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Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB

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The Amt proteins are ammonium transporters that are conserved throughout all domains of life, being found in bacteria, archaea and eukarya. In bacteria and archaea, the Amt structural genes (*amtB*) are invariably linked to *glnK*, which encodes a member of the P_{II} signal transduction protein family, proteins that regulate enzyme activity and gene expression in response to the intracellular nitrogen status. We have now shown that in *Escherichia coli* and *Azotobacter vinelandii*, GlnK binds to the membrane in an AmtB-dependent manner and that GlnK acts as a negative regulator of the transport activity of AmtB. Membrane binding is dependent on the uridylylation state of GlnK and is modulated according to the cellular nitrogen status such that it is maximal in nitrogen-sufficient situations. The membrane sequestration of GlnK by AmtB represents a novel form of signal transduction in which an integral membrane transport protein functions to link the extracellular ammonium concentration to the intracellular responses to nitrogen status. The results also offer new insights into the evolution of P_{II} proteins and a rationale for their trigonal symmetry.

Keywords: ammonium transport/membrane sequestration/nitrogen regulation/P_{II} regulatory proteins/signal transduction

Introduction

The ammonium transport (Amt) family of proteins comprises a unique and ubiquitous group of integral membrane proteins found in all domains of life. They are present in bacteria, archaea, fungi, plants and animals, including humans, where they are represented by the Rhesus proteins (Saier *et al*., 1999; Howitt and Udvardi, 2000; Marini *et al*., 2000a; Thomas *et al*., 2000b). The Amt proteins are high-affinity ammonium transporters that probably function to scavenge ammonium and to recapture ammonium lost from cells by diffusion across the cell membrane. In *Saccharomyces cerevisiae* and *Rhodobacter capsulatus*, Amt proteins have also been implicated in sensing ammonium in the external medium (Lorenz and Heitman, 1998; Yakunin and Hallenbeck, 2000).

Analysis of the predicted primary amino acid sequences of Amt proteins suggests that all members of the family adopt a similar topology within the cell membrane. A combination of *in silico*, genetic and biochemical analyses suggests that most Amt proteins are likely to have 11 transmembrane (TM) helices, with the N-terminus being extracytosolic and the C-terminus being located in the cytoplasm (Marini and Andre, 2000; Thomas *et al*., 2000b). Some bacteria, including *Escherichia coli*, appear to have an AmtB protein with a twelfth TM at the N-terminus such that in these organisms the N-terminus would also be cytosolic (Thomas *et al*., 2000b). The C-terminal region of the Amt proteins is very variable in length, comprising a minimum of ~30 residues but in some cases extending to >150 residues. As such, this region is predicted to comprise by far the largest part of the protein exposed to the cytosol.

Almost all bacteria and archaea encode at least one Amt protein, and with very few exceptions the gene encoding the transporter (*amtB*) is found in an operon together with a second gene (*glnK*) encoding a small signal transduction protein (Thomas *et al*., 2000a; Arcondéguy *et al*., 2001). GlnK is a member of the P_{II} protein family, which act as sensors of the cellular nitrogen status in prokaryotes and which have also been identified in plants (Hsieh *et al*., 1998; Arcondéguy *et al*., 2001). The P_{II} proteins have been studied in most detail in the Proteobacteria, especially in *E.coli*, which encodes two P_{II} proteins, GlnK and GlnB. GlnK is homologous to GlnB at the primary sequence level and this is reflected in the very similar tertiary and quaternary structures adopted by both proteins (Carr *et al*., 1996; Xu *et al*., 1998). They are trimers and take the form of a squat barrel ~50 Å in diameter and 30 Å high, above the surface of which protrude three loops (the T-loops). In response to nitrogen deprivation, the proteins are covalently modified by uridylylation of residue Tyr51 at the apex of the T-loop, and this process is reversed in nitrogen sufficiency (Jaggi *et al*., 1996; Atkinson and Ninfa, 1999). P_{II} proteins regulate the activities of other proteins by protein–protein interaction, as exemplified by the role of GlnB in modulating the activity of the transcriptional activator NtrC by interaction with the histidine protein kinase NtrB (Pioszak *et al*., 2000). Whilst *E.coli* GlnK can substitute to some degree for GlnB, the primary function of GlnK has yet to be determined (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1998).

The conservation of genetic linkage in prokaryotes is characteristic of operons that encode either essential cellular components or proteins that physically interact (Dandekar *et al*., 1998). Neither *glnK* nor *amtB* is essential for growth in *E.coli* (Atkinson and Ninfa, 1998; Soupene *et al*., 1998), and this has led us to propose that their conserved association reflects a physical interaction and a related function between GlnK and AmtB (Thomas *et al*.,

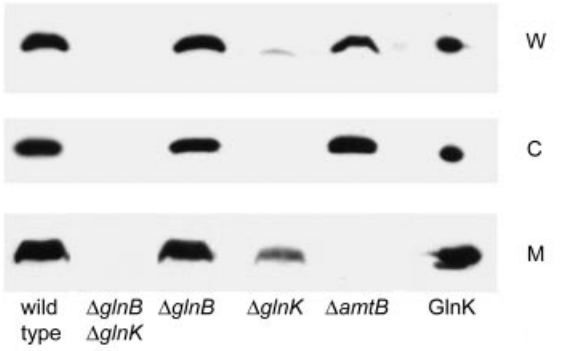

Fig. 1. GlnK and GlnB associate with the membrane in an AmtB-dependent manner. Whole-cell extracts (W), cytoplasmic (C) and membrane (M) fractions were subjected to SDS–PAGE followed by western blotting using an anti-P_{II} antibody. Extracts and fractions were prepared and loaded as described in Materials and methods. The strains used are indicated below each lane: wild type (ET8000), Δ*glnB,*Δ*glnK* (FT8000), Δ*glnB* (PT8000), Δ*glnK* (GT1002), Δ*amtB* (GT1001). The control lane contained 0.3 μg of purified *E.coli* GlnK.

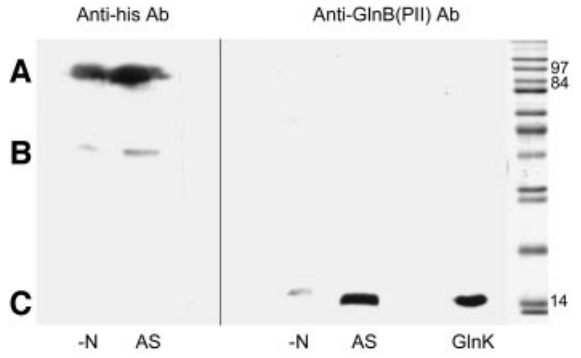

Fig. 2. The membrane association of P_{II} is affected by the cellular nitrogen status. Membrane fractions of strain GT1000 (pMM280) [Δ*glnK amtB* (*glnK amtB3*)] were subjected to SDS–PAGE followed by western blotting using either an anti-His tag antibody to detect AmtB-His_6 (**A**; **B** is a non-specific cross-reaction) or an anti-P_{II} antibody to detect P_{II} (**C**). Extracts were prepared from cells grown in nitrogen-limiting conditions (–N) and 15 min after ammonia shock (AS). The control lane contained 0.3 μg of purified *E.coli* GlnK. Molecular weight markers (Sigma, wide molecular weight standards M6539) run on the same 12.5% SDS–polyacrylamide gel are shown for calibration.

2000a). We now report that in *E.coli* and *Azotobacter vinelandii* this is indeed the case. We show that GlnK is sequestered to the cell membrane in response to nitrogen shock in an AmtB-dependent manner and that this interaction is dependent on the C-terminal cytoplasmic domain of AmtB. We discuss the potential role of this interaction and the implications of its conservation throughout bacteria and archaea.

