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Antagonism of PII signalling by the AmtB protein of *Escherichia coli*

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Summary

Escherichia coli **AmtB is a member of the MEP/Amt family of ammonia transporters found in archaea, eubacteria, fungi, plants and animals. In prokaryotes, AmtB homologues are co-transcribed with a PII paralogue, GlnK, in response to nitrogen limitation. Here, we show that AmtB antagonizes PII signalling through NRII and that co-expression of GlnK with AmtB overcomes this antagonism. In cells lacking GlnK, expression of AmtB during nitrogen starvation prevented deinduction of Ntr gene expression when a nitrogen source became available. The absence of AmtB in cells lacking GlnK allowed rapid reduction of Ntr gene expression during this transition, indicating that one function of GlnK is to prevent AmtB-mediated antagonism of PII signalling after nitrogen starvation. Other roles of GlnK in controlling Ntr gene expression and maintaining viability during nitrogen starvation were unaffected by AmtB. Expression of AmtB from a constitutive promoter under nitrogen-rich conditions induced full expression of *glnALG* and elevated expression of *glnK* in wild-type and *glnK* cells; thus, the ability of AmtB to raise Ntr gene expression did not require a factor found only in nitrogen-starved cells. Experiments with intact cells showed that AmtB acted downstream of a uridylyl transferase uridylyl-removing enzyme (UTase/UR) and upstream of NRII, suggesting that the target was PII. AmtB also slowed the deuridylylation of PII~UMP upon ammonia addition, showing that multiple PII interactions were affected by AmtB. Our data are consistent with a hypothesis that AmtB interacts with PII and GlnK, and that co-transcription of *glnK* and *amtB* prevents titration of PII when AmtB is highly expressed.**

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Introduction

The nitrogen regulatory (Ntr) system of *Escherichia coli* facilitates the efficient assimilation of nitrogen atoms from a variety of compounds containing reduced nitrogen into glutamine and glutamate (for reviews, see Merrick and Edwards, 1995; Ninfa *et al*., 2000). At high concentrations, ammonia enters cells without the help of Ntr gene products, and basal levels of glutamine synthetase (GS; encoded by *glnA*) convert the ammonia to glutamine at a rate that does not limit the growth rate. The intracellular glutamine concentration is detected by the product of the *glnD* gene, a bifunctional uridylyl-transferase/uridylyl-removing enzyme (UTase/UR) that catalyses the addition and removal of uridylyl groups from the signal transduction protein PII (encoded by *glnB*). The non-uridylylated form of PII, present when the glutamine concentration is high, activates the phosphatase activity of the two-component system 'transmitter' protein NRII (NtrB, the product of *glnL*), which in turn dephosphorylates the 'receiver' protein NRI (NtrC, the product of *glnG*), thereby preventing the activation of Ntr genes by NRI~P. During nitrogen limitation, the intracellular concentration of glutamine is low (Ikeda *et al*., 1996). Uridylylation of PII by the UTase/UR at low intracellular glutamine concentrations prevents PII from activating the phosphatase activity of NRII. Under these conditions, kinase activity of NRII phosphorylates NRI, which activates the transcription of genes whose products facilitate the scavenging of nitrogen atoms from various compounds containing reduced nitrogen (Zimmer *et al*., 2000).

The Ntr promoters exhibit different sensitivities to transcriptional activation by NRI~P, resulting in a defined sequence of activation with increasing NRI~P concentrations (Feng *et al*., 1995; Atkinson *et al*., 2002a). *glnALG* transcription is the most sensitive to activation by NRI~P and, at low intracellular glutamine concentrations, a positive feedback loop results, in which the levels of NRII and NRI are rapidly increased and NRI is phosphorylated. Elevated NRI~P levels are required for the transcription of operons such as *glnKamtB* and *nac* that have less efficient enhancers than *glnALG* (Atkinson *et al*., 2002a). Expression of *glnK* under conditions of nitrogen limitation results in the appearance of GlnK, a PII paralogue that

interacts with NRII similar to PII (Atkinson and Ninfa, 1999; Atkinson *et al*., 2002b) and is reversibly uridylylated by UTase/UR similar to PII (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1999).

