GlnZ is absent.

No differences in the anaerobic induction of NifH modification were observed in the *glnZ* mutant compared with the wild-type (data not shown).

Azospirillum brasilense GlnB and GlnZ are membrane-sequestered in an AmtB-dependent manner

The almost invariant genetic linkage of *glnK* and *amtB* in prokaryotes led to the hypothesis that GlnK and AmtB physically interact (Thomas *et al.*, 2000) and it has since been shown that P_{II} proteins interact with the cell membrane in an AmtB-dependent manner in a variety of microorganisms (Coutts *et al.*, 2002; Detsch and Stulke, 2003; Strosser *et al.*, 2004). In *E. coli*, GlnK has been shown to interact directly with AmtB and this interaction, which only occurs when GlnK is in its deuridylylated form, leads to AmtB inactivation (Coutts *et al.*, 2002; Javelle *et al.*, 2004).

Azospirillum brasilense *amtB* is one of the few exceptions where the *amtB* gene is expressed as a monocistronic unit and is unlinked to a P_{II} gene (van Dommelen *et al.*, 1998). We therefore examined whether the ammonium shock-induced membrane sequestration of GlnB and GlnZ observed in wild-type *A. brasilense* (Fig. 1E and F) was AmtB-dependent and we found that this was indeed the case (Fig. 4). The *amtB* mutant could potentially have a slower ammonium uptake rate than wild-type, but this mutant has been shown by others to have ammonium uptake rates comparable with wild-type (van Dommelen *et al.*, 1998). Nevertheless we used a high (20 mM) ammonium shock in these experiments with the intention of ensuring that the ammonium flux into the cell was *amtB*-independent and indeed the shock caused comparable deuridylylation of GlnB and GlnZ in both the wild-type and the *amtB* mutant (Fig. 4).

NifH modification and nitrogenase inactivation in response to ammonium is dependent on AmtB

The ammonia channel proteins (AmtB) have been shown to be necessary for the ammonium-induced ADP-ribosylation of NifH in *R. capsulatus* and in *Azoarcus* sp. (Martin and Reinhold-Hurek, 2002; Yakunin and Hallenbeck, 2002). To examine whether AmtB is also involved in ADP-ribosylation of NifH in *A. brasilense*, we performed the same experiments described for the wild-type (Fig. 1)

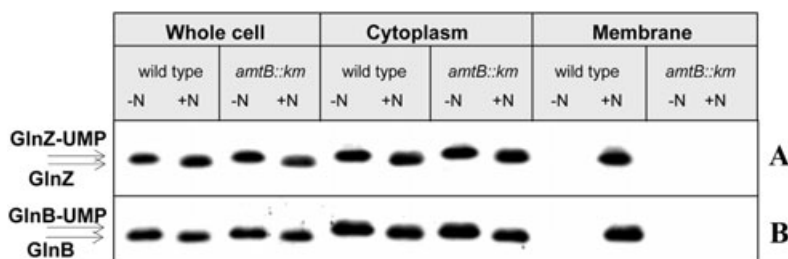


Fig. 4. GlnB and GlnZ are membrane-associated in an AmtB-dependent manner after an ammonium shock. Wild-type (FP2) or *amtB* mutant (FAJ310) cells derepressed for nitrogenase were collected before (–N) or 20 min after (+N) the addition of 20 mM NH₄Cl. Whole cell extracts, cytoplasmic and membrane fractions were subjected to SDS-PAGE followed by Western blotting with anti-GlnB antibody (A) and anti-GlnZ antibody (B). In contrast to Native PAGE (Fig. 1C and D), on SDS-PAGE the GlnB and GlnZ monomers migrate according to their mass so that the non-uridylylated form has the higher mobility.

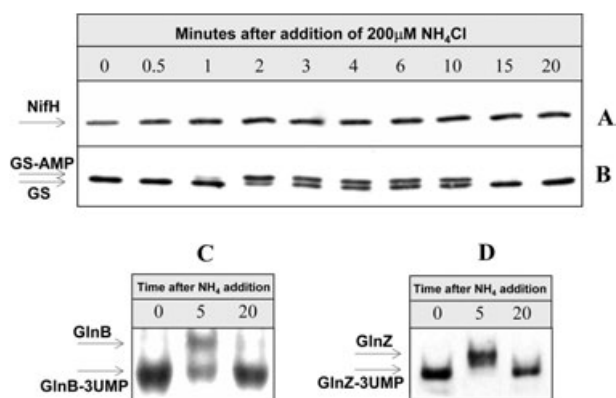


Fig. 5. Modification of NifH is impaired in *A. brasilense* FAJ310 (*amtB::km*). *A. brasilense* FAJ310 (*amtB::km*) cells derepressed for nitrogenase were subjected to an ammonium shock (200 μ M NH_4Cl) at time zero. Cell extracts were subjected to SDS-PAGE followed by Western blotting using anti-NifH antibody (A) or anti-GS antibody (B). Whole cell extracts were subjected to Native PAGE followed by Western blotting with anti-GlnB antibody (C) or anti-GlnZ antibody (D).