Results

GlnK and GlnB associate with the membrane in an AmtB-dependent manner

The highly conserved genetic linkage between the *glnK* and *amtB* genes in both bacteria and archaea led us to hypothesize that GlnK was functionally associated with AmtB and that the two proteins might physically interact (Thomas *et al*., 2000a). We therefore used a variety of wild-type and mutant strains of *E.coli* to assess whole-cell extracts, cytoplasmic fractions and membrane fractions for the presence of P_{II} proteins (GlnK or GlnB) using SDS–PAGE and western blotting. The cells were grown in nitrogen-limiting media with glutamine as the nitrogen source (M9Gln medium), conditions under which the *glnK–amtB* operon is highly expressed (Atkinson and Ninfa, 1998). In initial experiments, the membrane fractions isolated by ultracentrifugation were subjected to repeated washes with 50 mM sodium phosphate buffer.

Cellular fractions prepared from wild-type cells (strain ET8000) indicated that P_{II} protein was present in both the cytoplasmic and the membrane fractions, and control experiments with strain FT8000 (Δ*glnB,*Δ*glnK*) confirm that these signals are P_{II} specific (Figure 1). The data confirm that some P_{II} protein is membrane associated under these growth conditions but do not distinguish between GlnB and GlnK. To clarify the nature of P_{II} association with the membrane and the role of AmtB in this association, three isogenic mutant strains were employed (Δ*glnK*, GT1002; Δ*glnB*, PT8000; and Δ*amtB*, GT1001). The Δ*glnK* mutation is an in-frame deletion within *glnK* that is not polar on *amtB* (Arcondéguy *et al*., 1999).

Analysis of the Δ*glnB* strain gave an identical pattern to that seen with the wild type, demonstrating that GlnK does indeed associate with the membrane (Figure 1). In the Δ*glnK* strain, the pattern was somewhat different and GlnB was only detected in the whole-cell lysate and the membrane fraction. Consequently, GlnB also appears to associate with the membrane, at least in the absence of GlnK; however, the signal was considerably weaker compared with that seen in the Δ*glnB* strain, suggesting that under these growth conditions there is considerably less GlnB than GlnK in the cell. Control experiments indicate that the anti-P_{II} antibody used in these experiments is significantly less active against GlnK than GlnB (R.Little and T.Arcondéguy, personal communication), hence the GlnK:GlnB ratio is actually underestimated. When the localization of the P_{II} proteins was examined in extracts prepared from the Δ*amtB* strain GT1001, there was a striking absence of signal from the membrane fraction, while the proteins were just as abundant in the cytoplasmic fraction as in the wild type (Figure 1). Hence AmtB is essential for the association of the P_{II} proteins with the membrane, most probably through a direct physical interaction between P_{II} and AmtB.

The membrane association of P_{II} is affected by the cellular nitrogen status

P_{II} proteins typically function to regulate the activity of those proteins with which they interact in response to the nitrogen status of the cell (Arcondéguy *et al*., 2001). We therefore examined what effect, if any, a change in the cellular nitrogen status would have on the observed association between P_{II} (in particular GlnK) and AmtB. Previous studies of this nature have investigated the role of GlnB in regulating adenylyltransferase, and thereby the adenylylation of glutamine synthetase, in response to a rapid addition of ammonium to a nitrogen-limited culture (termed 'ammonia shock') (Bender *et al*., 1977;

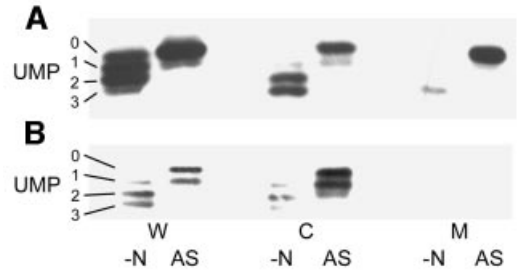

Fig. 3. The membrane association of GlnK reflects the uridylylation state of the protein. Whole-cell extracts (W), and cytoplasmic (C) and membrane (M) fractions were prepared from strains (**A**) Δ*glnB* (PT8000) and (**B**) Δ*glnB,*Δ*amtB* (AT8000) both before (–N) and after (AS) ammonia shock. Extracts and fractions were prepared as described in Materials and methods and subjected to native PAGE followed by western blotting using an anti-P_{II} antibody. Under these conditions, all four possible forms of GlnK (UMP 0–3) can be identified.

van Heeswijk *et al*., 1996). We therefore used the same ammonia shock conditions to investigate effects on GlnK–AmtB association.

For these experiments, we used strain GT1000 (pMM280), which has chromosomal deletions of both *glnK* and *amtB* and contains *glnK* and *amtB in trans* with a modified version of *amtB* that encodes a C-terminally His-tagged version of the protein (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation). The modified AmtB protein retains methylammonium transport activity at a level comparable to the wild-type protein (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation). The cells were grown in M9Gln medium and then subjected to ammonia shock with 30 mM NH_4Cl. Samples were taken immediately prior to addition of ammonium and 15 min later, and then fractionated as before. We monitored the membrane fractions for the presence of both P_{II} and AmtB, the latter being detected using an anti-His tag antibody (Qiagen) to detect the C-terminal His tag on the modified version of AmtB. The levels of AmtB in the membrane fraction remained constant both pre- and post-ammonia shock (Figure 2). In contrast, there was a very significant increase in the amount of P_{II} protein associated with the membrane after ammonia shock, indicating that membrane association is modulated by the nitrogen status (Figure 2). The levels of P_{II} (GlnB + GlnK) in the cytoplasmic fractions showed a significant reduction after ammonia shock (data not shown).

Membrane association of GlnK reflects the uridylylation state of the protein

Given that GlnK is a trimer, the protein can exist in four states in the cell depending upon the number of subunits that are modified by uridylylation. Ammonia shock is known to cause rapid deuridylylation of both GlnB and GlnK (van Heeswijk *et al*., 1996; Jiang *et al*., 1998) and the uridylylation states of the P_{II} trimers can be assessed by separating the different forms on native polyacrylamide gels (Forchhammer *et al*., 1999; van Heeswijk *et al*., 2000). Under these conditions, the non-uridylylated form of the protein has the lowest mobility and the fully uridylylated form the highest (Jaggi *et al*., 1996). For these experiments, we used a Δ*glnB* strain (PT8000) and a Δ*glnB,*Δ*amtB* strain (AT8000) in order to assess specifically the membrane association of GlnK. We also subjected the membrane fractions to a more stringent wash using 50 mM sodium phosphate supplemented with 600 mM NaCl to ensure that only those proteins that were tightly associated with the membrane were detected.

In whole-cell lysates from Δ*glnB* cells grown in M9Gln medium, four bands were detected corresponding to the uridylylated forms of GlnK (GlnK-UMP_1, GlnK-UMP_2 and GlnK-UMP_3) and the unmodified protein (Figure 3). Following ammonia shock, this profile changes to reveal only two bands, the major one being the non-uridylylated form of GlnK and the minor species being GlnK-UMP_1. The cytoplasmic fraction showed a very similar pattern, although GlnK-UMP_1 was apparently less abundant than GlnK-UMP_2 and GlnK-UMP_3 pre-ammonia shock. Most strikingly, analysis of the membrane fractions gave a relatively weak signal pre-ammonia shock, with only GlnK-UMP_3 being revealed, and a very much stronger signal post-ammonia shock, which was almost exclusively the non-uridylylated form of GlnK (Figure 3A). Identical amounts of membrane material were loaded in each lane and, as shown earlier, the amount of AmtB present pre- and post-ammonia shock did not vary significantly. Parallel analyses of the Δ*glnB,*Δ*amtB* strain produced very similar profiles for the whole-cell lysate and for the cytoplasmic fraction, but there was no membrane-associated GlnK either pre- or post-ammonia shock (Figure 3B). In similar experiments using the less stringent salt wash, we detected membrane association of other uridylylated species of GlnK in the pre-ammonia shock samples, suggesting that whilst there is association of GlnK in nitrogen-limited conditions, most of this protein is only weakly bound (data not shown).