Previous work with an in frame *glnK* deletion mutant (*glnK*$^-$*amtB*$^+$) identified several roles for the GlnK protein during the transition between various states of nitrogen availability (Blauwkamp and Ninfa, 2002a). Wild-type cells that became starved for nitrogen upon consumption of a limited amount of ammonia expressed Ntr genes for only about 4 h after the onset of nitrogen starvation. In the absence of GlnK, cells continued to express Ntr genes for at least 16 h after the onset of nitrogen starvation, suggesting that GlnK is required for the regulation of Ntr genes during nitrogen starvation. Furthermore, in the absence of GlnK, cells lost viability much faster than wild-type cells during nitrogen starvation and were unable to turn off Ntr gene expression when a nitrogen source(s) became available (Blauwkamp and Ninfa, 2002a). This persistent Ntr gene expression resulted in a Nac-mediated growth defect that lasted for at least 8 days (Blauwkamp and Ninfa, 2002b). In other words, GlnK prevents a very strong and debilitating memory of nitrogen starvation in *E. coli*.

The inclusion of *glnK* and *amtB* in a single operon in a number of prokaryotes and the nitrogen-regulated transcription of this operon suggest a possible interaction between GlnK and AmtB during nitrogen limitation (Dandekar *et al*., 1998; Thomas *et al*., 2000). AmtB is a member of the Mep/Amt family of membrane transporters, and homologues have been identified in eubacteria, archaea, fungi, plants, protists and recently humans; however, the exact role(s) in *E. coli* remain(s) unclear (Saier *et al*., 1999; Marini *et al*., 2000; Thomas *et al*., 2000). The *Saccharomyces cerevisiae* AmtB homologues, *MEP1*, *MEP2* and *MEP3*, have been shown to be high-affinity ammonia transporters (Marini *et al*., 1994; 1997). *Corynebacterium glutamicum* contains two Mep/Amt proteins, Amt and AmtB, which transport (methyl)ammonium (charged species only) and ammonium or ammonia respectively (Meier-Wagner *et al*., 2001). Soupene *et al*. (2002a) have presented evidence that *Salmonella typhimurium* AmtB facilitates diffusion of ammonia (uncharged species only) across the cytoplasmic membrane and that *E. coli* AmtB is required for growth in liquid culture at ammonia (uncharged species) concentrations <50 nM. In addition to roles in ammonia transport, roles for Mep/Amt proteins in signal transduction have also been suggested for *MEP2* in *S. cerevisiae* and for AmtB in *Rhodobacter capsulatus* (Lorenz and Heitman, 1998; Yakunin and Hallenbeck, 2002). Recently, a nitrogen-regulated interaction between GlnK and AmtB in *E. coli* has been reported based on the observation that GlnK associated with the membrane fraction of cell lysates in an AmtB-dependent manner (Coutts *et al*., 2002). These results led the authors to hypothesize that AmtB interacts directly with GlnK (Coutts *et al*., 2002).

In this work, we explored the roles of AmtB in the memory of nitrogen starvation, as well as its roles in ammonia sensing and signalling through the Ntr system. Our data show that AmtB expression is the cause of the debilitating memory of nitrogen starvation in cells lacking GlnK, and that AmtB antagonizes PII and GlnK signalling through NRII independent of the presence of ammonia. AmtB had no role in limiting Ntr gene expression during nitrogen starvation or in maintaining cell viability during nitrogen starvation in these experiments. Our results provide a molecular explanation for the conserved stoichiometric expression of *glnK* and *amtB* observed in all prokaryotes that have an AmtB homologue.