using *A. brasilense* strain FAJ310 (*amtB::km*). No modification of NifH was observed after the addition of 200 μ M NH_4Cl (Fig. 5A) and complementation of strain FAJ310 using a low copy number plasmid (pFAJ309) expressing *A. brasilense amtB* restored NifH modification (data not shown). After ammonium shock neither the GS modification profile nor the profile of GlnB and GlnZ deuridylylation were significantly altered in the absence of AmtB (compare Figs 1B and 3B and Fig. 1C and D with Fig. 3C and D). Hence we conclude that the absence of AmtB does not substantially change ammonium uptake under the conditions tested. As predicted from these data we found that addition of 20 mM NH_4Cl completely inhibited nitrogenase activity in the wild-type strain but not in the *amtB* mutant (Fig. 6). However, anaerobically induced NifH modification was similar in the wild-type and the *amtB* mutant (data not shown) corroborating a previous report that ammonium-induced and anaerobiosis-induced NifH modification use different signalling pathways (Zhang *et al.*, 1994).

DraG is sequestered to the membrane after an ammonium shock

As discussed so far it is clear that in *A. brasilense*, as in *R. capsulatus* and *Azoarcus* sp., the ammonia channel protein AmtB is completely necessary for ammonium-induced NifH modification. These facts reinforce the idea that AmtB acts as an ammonium sensor but how does AmtB transduce the signal of ammonium presence in the medium to alter the activities of the DraT and/or DraG enzymes? It has been postulated that a critical element for DraG regulation in *R. rubrum* might be through its membrane association, with membrane binding causing DraG inactivation (Noren and Nordlund, 1997); a sugges-

tion derived from the observation that *R. rubrum* DraG is found in the membrane fraction of cell extracts (Saari *et al.*, 1984).

To examine whether the membrane association of DraG could act as a possible mechanism for its inactivation, we assessed the cellular localization of DraG in relation to the cellular nitrogen status. DraT and DraG enzymes are predicted to be expressed at very low levels (Saari *et al.*, 1984) and we could not detect them in cell extracts by Western blot. To increase their expression levels we used a plasmid (pLHThisGMP) that expresses a version of DraT with an N-terminal His tag (His-DraT) and native DraG from the IPTG induced *ptac* promoter (Huergo *et al.*, 2005). We introduced pLHThisGMP into *A. brasilense* strain UB2 (*draT::km* which is polar on *draG*) and to avoid any potential titration effect of a regulator of DraT and/or DraG activities we did not induce the *ptac* promoter as we could detect both proteins in the uninduced state. We have shown previously that the increased expression of both His-DraT and DraG from this plasmid did not affect the pattern of NifH modification in response to ammonium addition (Huergo *et al.*, 2005). Furthermore, as predicted, after the addition of 200 μ M ammonium, strain UB2 (pLHThisGMP) showed a post-translational modification profile for NifH, GS, GlnB and GlnZ similar to that observed in the wild-type strain (data not shown).

We then monitored whole cell extracts, cytoplasmic and membrane fractions for the presence of GlnB, GlnZ, DraT and DraG before and after the ammonium shock. The profiles of membrane association for both GlnB and GlnZ were similar to that observed in the wild-type (compare Fig. 1E and F with Fig. 7A and B). DraT was distributed

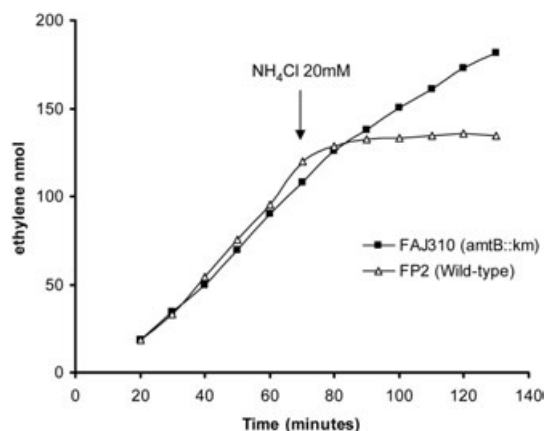


Fig. 6. AmtB is necessary for ammonium-induced nitrogenase switch-off. Wild-type (FP2) or *amtB* mutant (FAJ310) cells were assayed for *in vivo* nitrogenase activity by acetylene reduction. At the time points indicated aliquots of the gas phase were withdrawn and analysed by gas chromatography (data indicate the average of the ethylene measured in two different 0.5 ml aliquots, the standard deviation was less than 5%). The arrow indicates the time point where 20 mM NH_4Cl was injected.