We carried out experiments comparable to those described above using the Δ*glnK* strain (GT1002) to examine the behaviour of GlnB in the absence of GlnK. Our initial experiments on nitrogen-limited cells had indicated that in the absence of GlnK essentially all GlnB was membrane associated (Figure 1). Nevertheless, we saw a very marked apparent increase in the amount of GlnB in the membrane fraction after ammonia shock (data not shown). We interpret this to reflect an increased affinity of GlnB for AmtB in response to ammonia shock so that membrane-associated GlnB that was lost from pre-ammonia shock samples during the salt wash was retained in post-ammonia shock samples.

Finally, we repeated the experiment with wild-type cells, but in this case interpretation of the results is complicated due to the potential presence of heterotrimers between GlnB and GlnK whereby at least 16 different species of P_{II} protein can occur in principle (van Heeswijk *et al*., 2000). Using native gel electrophoresis, we did indeed observe more bands in western blots of whole-cell extracts, cytoplasmic and membrane fractions both pre- and post-ammonia shock than we detected in either the Δ*glnB* or Δ*glnK* strains (data not shown). Assignment of these bands was assisted by the previously reported differential migration of the homotrimeric GlnK and GlnB proteins (van Heeswijk *et al*., 2000). The membrane fractions from post-ammonia shock samples contained

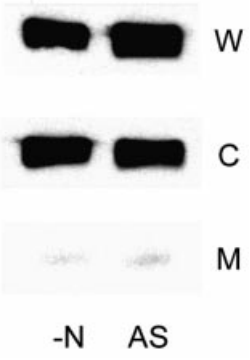

Fig. 4. The C-terminal domain of AmtB is required for GlnK association. Whole-cell extracts (W), and cytoplasmic (C) and membrane (M) fractions were prepared from strain GT1000 (pGC4) [Δ*glnK amtB* (*glnK amtB4*)] both before (–N) and after (AS) ammonia shock. Extracts and fractions were prepared as described in Materials and methods and were subjected to SDS–PAGE followed by western blotting using an anti-P_{II} antibody.

three major species of which the dominant form had a mobility consistent with that of the non-uridylylated GlnK homotrimer, the other species most probaby being heterotrimers containing predominantly GlnK subunits. Hence it would appear that although GlnB can associate with AmtB in the absence of GlnK, it does not compete significantly with GlnK in wild-type cells.

The C-terminal domain of AmtB is required for GlnK association

The C-terminal 32 residues of *E.coli* AmtB are predicted to be located on the cytoplasmic side of the membrane and to constitute the most extensive part of the protein exposed to the cytoplasm (Thomas *et al*., 2000b). The region contains a number of residues that are highly conserved amongst members of the Amt family and is therefore likely to be either functionally and/or structurally important. We reasoned that this region constitutes a potential interaction domain for GlnK and we therefore examined the consequences of deleting the C-terminus of the transporter.

The *amtB* gene was truncated (*amtB4*) so that 25 residues were removed from the C-terminus and the encoded protein terminated at residue 403. The activity of the truncated protein was compared with that of wild-type AmtB by introducing the wild-type *glnK–amtB* operon (on pGC2) and the derivative operon *glnK–amtB4* (on pGC4) into the Δ*glnK,amtB* strain GT1000. The truncated transporter had 28% of the wild-type AmtB activity as measured by [^{14}C]methylamine transport. We used a polyclonal antibody raised against purified *E.coli* AmtB (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation) to assess the presence of the truncated AmtB in the cell membrane and found levels comparable to the wild-type protein both pre- and post-ammonia shock (data not shown). Hence the reduced [^{14}C]methylamine transport activity of the truncated protein apparently is not due to reduced levels of the protein in the membrane, but rather to decreased activity of the protein.

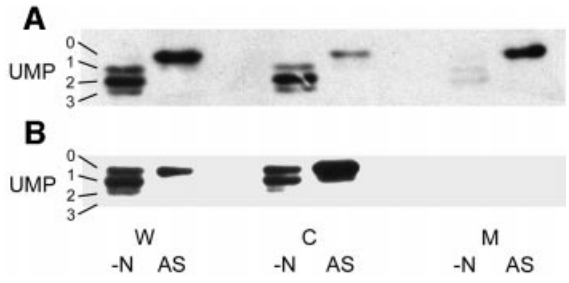

Fig. 5. GlnK–AmtB association is found in other bacteria. Whole-cell extracts (W), and cytoplasmic (C) and membrane (M) fractions were prepared from *A.vinelandii* strains (**A**) UW136 (wild type) and (**B**) MV560 (Δ*amtB1*::KIXX) both before (–N) and after (AS) ammonia shock. Extracts and fractions were prepared as described in Materials and methods and subjected to native PAGE followed by western blotting using an anti-P_{II} antibody. Under these conditions, all four possible forms of GlnK (UMP 0–3) can be identified.

Using strain GT1000 (pGC4), which synthesizes only the C-terminally truncated form of AmtB, we analysed the location of P_{II} in cell fractions both pre- and post-ammonia shock using western blots after denaturing SDS–PAGE. The protein was detected in whole-cell lysates and cytoplasmic fractions at levels comparable to those observed previously, but there was almost no signal in the membrane fractions (Figure 4). Although this strain synthesizes both GlnK and GlnB, a combination of the differential expression of *glnB* and *glnK*, together with the multicopy effect of the plasmid-borne *glnK* gene, means that nearly all of the P_{II} in these cells is expected to be GlnK. These data strongly suggest that the C-terminal domain of AmtB either constitutes the site of interaction between GlnK and AmtB or is required to allow AmtB to adopt a conformation required for GlnK association.

GlnK–AmtB association is found in other bacteria

We have proposed previously that the conservation of linkage between the *glnK* and *amtB* genes in both bacteria and archaea reflects a highly conserved interaction between the two proteins (Thomas *et al*., 2000a). Having demonstrated evidence for such an association in *E.coli*, we sought to support the concept that this is a general phenomenon by carrying out a similar analysis in another species, namely the γ-proteobacterium *A.vinelandii*. *Azotobacter vinelandii* contains a single *glnK–amtB* operon and, unlike *E.coli*, is proposed to encode only a single P_{II} protein, namely GlnK (Meletzus *et al*., 1998). We used a wild-type strain (UW136) and an *amtB* mutant (MV560) that has been shown to lack [^{14}C]methylamine transport activity (Meletzus *et al*., 1998). Cells were grown in a nitrogen-limited medium and then subjected to ammonia shock in a similar manner to that used for *E.coli*. Membrane fractions were isolated pre- and post-ammonia shock, subjected to stringent washing and then run on native polyacrylamide gels prior to western blotting with antibodies against *E.coli* GlnB and AmtB. As in *E.coli*, GlnK was found to be associated with the membrane in an AmtB-dependent manner and the level of membrane-associated GlnK increased post-ammonia shock whilst the level in the cytoplasm was reduced (Figure 5). The AmtB antibody confirmed that the levels of AmtB present in the membrane do not vary pre- and post-ammonia shock and that membranes from the *amtB* mutant