Results

AmtB imposes a GlnK requirement for recovery from nitrogen starvation

Previous results have shown that GlnK was essential for controlling Ntr gene expression during nitrogen starvation and lowering Ntr gene expression when nitrogen-starved cells were fed ammonia (Blauwkamp and Ninfa 2002a; Fig. 1). Here, we examined the role of AmtB in these processes. In order to probe the effects of GlnK and AmtB on Ntr induction during nitrogen-rich, nitrogen-starved and post-starvation nitrogen-rich conditions, cells were grown with a growth yield limiting the concentration of ammonia (0.005%), held in the starved state and then provided with ammonia by dilution into fresh medium, and the expression of a *glnKp–lacZYA* transcriptional fusion (*glnKp*ϕ) was monitored during the various growth phases. As shown in Fig. 1, wild-type cells grew rapidly without Ntr induction to an OD_{600} of $\approx$ 0.2, after which cell growth slowed as a result of the consumption of the ammonia, and Ntr gene transcription was induced for $\approx$ 4 h before ceasing again (Fig. 1, circles and solid bars). Similar to the wild type, cells containing an in frame deletion of *glnK*, strain K_3 (*glnK*$^-$*amtB*$^+$), also grew rapidly without Ntr induction to an OD_{600} of $\approx$ 0.2, but thereafter expressed Ntr genes continuously for at least 16 h (Fig. 1, squares and hollow bars). The rate of β-galactosidase accumulation did not differ significantly between wild type and K_3 (*glnK*$^-$*amtB*$^+$) cells (Table 1). However, as strain K_3 (*glnK*$^-$*amtB*$^+$) did not shut off Ntr gene expression, it accumulated *glnK* promoter-driven β-galactosidase to levels approximately five times higher than those observed in wild-type cells (Fig. 1). When diluted into fresh media containing the same initial concentration of ammonia (0.005%), wild-type cells immediately resumed rapid growth and shut off Ntr gene expression, whereas strain K_3 (*glnK*$^-$*amtB*$^+$) lagged before resuming growth at a

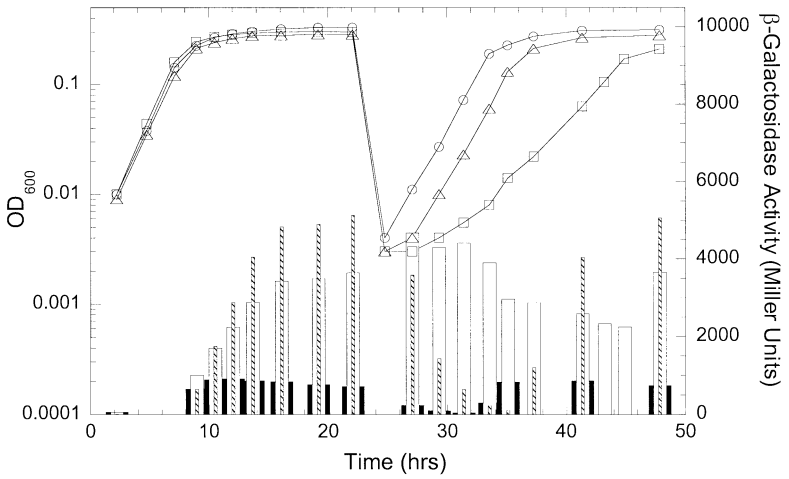

Fig. 1. Expression of *glnK*pϕ as the availability of ammonia changes. The optical density (line graph) and β-galactosidase activity (bar graph) of cells containing a single copy of the *glnK*pϕ were plotted as a function of time for cultures grown with 0.005% ammonium sulphate as the sole nitrogen source. Cells became nitrogen starved at ≈ 9 h, were held in the nitrogen-starved state for ≈ 13 h and subcultured into identical media (nitrogen-rich) near 25 h as shown by the drop in optical density. Wild type, circles and solid bars; K_3 (*glnK*$^-$*amtB*$^+$), squares and hollow bars; K_2 (*glnK*$^-$*amtB*$^-$), triangles and hatched bars.

much slower rate and did not turn off Ntr gene expression (Table 1; Fig. 1). Strain K_2, containing a deletion from the middle of *mdl* to the middle of *amtB* (*glnK*$^-$*amtB*$^-$), behaved similar to strain K_3 (*glnK*$^-$*amtB*$^+$) before dilution in that it grew rapidly without Ntr induction on the limited ammonia supply and expressed Ntr genes for at least 16 h after consumption of the ammonia (Fig. 1, triangles and hatched bars). Unlike strain K_3 (*glnK*$^-$*amtB*$^+$), strain K_2 (*glnK*$^-$*amtB*$^-$) rapidly lowered Ntr gene expression after dilution into fresh media and resumed rapid growth at the wild-type rate after a brief lag (Fig. 1; Table 1). Thus, GlnK was required in all cases to control Ntr gene expression during nitrogen starvation, but was only required to reduce Ntr gene expression and permit rapid growth after nitrogen starvation when AmtB was present.