between the membrane and cytoplasm, but the relative amounts in these fractions did not change according to the cellular nitrogen status (Fig. 7C). By contrast DraG was substantially sequestered to the membrane 5 min after ammonium addition and returned to the cytoplasmic fraction after 20 min (Fig. 7D). The ratio of DraG cellular distribution between cytoplasm:membrane (based on two independent experiments) was 88:12, 26:74 and 73:27 at time points 0, 5 and 20 min respectively. As DraG is overexpressed under these conditions, the fact that DraG is not completely sequestered to the membrane after 5 min may be caused by titration of the membrane-associated receptor; alternatively, it may be due to dissociation from the membrane during sample processing.

The membrane association of DraG clearly correlates with the modification profile of NifH. At time points 0 and 20 min, when NifH is completely unmodified (conditions where DraG is active) DraG is predominantly in the cytoplasm, and at 5 min, when NifH is completely modified (the condition where DraG is inactive) most DraG is membrane-associated. As NifH is only present in the cytoplasmic fractions, these data clearly suggest that membrane sequestration of DraG may affect its apparent activity simply by physical separation from its substrate (ADP-ribosylated NifH).

So what causes DraG to bind to the membrane after the ammonium shock? The membrane association of GlnB, GlnZ and DraG are clearly co-ordinated (Fig. 7A, B and D) suggesting that DraG membrane sequestration could be mediated by GlnB or GlnZ through direct protein-protein interaction. As AmtB is necessary for GlnB and GlnZ membrane association we examined whether membrane association of DraG was also dependent on AmtB.

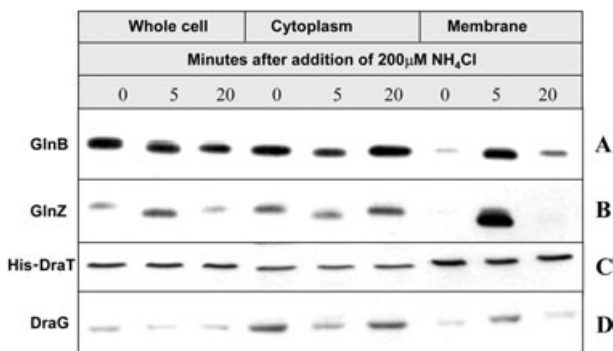


Fig. 7. *Azospirillum brasilense* DraG is membrane-associated after an ammonium shock. UB2 (*draT::km*) cells carrying pLHThisGMP were derepressed for nitrogenase and subjected to an ammonium shock (200 μ M NH_4Cl) at time zero, samples were collected, processed and analysed by Western blotting at the time point indicated. Whole cell extracts, cytoplasmic and membrane fractions were subjected to SDS-PAGE followed by Western blotting with anti-GlnB antibody (A), anti-GlnZ antibody (B), anti-DraT antibody (C) and anti-DraG antibody (D).

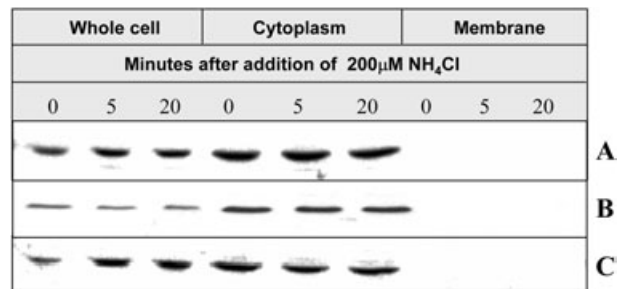


Fig. 8. DraG membrane sequestration requires AmtB, GlnB and GlnZ. FAJ310 (*amtB::km*) (A), LFH3 (*glnB* non-polar mutant) (B) or 7611 (*glnZ::Δ*) cells (C) carrying the pLHThisGMP plasmid were derepressed for nitrogenase and subjected to an ammonium shock (200 μ M NH_4Cl) at time zero, samples were collected, processed and analysed by Western blot at the time point indicated. Whole cell extracts, cytoplasmic and membrane fractions were subjected to SDS-PAGE followed by Western blot with anti-DraG antibody.