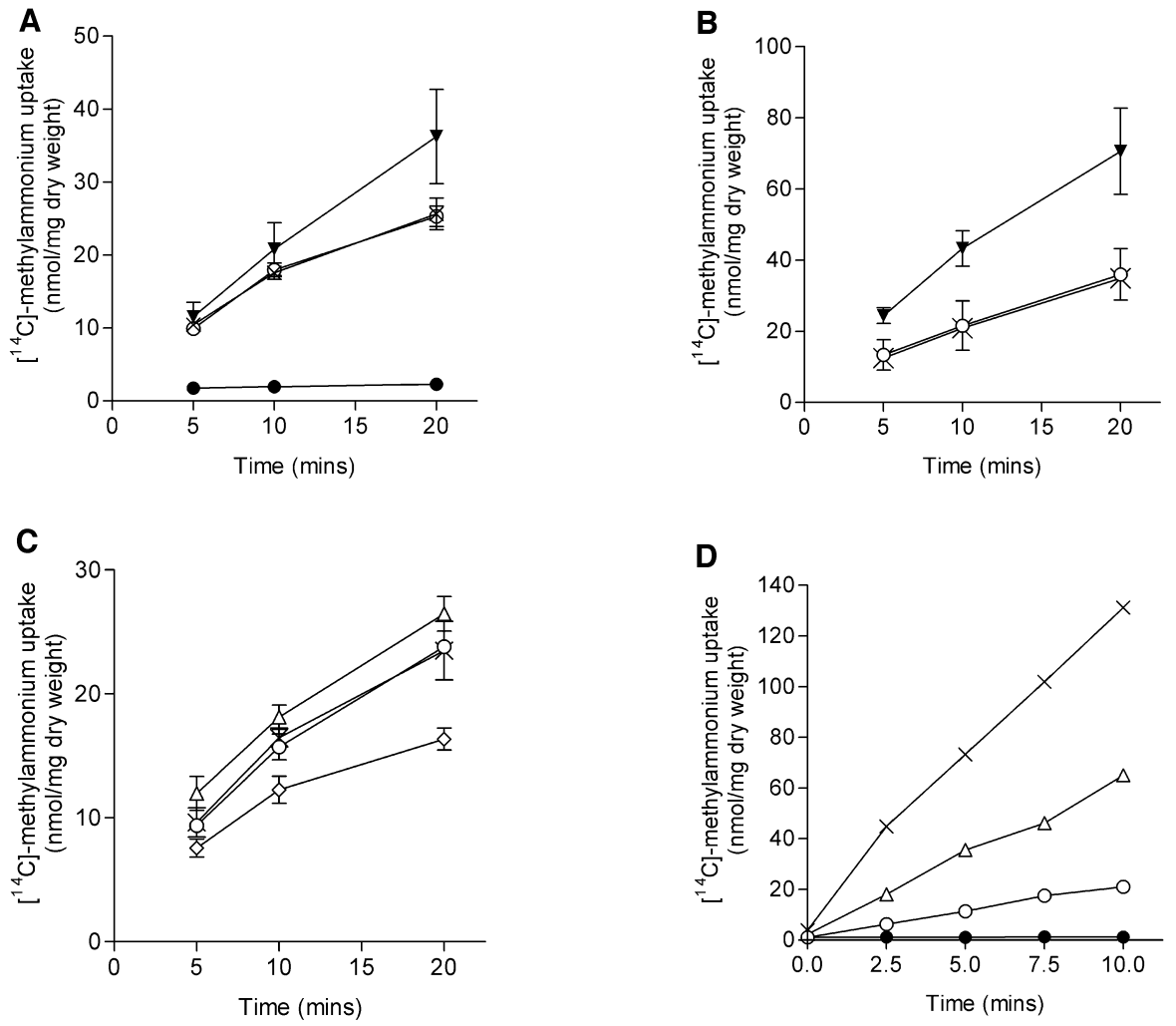

Fig. 6. The GlnK protein affects the methylammonium transport activity of AmtB. [^{14}C]methylammonium transport activities were determined as described in Materials and methods. Strains used were as follows. (**A**) ET8000, wild type (open circles); PT8000, Δ*glnB* (crosses); GT1002, Δ*glnK* (inverted trangles); and GT1000, Δ*glnK amtB* (filled circles). (**B**) YMC10, wild type (open circles); RB9060, Δ*glnB* (crosses); and WCH30, Δ*glnK* (inverted triangles). (**C**) ET8000, wild type (open circles); ET8000 (pWSK29), wild type (upright triangles); ET8000 (pTA40), wild type (p*glnK*$^+$) (crosses); and ET8000 (pTA43), wild type (p*glnKY51F*) (diamonds). (**D**) ET8000, wild type (open circles); GT1000, Δ*glnK amtB* (filled circles); GT1000 (pGT11), Δ*glnK amtB* (*glnK*$^+$ *amtB*$^+$) (upright triangles); and GT1000 (pGT14), Δ*glnK amtB* (*amtB*$^+$) (crosses).

showed no cross-reacting material (data not shown). Hence the GlnK and AmtB proteins of *A.vinelandii* show very similar behaviour to that seen in *E.coli* in response to a rapid change in the cellular nitrogen status.

The GlnK protein negatively regulates the transport activity of AmtB

Having found evidence for an interaction between P_{II} proteins and the ammonium transporter AmtB, we considered what functions such an interaction might serve. One possibility is that the interaction serves to regulate the activity of the transporter and, with this in mind, we carried out a series of experiments comparing the transport activity of AmtB in the wild type with that in strains either lacking or overexpressing *glnK*. The substrate in these assays is [^{14}C]methylammonium, an analogue of ammonium, and as transport of methylammonium by AmtB is competitively inhibited by micromolar concentrations of ammonium it is not possible to assess the effect of GlnK under those conditions where its interaction with AmtB is maximal, i.e. after ammonia shock. Therefore, all these experiments were carried out using cells grown in nitrogen limitation, a condition where any effects of GlnK on AmtB activity were expected to be relatively small.

We examined two isogenic sets of strains, one derived from ET8000 and one from YMC10, for the effects of Δ*glnK* and Δ*glnB* mutations on uptake (Figure 6A and B). In each case, the Δ*glnB* strain was indistinguishable from the wild type, whereas the activity of the Δ*glnK* strain was considerably elevated (50% in the ET8000 background, Figure 6A; and 100% in the YMC10 background, Figure 6B). In principle, this effect of the Δ*glnK* mutation could arise in a number of ways. It could be due to a direct effect on the activity of AmtB or to an indirect effect (through NtrB and NtrC) leading to elevated *glnK–amtB*

transcription. Alternatively, as methylammonium is metabolized by glutamine synthetase to methylglutamine and the transport assay effectively measures metabolically trapped methylammonium (Soupene *et al*., 1998), there is also the potential for effects on the activity of glutamine synthetase. The effects of *glnK* mutants of *E.coli* on both *glnK–amtB* transcription and glutamine synthetase activity have been assessed previously and, in each case, the mutant was shown to have little if any effect (Atkinson and Ninfa, 1998). Indeed, the most marked effect is on *glnK–amtB* transcription where the *glnK* mutant decreases, rather than increases, expression. To confirm independently that the levels of AmtB are unaffected by the *glnK* mutation, we used western blots to assess the levels of AmtB present in whole-cell extracts of wild type, and Δ*glnK* and Δ*glnB* mutants. We found no significant differences in the levels of AmtB in each of the strains (data not shown). We therefore conclude that the elevated transport activity of the *glnK* mutant is attributable to an increase in the activity of AmtB.

The deduced negative effect of GlnK on AmtB was confirmed by examining the activities of strains in which GlnK levels were elevated. We introduced into a wild-type strain low-copy-number plasmids on which either *glnK* (pTA40) or a mutant *glnK* allele (*glnKY51F*, carried on pTA43) is expressed from the *lacZ* promoter. AmtB activity was unchanged in the presence of wild-type GlnK, but a non-uridylylatable form of the protein, GlnKY51F, which is expected to associate strongly with AmtB, significantly depressed activity (Figure 6C).

The negative effect of GlnK on AmtB activity was also apparent when we compared plasmids expressing either *amtB* alone (pGT14) or the complete *glnK–amtB* operon (pGT11) into strain GT1000, which carries a chromosomal *glnK–amtB* deletion (Figure 6D). The presence of multicopy *glnK–amtB* gave an activity 4-fold greater than wild type, but when *glnK* was deleted from the plasmid and *amtB* was expressed alone from the same native promoter, the activity was elevated 9-fold. Hence AmtB is significantly more active in the absence of GlnK.