Indeed no membrane association of DraG was observed in the *amtB* mutant after an ammonium shock (Fig. 8A), whilst the pattern of DraT cellular distribution was identical to the one shown in Fig. 7C (data not shown). Hence AmtB is necessary for membrane sequestration of DraG after an ammonium shock and this is consistent with the absence of NifH modification in an *amtB* mutant because in this case DraG remains in the cytoplasm after ammonium addition.

We then examined whether membrane association of DraG is dependent on the presence of either GlnB or GlnZ. We detected no membrane association of DraG in either mutant following an ammonium shock (Fig. 8B and C) although, as discussed earlier, whilst NifH modification was completely absent in the *glnB* mutant it did occur in the *glnZ* mutant (Fig. 3A and D). The pattern of DraT cellular distribution in both the *glnB* and *glnZ* mutants was identical that seen in the wild-type (data not shown).

Discussion

Although several lines of evidence have suggested that the P_{II} and Amt proteins are involved in regulation of DraT and/or DraG activities in a number of diazotrophs, there was until now no conclusive indication of how such regulation might take place. In order to address this, we have established a set of conditions to assess the modification cycles of NifH, GS, GlnB and GlnZ in *A. brasilense* after an ammonium shock. Our results show that post-translational modification of these proteins and the membrane association of GlnB and GlnZ are closely synchronized in wild-type cells. Mutants lacking either GlnB or GlnZ showed a different NifH modification pattern from that observed in wild-type cells, but other ammonium-induced responses such as GS adenylylation and membrane association of the other P_{II} family member were also sig-

nificantly altered. Consequently the pleiotropic nature of the *glnB* and *glnZ* mutations means that it is necessary to distinguish between direct and indirect effects of the mutations.

We have shown that in *A. brasilense*, as reported for *R. capsulatus* and *Azoarcus* sp., AmtB is necessary for ammonium-induced NifH modification. The absence of AmtB did not change the pattern of modification for GS, GlnB and GlnZ, indicating that in these experiments ammonium uptake was not significantly impaired in the *amtB* mutant. In *E. coli*, the AmtB channel is apparently active at extracellular levels of ammonium below 50 μ M, and at higher concentrations it is inactivated by complex formation with GlnK (Javelle *et al.*, 2004). If the situation is similar in *A. brasilense*, then at the concentration of 200 μ M used in our experiments ammonium might be expected to enter the cells by an alternative route; either by another protein or by membrane diffusion.

As we could observe very faint bands of partially modified NifH after a high ammonium shock (20 mM NH₄Cl) in the *amtB* mutant (data not shown), we assume that in the absence of AmtB, DraT is still regulated but DraG loses its regulation leading to a futile cycle of ADP-ribosylation. It is well documented that the absence of the transcriptional activator NtrC impairs ammonium-induced nitrogenase switch-off (Liang *et al.*, 1993; Zhang *et al.*, 1994) and we propose that this phenotype may be a consequence of the absence of AmtB because *amtB* expression is NtrC-dependent (van Dommelen *et al.*, 1998). If that is the case, it is also important to emphasize that an *ntrC* mutant strain is clearly defective in DraG regulation because a double *ntrC*, *draG* mutant strain is capable of inducing NifH ADP-ribosylation after an ammonium shock, suggesting that DraT is still properly regulated (Zhang *et al.*, 1994).

We have clearly demonstrated that in *A. brasilense* following extracellular addition of ammonium, GlnB, GlnZ and DraG are all sequestered to the membrane in a reversible, AmtB-dependent fashion. This is the first time that AmtB has been implicated in the membrane sequestration of a protein other than a member of the P_{II} family and it consequently extends the potential roles of Amt proteins in regulating bacterial nitrogen metabolism. The AmtB-dependent membrane sequestration of DraG after the ammonium shock could potentially be driven by direct interaction between AmtB and DraG or, alternatively, DraG could be associated to AmtB indirectly by interaction with the P_{II} proteins GlnB and/or GlnZ in a ternary protein complex. However, we have shown that membrane association of DraG is impaired in both *glnB* and *glnZ* mutant strains (Fig. 8B and C), suggesting that AmtB is indirectly involved in this process.

The phenotypes of the *glnB* and *glnZ* mutants differ, such that whilst there is no GlnZ membrane association

in a *glnB* mutant, consistent with the failure of GlnZ to be deuridylylated in this mutant, there is GlnB membrane association in a *glnZ* mutant where GlnB deuridylylation is normal. These data are compatible with a model in which deuridylylated GlnZ is required for DraG membrane association, possibly by direct protein interaction between deuridylylated GlnZ and DraG. This would be consistent with the absence of NifH modification in the *glnB* mutant (Fig. 3A), where DraG would remain in the cytoplasm and active after ammonium addition. In the absence of GlnZ, deuridylylated GlnB could be capable of associating with DraG and mediating its membrane association after an ammonium shock. We would favour a model in which this GlnB–DraG interaction only occurs as a consequence of the absence of GlnZ, in which case it might be expected to be significantly less stable than a GlnZ–DraG interaction and be liable to disruption during the sample preparation. This would explain the NifH modification observed in a *glnZ* mutant because DraG would be membrane-associated, albeit weakly, but would not be detected in membrane preparations. Such a situation is comparable with the interactions of the P_{II} proteins GlnK and GlnB with AmtB in *E. coli*. In that case GlnK is sequestered to the membrane by AmtB in response to an ammonium shock but GlnB, which has a much lower affinity for AmtB, can be seen to be membrane-sequestered when GlnK is absent, i.e. in a *glnK* mutant (Javelle *et al.*, 2004; A. Durand, pers. comm.).

Our findings suggest a model for the regulation of DraG activity in *A. brasilense* (Fig. 9) that we believe could also offer insight into the analogous process in other diazotrophs. Under nitrogen-fixing conditions, GlnB and GlnZ are fully uridylylated and are located in the cytoplasm. In this situation DraT is inactive and DraG is in the cytoplasm and active, consequently NifH is not modified allowing nitrogenase activity. As the extracellular ammonium concentration rises, GS activity increases the intracellular glutamine pool leading to deuridylylation of GlnB and GlnZ both of which become membrane-associated with AmtB. At this point DraT is somehow activated, possibly by interaction with a P_{II} protein as suggested for *R. capsulatus* (Pawlowski *et al.*, 2003), leading to NifH modification. DraG is sequestered to the membrane, possibly by interaction with deuridylylated GlnZ and/or GlnB, in an AmtB-dependent manner. The location of DraG potentially physically separates it from its cytoplasmic substrate NifH thereby inhibiting the ADP-ribosyl-removing process. Such physical separation could be sufficient to prevent DraG acting on NifH but it is of course possible that membrane sequestration also inactivates DraG.

We have not investigated whether DraG is also membrane-associated during anaerobically induced NifH modification. DraG has been found in the membrane frac-