Discussion

In almost all members of the bacteria and archaea, genes encoding the high-affinity ammonium transporter AmtB are linked to and potentially co-transcribed with *glnK*, which encodes a member of the P_{II} signal transduction protein family (Thomas *et al*., 2000a; Arcondéguy *et al*., 2001). We have now shown that in *E.coli* and in *A.vinelandii* this genetic linkage almost certainly reflects a physical interaction between the two proteins such that GlnK can be sequestered specifically to the cell membrane by AmtB. The affinity of non-uridylylated GlnK for AmtB appears to be considerably greater than that of the uridylylated protein, such that the interaction of GlnK with AmtB is promoted by an increase in the intracellular nitrogen status. AmtB activity increases in the absence of GlnK, indicating that GlnK acts as a negative regulator of the transporter.

We propose that the physiological role of this interaction is to modulate the activity of the transporter so as to optimize ammonium uptake under all growth conditions, and a model for this regulation is outlined in Figure 7. The

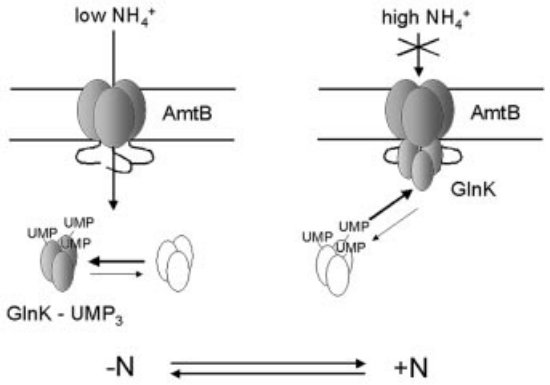

Fig. 7. Model for regulation of the interaction between GlnK and AmtB. In conditions of nitrogen limitation (–N), GlnK is predominantly in its fully uridylylated state and is not strongly membrane associated (non-shaded GlnK indicates the minority species in each situation). AmtB is active and will effectively scavenge ammonium from the surrounding medium. When the cellular nitrogen status changes, e.g. as a consequence of a sudden rise in the availability of extracellular ammonium, GlnK is deuridylylated rapidly and the unmodified form of the protein associates tightly with AmtB in the inner membrane. This association reduces the activity of AmtB and ammonium is no longer transported actively into the cell. This process is rapidly reversible, but in the event that the nitrogen status remains high, transcription of the *glnK–amtB* operon will cease due to dephosphorylation of the activator protein NtrC.

transcriptional control of *amtB* by the nitrogen regulation (NtrB/NtrC) system ensures that *amtB* is only expressed in nitrogen-limited growth conditions. However, during growth in nitrogen limitation, GlnK can fine-tune the activity of AmtB in response to minor or transient fluctuations in ammonium availability. In nitrogen-limited conditions, GlnK is predominantly in its fully uridylylated state and is not strongly membrane associated. AmtB is then active and will effectively scavenge ammonium from the surrounding medium. When the cellular nitrogen status changes, e.g. as a consequence of a sudden rise in the availability of extracellular ammonium, GlnK is deuridylylated rapidly and the unmodified form of the protein then associates tightly with AmtB in the inner membrane. This association reduces the activity of AmtB, and ammonium is no longer actively transported into the cell. This process is rapidly reversible, but in the event that the nitrogen status remains high, transcription of the *glnK–amtB* operon will cease due to dephosphorylation of the activator protein NtrC. GlnK previously has been proposed to act as a negative regulator of AmtB activity in the α-proteobacterium *Azospirillum brasilense* (de Zamaroczy, 1998). In this organism, a mutation in *glnZ* (the *A.brasilense* homologue of *glnK*) more than doubles the rate of [^{14}C]methylammonium uptake and the presence of additional copies of *glnZ* in either wild-type or *glnZ* mutant strains reduces uptake. These data are entirely consistent with the data and the model proposed here for *E.coli*.

Whilst we consider that regulation of AmtB activity is likely to be the primary function of the GlnK–AmtB interaction, another significant consequence of GlnK sequestration will be to deplete the cytoplasmic GlnK pool rapidly in response to an increase in extracellular ammonium. This would have the effect of amplifying the regulatory properties of GlnK with respect to any

Table I. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype or phenotype	Reference
E.coli		
ET8000	*rbs lacZ::IS gyrA hutC$_K$*	Jayakumar *et al.* (1986)
AT8000	*rbs lacZ::IS gyrA hutC$_K$ ΔglnB1, ΔamtB*	Reyes-Ramirez *et al.* (2001)
FT8000	*rbs lacZ::IS gyrA hutC$_K$ ΔglnB1, ΔglnK1*	Reyes-Ramirez *et al.* (2001)
GT1000	*rbs lacZ::IS gyrA hutC$_K$ ΔglnKamtB*	this work
GT1001	*rbs lacZ::IS gyrA hutC$_K$ ΔamtB*	Thomas *et al.* (2000b)
GT1002	*rbs lacZ::IS gyrA hutC$_K$ ΔglnK1*	this work
PT8000	*rbs lacZ::IS gyrA hutC$_K$ ΔglnB1*	Reyes-Ramirez *et al.* (2001)
RB9060	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutC$_K$ ΔglnB2306*	Bueno *et al.* (1985)
WCH30	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutC$_K$ ΩGm^r ΔglnK1*	Arcondéguy *et al.* (1999)
YMC10	*ΔlacU169 endA1 thi-1 hsdR17 supE44 hutC$_K$*	Chen *et al.* (1982)
A.vinelandii		
UW136	Rif^r	Bishop and Brill (1977)
MV560	Rif^r Δ*amtB1*::KIXX	Meletzus *et al.* (1998)
Plasmids		
pGC2	*glnK amtB1* in pACYC184	this work
pGC4	*glnK amtB4* in pACYC184	this work
pGT11	*glnK amtB* in pWSK29	this work
pGT14	*amtB* in pWSK29	Thomas *et al.* (2000b)
pMM280	*glnK amtB3* in pACYC184	this work
pTA40	*glnK* in pWSK30	this work
pTA43	*glnKY51F* in pWSK30	this work

cytoplasmic targets. To date, only one specific cytoplasmic target of GlnK is known, the nitrogen fixation regulatory protein complex NifLA (Jack *et al*., 1999). In *Klebsiella pneumoniae*, GlnK is required to relieve the inhibitory effects of NifL on the activator protein NifA and, somewhat surprisingly, uridylylation of GlnK is not required to regulate this process (He *et al*., 1998). This led to the question of how NifL-mediated inhibition could be restored when ammonium is added back to a nitrogen-limited medium and to the suggestion that GlnK might be proteolysed or covalently modified by a mechanism other than uridylylation upon replenishment of ammonium (He *et al*., 1998). Our present observations offer an alternative explanation in that the sequestration of deuridylylated GlnK by AmtB would rapidly lower the cytoplasmic GlnK pool and thereby release NifL to inhibit NifA activity. A further implication of this mechanism is that the pivotal role of AmtB in such a process would give the transporter the appearance of acting as a sensor of extracellular ammonium, a role that had been suggested previously for Amt proteins in *R.capsulatus* (Yakunin and Hallenbeck, 2000) and in *S.cerevisiae* (Lorenz and Heitman, 1998).

The C-terminal region of *E.coli* AmtB is required for the membrane sequestration of GlnK and potentially could constitute the specific site of interaction. In *S.cerevisiae* Mep proteins, a point mutation in this region can have a transdominant phenotype, suggesting that C-terminal residues are involved in interactions between subunits of the transporter (Marini *et al*., 2000b). In *E.coli*, deletion of this region does not abolish transport, though it does significantly reduce it. Hence, one function of this region of the transporter could be regulatory, and it is notable that whilst the C-terminal region in *E.coli* AmtB is conserved in Amt proteins, many members of the family have considerably extended C-terminal domains of up to 150 residues. These domains could potentially constitute interaction sites for regulatory proteins.