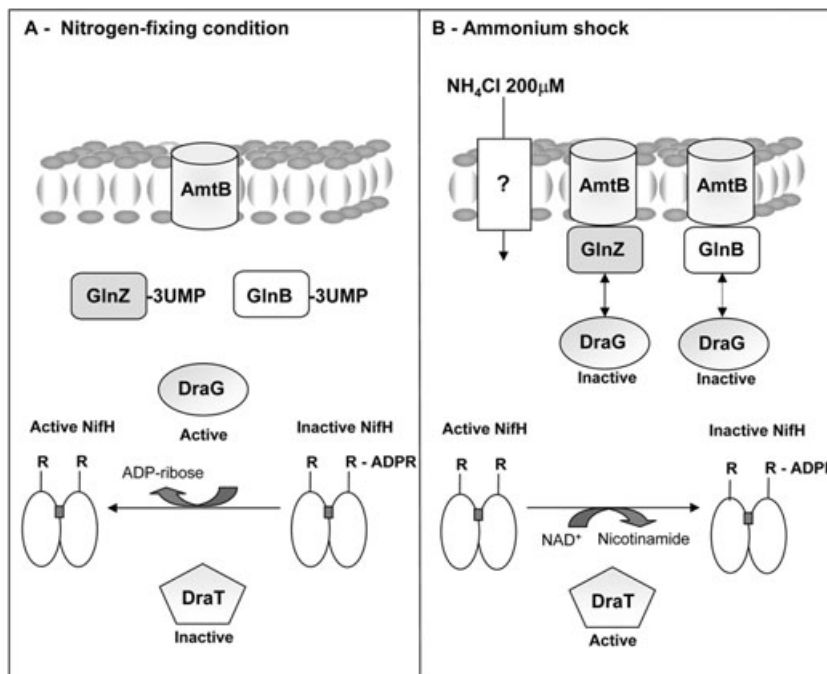


Fig. 9. Model for ammonium-induced DraG inactivation. Under nitrogen-fixing conditions GlnB and GlnZ are fully uridylylated and are located in the cytoplasm. In this situation DraT is inactive, DraG is located in the cytoplasm and active, consequently NifH is not modified allowing nitrogenase activity. Upon an ammonium shock of 200 μM NH_4Cl ammonium enters the cells by an unidentified transport system or by free diffusion, GS activity increases the intracellular glutamine pool leading to deuridylylation of GlnB and GlnZ by activation of the uridylyl-removing activity of GlnD. Non-uridylylated GlnB and GlnZ are membrane-associated with AmtB and at this point DraT is somehow activated leading to NifH modification whilst DraG is sequestered to the membrane possibly by interaction with free GlnZ and/or GlnB in an AmtB-dependent manner. The location of DraG physically separates it from its cytoplasmic substrate NifH thereby inhibiting the ADP-ribosylation process. The solid arrow between GlnZ and DraG indicates strong interaction; the light arrow between GlnB and DraG indicates weak interaction.

tion of large-scale cell preparations, conditions where the cells are subjected to anaerobic conditions during the process of cell harvesting and NifH is found to be ADP-ribosylated (Ljungstrom *et al.*, 1989). It is therefore quite possible that during anaerobically induced NifH modification, DraG is also inactivated by membrane association but, as we report here, this process would not be dependent on GlnB, GlnZ or AmtB.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used are listed in Table 1.

Media and growth conditions

Escherichia coli was grown in Luria–Bertani (LB) medium at

Table 1. Strains and plasmids.

Strain/plasmid	Genotype/phenotype	Reference or source
<i>A. brasilense</i>		
FP2	Nal ^r Sm ^r Nif ⁺ Sp7 (ATCC 29145)	Pedrosa and Yates (1984)
7611	Nal ^r Sm ^r <i>glnZ::</i> Ω Nif ⁺	de Zamaroczy <i>et al.</i> (1998)
7628	Nal ^r Km ^r <i>glnB::km</i> Nif ⁺	de Zamaroczy <i>et al.</i> (1996)
LFH3	Nal ^r Δ <i>glnB</i> Nif ⁺	This work
FAJ310	Nal ^r Km ^r <i>amtB::km</i>	van Dommelen <i>et al.</i> (1998)
UB2	Nal ^r Km ^r <i>draT::km</i>	Zhang <i>et al.</i> (1992)
Plasmids		
pAB448	Tc ^r (pBR322) <i>A. brasilense glnBA</i> 5.3 kb EcoRI-PstI fragment	Bozouklian and Elmerich (1986)
pCP20	Cb ^r Cm ^r <i>FLP⁺</i> , λ cl857 ⁺ , λ p _R Rep ^{ts}	Cherepanov and Wackernagel (1995)
pEMS136	Tc ^r (pLAFR3.18) expresses N-truncated <i>H. seropedicae nifA</i> from the <i>lacZ</i> promoter	Souza <i>et al.</i> (1999)
pFAJ309	Tc ^r pLAFR3 containing <i>A. brasilense amtB</i>	van Dommelen <i>et al.</i> (1998)
pIJ773	Apra ^r P1-FRT- <i>oriT-aac(3)IV</i> -FRT-P2	Gust <i>et al.</i> (2003)
pIJ790	Cm ^r λ -RED (<i>gam</i> , <i>bet</i> , <i>exo</i> , <i>cat</i> , <i>araC</i> , <i>rep101</i> ^{ts})	Gust <i>et al.</i> (2003)
pIJ784	Apra ^r <i>bla-oriT-aac(3)IV-bla</i>	K. F. Chater
pJC1	Tc ^r (pLAFR3.18) expresses <i>A. brasilense glnB</i> from the <i>lacZ</i> promoter	Vitorino <i>et al.</i> (2001)
pLHdraThisGMP	Cm ^r Tc ^r expresses <i>A. brasilense</i> N-terminal 6-His tagged DraT (His-DraT) and native DraG from <i>ptac</i> promoter	Huergo <i>et al.</i> (2005)
pLFH7	Cb ^r (pTZ19R) <i>A. brasilense glnBA</i> 5.3 kb EcoRI-PstI fragment from pAB448	This work
pLFH8	Cb ^r Apra ^r pLFH7 containing an <i>apra oriT</i> cassette inserted into the <i>glnB</i> coding region	This work
pLFH9	Cb ^r pLFH8 containing the scar version of <i>glnB</i>	This work
pLFH10	Apra ^r apramycin resistant version of pLFH9	This work
pTZ19R	Cb ^r cloning vector	GE healthcare

37°C. *A. brasilense* was grown at 30°C in NFbHP medium (Machado *et al.*, 1991), containing 5 mM sodium glutamate or 20 mM NH₄Cl and any required antibiotics. The antibiotics used were kanamycin (100 µg ml⁻¹), nalidixic acid (5 µg ml⁻¹), carbenicillin (100 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹), apramycin (50 µg ml⁻¹), tetracycline (10 µg ml⁻¹ or 1 µg ml⁻¹ to maintain the plasmids during nitrogen fixing conditions) and streptomycin (20 µg ml⁻¹ for *E. coli*, 100 µg ml⁻¹ for *A. brasilense*). Bacterial conjugation was performed according to the protocol described by Pedrosa and Yates (1984) using *E. coli* S17-1 as the donor.

Construction of an *A. brasilense* non-polar *glnB* mutation

Strain LFH3 carrying a non-polar *glnB* mutation was constructed using the λ-Red system as described (Gust *et al.*, 2003), a detailed protocol is available at http://streptomyces.org.uk/redirect/protocol_V1.3.pdf. The 5.3 kb EcoRI/PstI fragment containing the *glnB* coding region from pAB448 was ligated to pTZ19R digested with the same enzymes thereby generating pLFH7. This plasmid was transferred to *E. coli* strain BW25113 carrying pIJ790 that expresses the λ-Red proteins. The *glnB* gene in pLFH7 was replaced by an Apra^R cassette using polymerase chain reaction (PCR) targeting. The primers used were: GlnB apraF 5'-**GCCTGC CGACGTTGGCACCTCGTACACGAGAGACCCATG**ATTCC GGGGATCCGTCGACC-3' and GlnB apraR 5'-**TTCATG GTGGCCGGTGGTGGGTTCTCGGGACGCGATCA**TGTA GGCTGGAGCTGCTTC-3'. The 5' end marked in bold in each primer matches the flanking regions of *A. brasilense glnB* including the initiation and termination codons (underlined). The 3' ends of the primers match the ends of the disruption cassette. These primers were used to amplify the Apra^R disruption cassette from pIJ773 and the PCR product was used to transform BW25113 carrying pLFH7 and pIJ790. Cells were plated on media containing Cb (resistance of pLFH7) and Apra (resistance of the cassette) in order to select cells carrying plasmids where *glnB* was replaced by the Apra cassette. Correct replacement was verified by PCR using primers that flank the *glnB* coding region and one plasmid (pLFH8) was selected.

To obtain a 'scar' version of *glnB*, pLFH8 was transferred to *E. coli* DH5α carrying pCP20 which codes for the FLP-recombinase that removes the central part of the disruption cassette, leaving behind a 81 bp 'scar' sequence creating an in-frame deletion. Correct excision of the cassette was verified by PCR and DNA sequencing, one plasmid was selected and named pLFH9. A mobilizable version of pLFH9 was constructed by replacing the Cb resistance gene by an *apraoriT* cassette using PCR targeting. The primers bla forward 5'-CCTGATAAATGCTTCAATAATATTGAAAAAGGAAG-3' and bla reverse 5'-AATCAATCTAAAGTATATATGAGTAACTTGGTCTGACAG-3' were used to amplify the *apraoriT* cassette using pIJ784 as template. The PCR product was transformed into BW25113 carrying pLFH9 and pIJ790, and Cb^s, Apra^R cells were selected. Replacement of resistance markers was confirmed by restriction analyses and one plasmid, pLFH10, was selected.

The suicide plasmid pLFH10 was transferred to *A. brasilense* strain 7628 (*glnB::km*) using *E. coli* S17.1 as donor. Single recombinants were selected by plating on Km

and Apra, giving strain LFH2. Resolution of the recombinant (loss of the Km cassette and of the integrated plasmid) was selected by cultivating LFH2 in non-selective liquid media to saturation, subculturing to fresh medium using a 1/10 000 dilution, and repeating this process four times before plating on non-selective media to obtain single colonies. Km^s and Apra^s cells were selected and analysed for the loss of the Km cassette and the presence of the 'scar' version of *glnB* by PCR using the *glnB* flanking primers and one primer with homology to the Km cassette Km^R 5'-GGTTGATGCGAGT GATTTTGGATGACG-3'. One colony was selected and designated strain LFH3.