We suggest that the interaction of GlnK and Amt proteins is a general phenomenon that is fundamental to the biology of bacterial and archaeal ammonium transporters. In those organisms in which multiple copies of the *amtB* gene are present, e.g. *Archaeoglobus fulgidus* and *Azoarcus*, a linked *glnK* homologue is also duplicated, suggesting that the two proteins co-evolved. The major exception to this situation is in the cyanobacteria, which have a single P_{II} gene (*glnB*) that is not *amt* linked and which can have multiple homologues of *amtB* (Montesinos *et al*., 1998). The conservation of the *glnK–amtB* operon leads us to suggest that P_{II} proteins originally evolved together with the Amt proteins and that the primary role of the P_{II} protein was to regulate the activity of the ammonium transporter. Based on this model, the P_{II} paralogues that are now found in the bacteria and the archaea, namely the GlnB proteins of the Proteobacteria and the NifI proteins in the nitrogen fixation gene clusters of some archaea (Arcondéguy *et al*., 2001), are likely to be the result of subsequent gene duplication and specialization. Of particular interest in this context is the fact that the P_{II} proteins are trimers and to date the reason for this quaternary structure has not been apparent, particularly as none of their known targets has trigonal symmetry. However, we have recently shown that the purified AmtB protein from *E.coli* is also trimeric (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation) and hence, if GlnK did evolve in concert with AmtB, its trimeric structure may reflect a symmetry required for optimal interaction between the two proteins.

In *E.coli*, the interaction of GlnK with AmtB apparently is modulated by uridylylation, and a generalization of our model would imply that covalent modification of the P_{II} protein is required to regulate interaction with the transporter. In the cyanobacteria, P_{II} is modified by phosphorylation of Ser49 rather than uridylylation of

Tyr51 (Forchhammer and Tandeau de Marsac, 1994) and this could serve the same purpose. However, it is not known whether the P_{II} proteins of archaea are subject to covalent modification. A uridylyltransferase (GlnD homologue) is not present in the archaea and their P_{II} proteins do not contain a potential phosphorylation site, i.e. a conserved serine residue, in the T-loop. It is also possible that the transporter plays an active role in the interaction with GlnK and that their association is also modulated by conformational changes in the transporter in response to increased levels of extracellular ammonium.

Dynamic changes in the subcellular localization of proteins are emerging as significant features in bacteria with respect to both cellular physiology and cell differentiation (Shapiro and Losick, 2000). A number of examples of membrane sequestration of regulatory proteins have been described. The transcriptional repressor of the *Salmonella typhimurium* proline utilization (*put*) operon, PutA, shuttles between the membrane and the cytoplasm depending on the intracellular concentration of proline (Muro-Pastor *et al*., 1997). Similarly, in the presence of glucose, the *E.coli* repressor protein Mlc binds to the activated glucose transporter PtsG, thereby becoming sequestered to the membrane and allowing derepression of Mlc-regulated promoters (Lee *et al*., 2000; Tanaka *et al*., 2000). In the case of GlnK and AmtB, the cytoplasmic component is a signal transduction protein rather than a transcriptional regulator but, as with Mlc, the system potentially provides a means not only of responding to the presence of an extracellular substrate but also of sensing how much is entering the cell. One can imagine that such mechanisms may be much more widespread and indeed the potential role of transport proteins as part of signal transduction pathways in bacteria and archaea is perhaps considerably greater than has been recognized so far. One possible example, which also involves a P_{II} protein, is in the cyanobacterium *Synechococcus*, where GlnB has been shown to regulate nitrate uptake and might interact with the C-terminus of one of the ATP-binding subunits of the nitrate transporter NrtC (Kobayashi *et al*., 1997; Hisbergues *et al*., 1999).

Materials and methods

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table I. The *E.coli* strains were grown routinely in Luria medium (Luria broth or Luria agar LA). For growth under nitrogen limitation, an altered M9 medium was used in which the ammonia was replaced by 200 μg/ml glutamine (M9Gln medium). Ammonia shock was achieved by addition of NH_4Cl to a final concentration of 30 mM. *Azotobacter vinelandii* was grown in Burk's sucrose medium supplemented with 25 mM ammonium acetate for +N conditions and 200 μg/ml glutamine for –N conditions. Antibiotics were used at the following concentrations: chloramphenicol 30 μg/ml and kanamycin 1 μg/ml.

Plasmid pGT11, which contains the complete *glnK–amtB* operon, was created by subcloning an *Ssp*I–*Bam*HI fragment from pWVH141 (van Heeswijk *et al*., 1996) into *Ssp*I–*Bam*HI-cut pWSK29 (Wang and Kushner, 1991). pGC2 was derived from pGT11 in two steps: first, the *Hin*dIII–*Bam*HI 1.8 kb fragment of pGT11 was cloned into pACYC184 (Chang and Cohen, 1978) to give pGC1. Secondly, using the primers AMT1 (GGGCCATCAGTTGCTGG) and AMT2 (CCGGATCC*TTA*C-AGATCTGCGTTATAGGCATTCTCGCC; *Bam*HI and *Bgl*II sites underlined and termination codon italicized), the 3′ end of *amtB* was amplified on a *Pvu*I–*Bam*HI fragment. This fragment then replaced the wild-type *Pvu*I–*Bam*HI fragment, giving pGC2 in which the *Bam*HI site was moved closer to the termination codon of *amtB* and a unique *Bgl*II site encoding two additional residues (aspartate and leucine) was introduced 5′ to the stop codon. The modified *amtB* gene in pGC2 was designated *amtB1*.

A short linking amino acid-coding sequence was added to the C-terminus of AmtB by the ligation of an oligonucleotide cassette into pGC2 cut with *Bgl*II and *Bam*HI. The cassette was produced by annealing AMT3 (GATCAAGCGCACCAGCCCGCTCAGGCAGATCTCTCGA-GCTAAG) and AMT4 (GATCCTTAGCTCGAGAGATCTGCCTGA-GCGGGCTGGTGCGCTT) to produce pGC5. A His_6 tag [made by annealing oligos AMT5 (GATCATCACCACCATCATCATCAC-TAAG) and AMT6 (GATCCTTAGTGATGATGGTGGTGAT)] was added to the C-terminus of the protein by the ligation of the oligonucleotide cassette between the *Bgl*II and *Bam*HI sites in pGC5 to give a further allele of *amtB*,*amtB3*, encoded on pMM280.

The plasmid pGC4 was created by using two primers Amt1 (GGGCCATCAGTTGCTGG) and Amt5 (CCGGATCC*TTA*GCTC-GAGGTCAAGATCCGCCAATTTGTAGCC; *Bam*HI and *Xho*I sites underlined and termination codon italicized) to amplify part of the end of *amtB*. This fragment was then ligated into pGC2 (*glnK amtB1*) on a *Pvu*I–*Bam*HI fragment to give pGC4 (*glnK amtB4*), which encodes GlnK and a truncated form of AmtB comprising the wild-type sequence to residue Thr402 with a newly introduced *Xho*I site resulting in one extra codon (Ser403) just prior to the stop codon.

Plasmid pTA40 was constructed by cloning an *Eco*RI–*Sac*I fragment carrying *glnK* from pWVH149 (van Heeswijk *et al*., 1996) into pWSK30 (Wang and Kushner, 1991) so that *glnK* is expressed from p*lacZ*. The derivative plasmid pTA43 is identical to pTA40 but contains a site-directed mutation that converts the TAC codon for Y51 to a TTC codon, giving F51.