Antibody production

Azospirillum brasilense His-GlnZ was purified as described previously (Araujo *et al.*, 2004) and used to raise polyclonal antibodies in rabbits as described (Harlow and Lane, 1988).

Western blot analysis

Protein concentration in cell extracts was determined by the Bradford assay (Sigma) using bovine serum albumin as a standard. Protein samples (5 µg) were subjected to Native PAGE (7.5% polyacrylamide) or SDS-PAGE (12.5% polyacrylamide for DraT, DraG, GlnB, GlnZ or 7.5% polyacrylamide for GS). All protein gels were prepared using 30% acrylamide (37.5/1 acrylamide:bisacrylamide) from Severn Biotech. The proteins were transferred to ECL nitrocellulose membranes (GE healthcare) and probed with anti *A. brasilense* NifH (1/5000), anti-DraT (1/5000) and anti-DraG (1/2500) (Huerigo *et al.*, 2005), anti-*E. coli* GS that cross reacts with *A. brasilense* GS (1/10 000) (from W. van Heeswijk), anti-*A. brasilense* GlnB (1/2500) (Araújo *et al.*, 2004) and anti-GlnZ (1/5000) (this work). Anti-GlnB and anti-GlnZ did not cross-react with GlnZ and GlnB respectively under the conditions used in this work. The primary antibody incubation was performed overnight at 4°C. An aliquot of whole cell extract from *E. coli* strain FT8000 (Δ *glnB* Δ *glnK*) was routinely used in a protein concentration of approximately 100 µg ml⁻¹ during the primary antibody incubation to reduce non-specific interactions. A horseradish peroxidase-conjugated anti-rabbit immunoglobulin (GE healthcare) was used as the secondary antibody at a 1:5000 dilution for 1 h at room temperature. The signals were detected by using the ECL development reagent (GE healthcare) and exposing the membrane to X-ray films (Kodak).

In order to quantify DraG membrane sequestration, signals were analysed by densitometry using the GeneTools program (SynGene, Frederick, USA). The relative amounts of protein in each cellular fraction were calculated by setting the total amounts in both cytoplasmic and membrane fractions as 100%, the values reported are the average of two independent experiments.

Cell extracts and cellular fractionation

A pre-culture of *A. brasilense* grown to saturation in NFbHP medium supplemented with 20 mM NH₄Cl was used to inoculate 110 ml of NFbHP containing 5 mM sodium glutamate

and 1% LB using a 1:50 dilution. The cells were grown at 120 rpm for 24 h at 30°C, the protein concentration after growth was between 0.35 and 0.4 mg ml⁻¹. Nitrogenase activity was determined in whole cells by the acetylene reduction method as described (Huergo *et al.*, 2005), nitrogenase activity varied between 10 and 25 nmol ethylene formed per minute per mg of protein in all the strains used. The ammonium shock was achieved by adding the appropriate amount of a 100 times concentrated solution of ammonium chloride.

Cellular fractions were obtained as follows: 10 ml of the culture were withdrawn aerobically at the time points indicated, the culture aliquot was immediately cooled by immersion in liquid nitrogen for 10 s and the cells were then harvested by centrifugation (5000 *g* for 5 min at 4°C). The cell pellet was frozen in liquid nitrogen and stored at -80°C. The cells were resuspended in 1 ml of SP buffer (40 mM K₂HPO₄, 22 mM KH₂PO₄, 150 mM NaCl, pH 7.2) and processed as described (Coutts *et al.*, 2002). The membrane fraction was washed only once with 1 ml of SP buffer. The purity of the membrane fractions was confirmed by Western blot with antibody against NifH which is only present in the cytoplasm.

To obtain a rapid whole cell extract 33 µl of nitrogenase, derepressed culture was mixed with 17 µl of SDS-PAGE sample buffer 3× (with freshly added β-mercaptoethanol) for 10 s and immediately frozen in liquid nitrogen and stored at -80°C. The samples were boiled for 1 min, centrifuged at 13 000 *g* for 3 min, and 6 µl of the supernatant was used for Western blot analysis. All data reported were confirmed in at least two independent experiments.

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Note added in proof

Since this manuscript was accepted we have learnt that AmtB-dependent membrane sequestration of DraG has also been observed in *Rhodospirillum rubrum*. Wang *et al.* (2005) *FEMS Microbiol Letts* (in press).

Reversible membrane association of dinitrogenase reductase activating glycohydrolase in the regulation of nitrogenase activity in *Rhodospirillum rubrum*, dependence on GlnJ and AmtB1.

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