Strain GT1000 containing an unmarked deletion that removes the majority of the *glnK–amtB* operon was constructed in a manner comparable with that used previously to construct the unmarked *amtB* deletion strain GT1001 (Thomas *et al*., 2000b). PCR primers GlnKNo (5′-CGCGGATCCGCTGGTCGAGACGCCGCAAA-3′) and GlnKNi (5′-CCCATCCTCTAGACTTAAACACAGGGA-3′) were used, with plasmid pWVH141 as template DNA, to amplify a 500 bp region upstream of *glnK*, which ends with the first six codons of *glnK*. A similar pair of primers, AmtBCi (5′-TGTTTAAGTCTAGAGGATGGGG-ATGTCAACAGCCACGGCGAG-3′) and AmtBCo (5′-CGCGGAT-CCGCGCAAATGGTAGC-3′), was used to amplify a 500 bp region extending from the last seven codons of *amtB* into the downstream *tesB* gene. The two PCR products were purified, mixed and used as templates for a second PCR using primers GlnKNo and AmtBCo. The resulting PCR product was digested with *Bam*HI and cloned into *Bam*HI-cut pKO3 (Link *et al*., 1997). This unmarked deletion of *glnK–amtB* was then crossed onto the chromosome of strain ET8000. Resolution of the recombination was checked extensively by PCR. The unmarked deletion of *glnK* (Δ*glnK1*) that is not polar on *amtB* was transduced from strain WCH30 into ET8000 selecting for gentamicin-resistant mutants. Candidate transductants were checked using PCR and the resultant strain was designated GT1002.

In vivo [^{14}C]methylamine transport assays

These assays were performed as described previously using cells grown in M9Gln medium (Jack *et al*., 1999; Thomas *et al*., 2000b). [^{14}C]methylamine hydrochloride (2.15 Gbq/mmol) was obtained from Amersham Pharmacia, UK.

Western blotting

Protein concentrations of various cell fractions were determined using the Sigma Bradford reagent/Bio-Rad protein assay system using bovine serum albumin as a standard. In all cases, 5 μg of total protein was separated by either SDS–PAGE (15% polyacrylamide) or native PAGE (7.5% polyacrylamide). After transfer to a nitrocellulose membrane (Hybond ECL nitrocellulose membrane; Amersham), the proteins were reacted with either anti-P_{II} antibody (initially raised against purified *E.coli* GlnB protein, but also cross-reactive against *E.coli* and *A.vinelandii* GlnK), anti-AmtB antibody or anti-(tetra-)His antibody (Qiagen). Antibody against *E.coli* AmtB was a polyclonal antibody raised in rats against purified protein prepared in this laboratory (D.Blakey, A.Leach, G.H.Thomas, G.Coutts, K.Findlay and M.Merrick, in preparation). A 100 μl aliquot of whole-cell extract from strain FT8000 (Δ*glnB*,Δ*glnK*) was added routinely to the anti-P_{II} primary incubations to reduce non-specific interactions. Signals were then detected using the ECL system (Amersham).

Fractionation

A pre-culture grown in Luria broth was used to inoculate 500 ml of modified M9 medium, M9Gln (or Burk's sucrose medium –N for *Azotobacter*) using a 1:50 dilution. The cells were grown to an OD_{650} of 1.3–1.4 when they were harvested by centrifugation at 5000 r.p.m. at 4°C for 10 min in an SLA-1500 rotor and resuspended in 0.75 ml of 50 mM sodium phosphate buffer pH 7.0. For ammonia shock experiments, an initial 1 l culture was spilt into two cultures of 500 ml just prior to shock and one was treated by addition of NH_4Cl to a final concentration of 30 mM and harvested 15 min later. Breakage was achieved by five 15 s sonications on ice in an MSE Soniprep 150 sonicator at an amplitude of 10 µm with 15 s intervals between bursts. The extract was clarified by centrifugation in a microfuge at 16 000 *g* for 4 min. A 1 ml aliquot of the supernatant was then transferred to an 11 × 34 mm polycarbonate ultracentrifuge tube (Beckman) and centrifuged at 250 000 *g* at 4°C for 30 min using a Beckman TLA-120.2 rotor in a Beckman TL-100 ultracentrifuge; the remaining low-speed supernatant was used as the whole-cell fraction. After ultracentrifugation, the top 0.1 ml was taken from the tube and used as the cytoplasmic fraction; the rest of the supernatant was disposed of. The membrane pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer and centrifuged again at 250 000 *g*. The supernatant was discarded, and the membrane pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer and centrifuged again at 250 000 *g*. The pellet from this third centrifugation was resuspended in 50 µl of 50 mM sodium phosphate buffer and used as the membrane fraction. The purity of the membrane fractions was confirmed by western blotting with an antibody against a known cytoplasmic protein, namely pyruvate formate lyase.

To wash the membranes stringently, the cells were treated as above but after the third centrifugation the membrane pellet was resuspended in 50 mM sodium phosphate, 600 mM NaCl buffer pH 7.0. This was then centrifuged twice at 250 000 *g* using the same high-salt buffer, after which the pellet was resuspended in 16 µl (or other suitable volume) of 50 mM sodium phosphate buffer.

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References

Arcondéguy,T., van Heeswijk,W.C. and Merrick,M. (1999) Studies on the roles of GlnK and GlnB in regulating *Klebsiella pneumoniae* NifL-dependent nitrogen control. *FEMS Microbiol. Lett.*, **180**, 263–270.

Arcondéguy,T., Jack,R. and Merrick,M. (2001) P_{II} signal transduction proteins: pivotal players in microbial nitrogen control. *Microbiol. Mol. Biol. Rev.*, **65**, 80–105.

Atkinson,M. and Ninfa,A.J. (1998) Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. *Mol. Microbiol.*, **29**, 431–447.

Atkinson,M. and Ninfa,A.J. (1999) Characterization of the GlnK protein of *Escherichia coli*. *Mol. Microbiol.*, **32**, 301–313.

Bender,R.A., Janssen,K.A., Resnick,A.D., Blumenberg,M., Foor,F. and Magasanik,B. (1977) Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. *J. Bacteriol.*, **129**, 1001–1009.

Bishop,P.E. and Brill,W.J. (1977) Genetic analysis of *Azotobacter vinelandii* mutant strains unable to fix nitrogen. *J. Bacteriol.*, **130**, 954–956.

Bueno,R., Pahel,G. and Magasanik,B. (1985) Role of *glnB* and *glnD* gene products in regulation of the *glnALG* operon of *Escherichia coli*. *J. Bacteriol.*, **164**, 816–822.

Carr,P.D., Cheah,E., Suffolk,P.M., Vasudevan,S.G., Dixon,N.E. and Ollis,D.L. (1996) X-ray structure of the signal transduction protein P_{II} from *Escherichia coli* at 1.9 Å. *Acta Crystallogr. D*, **52**, 93–104.

Chang,A.C. and Cohen,S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.*, **134**, 1141–1156.

Chen,Y.M., Backman,K. and Magasanik,B. (1982) Characterisation of a gene, *glnL*, the product of which is involved in the regulation of nitrogen utilisation in *Escherichia coli*. *J. Bacteriol.*, **150**, 214–220.

Dandekar,T., Snel,B., Huynen,M. and Bork,P. (1998) Conservation of gene order: a fingerprint of proteins that physically interact. *Trends Biochem. Sci.*, **23**, 324–328.

de Zamaroczy,M. (1998) Structural homologues P_{II} and P_Z of *Azospirillum brasilense* provide intracellular signalling for selective regulation of various nitrogen-dependent functions. *Mol. Microbiol.*, **29**, 449–463.

Forchhammer,K. and Tandeau de Marsac,N. (1994) The P_{II} protein in the cyanobacterium *Synechococcus* sp. strain PCC 7942 is modified by serine phosphorylation and signals the cellular N-status. *J. Bacteriol.*, **176**, 84–91.

Forchhammer,K., Hedler,A., Strobel,H. and Weiss,V. (1999) Heterotrimerization of P_{II}-like signalling proteins: implications for P_{II}-mediated signal transduction systems. *Mol. Microbiol.*, **33**, 338–349.

He,L., Soupene,E., Ninfa,A.J. and Kustu,S. (1998) Physiological role for the GlnK protein of enteric bacteria: relief of NifL inhibition under nitrogen-limiting conditions. *J. Bacteriol.*, **180**, 6661–6667.

Hisbergues,M., Jeanjean,R., Joset,F., Tandeau de Marsac,N. and Bedu,S. (1999) Protein P_{II} regulates both inorganic carbon and nitrate uptake and is modified by a redox signal in *Synechocystis* PCC 6803. *FEBS Lett.*, **463**, 216–220.

Howitt,S. and Udvardi,M. (2000) Structure, function and regulation of ammonium transporters in plants. *Biochim. Biophys. Acta*, **1465**, 152–170.

Hsieh,M.H., Lam,H.M., van de Loo,F.J. and Coruzzi,G. (1998) A P_{II}-like protein in *Arabidopsis*: putative role in nitrogen sensing. *Proc. Natl Acad. Sci. USA*, **95**, 13965–13970.

Jack,R., de Zamaroczy,M. and Merrick,M. (1999) The signal transduction protein GlnK is required for NifL-dependent nitrogen control of *nif* expression in *Klebsiella pneumoniae*. *J. Bacteriol.*, **181**, 1156–1162.

Jaggi,R., Ybarlucea,W., Cheah,E., Carr,P.D., Edwards,K.J., Ollis,D. and Vasudevan,S.G. (1996) The role of the T-loop of the signal transducing protein P_{II} from *Escherichia coli*. *FEBS Lett.*, **391**, 223–228.

Jayakumar,A., Schulman,I., Macneil,D. and Barnes,E.M. (1986) Role of the *Escherichia coli glnALG* operon in regulation of ammonium transport. *J. Bacteriol.*, **166**, 281–284.

Jiang,P., Peliska,J.A. and Ninfa,A.J. (1998) Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the P_{II} protein. *Biochemistry*, **37**, 12782–12794.

Kobayashi,M., Rodriguez,R., Lara,C. and Omata,T. (1997) Involvement of the C-terminal domain of an ATP-binding subunit in the regulation of the ABC-type nitrate/nitrite transporter of the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J. Biol. Chem.*, **272**, 27197–27201.

Lee,S.J., Boos,W., Bouche,J.P. and Plumbridge,J. (2000) Signal transduction between a membrane-bound transporter, PtsG, and a soluble transcription factor, Mlc, of *Escherichia coli*. *EMBO J.*, **19**, 5353–5361.

Link,A.J., Phillips,D. and Church,G.M. (1997) Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol.*, **179**, 6228–6237.

Lorenz,M.C. and Heitman,J. (1998) The MEP2 ammonium permease regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *EMBO J.*, **17**, 1236–1247.

Marini,A.-M. and André,B. (2000) *In vivo N*-glycosylation of the Mep2 high-affinity ammonium transporter of *Saccharomyces cerevisiae* reveals an extracytosolic N-terminus. *Mol. Microbiol.*, **38**, 552–564.

Marini,A.-M., Matassi,G., Raynal,V., André,B., Cartron,J.-P. and Chérif-Zahar,B. (2000a) The human Rhesus-blood-group associated RhAG protein and a novel kidney homologue promote ammonium transport in yeast. *Nature Genet.*, **26**, 341–344.

Marini,A.-M., Springael,J.Y., Frommer,W.B. and André,B. (2000b) Cross-talk between ammonium transporters in yeast and interference by the soybean SAT1 protein. *Mol. Microbiol.*, **35**, 378–385.

Meletzus,D., Rudnick,P., Doetsch,N., Green,A. and Kennedy,C. (1998)

Characterization of the *glnK–amtB* operon of *Azotobacter vinelandii*. *J. Bacteriol.*, **180**, 3260–3264.

Montesinos,M.L., Muro-Pastor,A.M., Herrero,A. and Flores,E. (1998) Ammonium/methylammonium permeases of a cyanobacterium. Identification and analysis of three nitrogen-regulated *amt* genes in *Synechocystis* sp. PCC 6803. *J. Biol. Chem.*, **273**, 31463–31470.

Muro-Pastor,A.M., Ostrovsky,P. and Maloy,S. (1997) Regulation of gene expression by repressor localization: biochemical evidence that membrane and DNA binding by the PutA protein are mutually exclusive. *J. Bacteriol.*, **179**, 2788–2791.

Pioszak,A.A., Jiang,P. and Ninfa,A.J. (2000) The *Escherichia coli* PII signal transduction protein regulates the activities of the two-component system transmitter protein NRII by direct interaction with the kinase domain of the transmitter module. *Biochemistry*, **39**, 13450–13461.

Reyes-Ramirez,F., Little,R. and Dixon,R. (2001) Role of *Escherichia coli* nitrogen regulatory genes in the nitrogen response of the *Azotobacter vinelandii* NifL–NifA complex. *J. Bacteriol.*, **183**, 3076–3082.

Saier,M.H.,Jr *et al.* (1999) Phylogenetic characterisation of novel transport protein families revealed by genome analysis. *Biochim. Biophys. Acta*, **1422**, 1–56.

Shapiro,L. and Losick,R. (2000) Dynamic spatial regulation in the bacterial cell. *Cell*, **100**, 89–98.

Soupene,E., He,L., Yan,D. and Kustu,S. (1998) Ammonia acquisition in enteric bacteria: physiological role of the ammonium/methylammonium transport B (AmtB) protein. *Proc. Natl Acad. Sci. USA*, **95**, 7030–7034.

Tanaka,Y., Kimata,K. and Aiba,H. (2000) A novel regulatory role of glucose transporter of *Escherichia coli*: membrane sequestration of a global repressor Mlc. *EMBO J.*, **19**, 5344–5352.

Thomas,G., Coutts,G. and Merrick,M. (2000a) The *glnKamtB* operon: a conserved gene pair in prokaryotes. *Trends Genet.*, **16**, 11–14.

Thomas,G., Mullins,J.G.L. and Merrick,M. (2000b) Membrane topology of the Mep/Amt family of ammonium transporters. *Mol. Microbiol.*, **37**, 331–344.

van Heeswijk,W.C., Hoving,S., Molenaar,D., Stegeman,B., Kahn,D. and Westerhoff,H.V. (1996) An alternative P_{II} protein in the regulation of glutamine synthetase in *Escherichia coli*. *Mol. Microbiol.*, **21**, 133–146.

van Heeswijk,W.C., Wen,D., Clancy,P., Jaggi,R., Ollis,D.L., Westerhoff,H.V. and Vasudevan,S.G. (2000) The *Escherichia coli* signal transducers P_{II} (GlnB) and GlnK form heterotrimers *in vivo*: fine tuning the nitrogen signal cascade. *Proc. Natl Acad. Sci. USA*, **97**, 3942–3947.

Wang,R.F. and Kushner,S.R. (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene*, **100**, 195–199.

Xu,Y., Cheah,E., Carr,P.D., van Heeswijk,W.C., Westerhoff,H.V., Vasudevan,S.G. and Ollis,D. (1998) GlnK, a P_{II}-homologue: structure reveals ATP binding site and indicates how the T-loops may be involved in molecular recognition. *J. Mol. Biol.*, **282**, 149–165.

Yakunin,A.F. and Hallenbeck,P.C. (2000) AmtB is necessary for NH_4^+ induced nitrogenase switch-off and ADP-ribosylation in *Rhodobacter capsulatus*. In Pedrosa,F.O., Hungria,M., Yates,M.G. and Newton,W.E. (eds), *Nitrogen Fixation: From Molecules to Crop Productivity*. Kluwer Academic, Dordrecht, The Netherlands, pp. 95–96.

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