To be sure that the GlnK and AmtB phenotypes were not specific to ammonia depletion and addition, we performed analogous experiments with glutamine as the sole nitrogen source. Glutamine at 0.04% (w/v) is not nitrogen limiting until the cells reach an OD_{600} of ≈ 0.2 (Atkinson *et al*. 2002a; this work). Cells were grown with 0.04% glutamine as the sole nitrogen source for ≈ 16 h to an OD_{600} ≈ 1.3 and diluted into fresh media containing 0.04% glutamine as the sole nitrogen source. As in the ammonia run-out experiments, strains lacking GlnK, regardless of AmtB presence, accumulated threefold higher levels of β-galactosidase activity expressed from the *glnK* promoter during the nitrogen starvation phase than wild-type cells (Fig. 2). Upon dilution into fresh media identical to the original growth media, wild-type cells and strain K_2 (*glnK*$^-$*amtB*$^-$) immediately resumed growth and turned off Ntr gene expression until they reached an OD_{600} of ≈ 0.2, after which Ntr gene transcription was activated again (Fig. 2; Table 2). In contrast, strain K_3 (*glnK*$^-$*amtB*$^+$) grew poorly and failed to lower Ntr gene expression when diluted into fresh media, analogous to the situation observed in the ammonia run-out experiments (Fig. 2; Table 2).

The poor growth of strain K_3 (*glnK*$^-$*amtB*$^+$) after starvation is also evident on solid defined media (Blauwkamp and Ninfa, 2002a). In order to determine whether AmtB contributed to the memory of nitrogen starvation in strain K_3 (*glnK*$^-$*amtB*$^+$) on solid media, samples of nitrogen-starved cells were plated on nitrogen-rich solid media and the colony sizes were observed. As observed in liquid media, strain K_3 (*glnK*$^-$*amtB*$^+$) grew poorly, producing pinpoint colonies barely visible in Fig. 3, and deletion of AmtB (strains K_2 and K_4, both *glnK*$^-$*amtB*$^-$) relieved the post-starvation growth defect (Fig. 3). Wild-type, K_2 and K_3 cells starved as described above were also plated on solid media containing either glutamine, ammonia and glutamine or glutamine and glutamate as the nitrogen source(s) and, in all cases, wild-type and K_2 (*glnK*$^-$*amtB*$^-$) cells grew rapidly, whereas K_3 (*glnK*$^-$*amtB*$^+$) cells grew as poorly as those shown in Fig. 2 (data not shown). As

Table 1. Effects of AmtB on *glnK* promoter firing rate during ammonia exhaustion.

	glnK promoter firing rate (Δβ-galactosidase units/ h/10^9 cells)[a]			Doubling time (min)
Strain	NH_3-rich (prestarv.)	Max. NH_3-poor (during starv.)	NH_3-rich (post-starv.)	NH_3-rich (post-starv.)
WT	<5	437	<5	92
K_3 (*glnK*$^-$*amtB*$^+$)	<5	515	550	153
K_2 (*glnK*$^-$*amtB*$^-$)	<5	836	<5	87

a. Values shown were calculated using the data in Fig. 1.

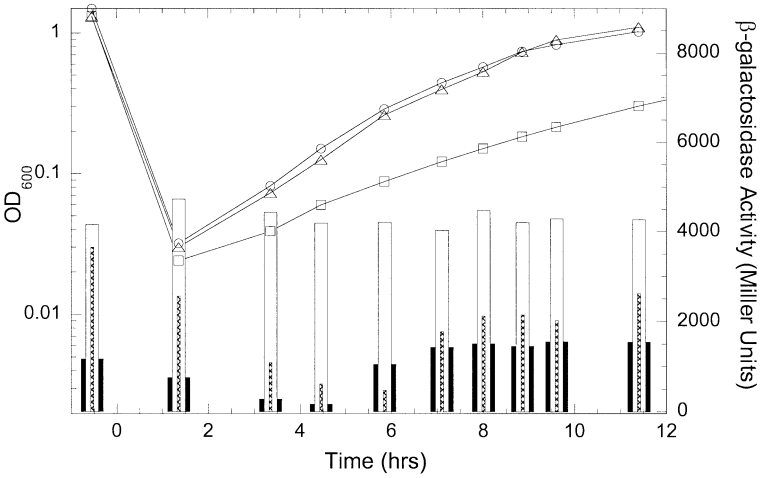

Fig. 2. Expression of *glnK*pϕ as the availability of glutamine changes. The optical density (line graph) and β-galactosidase activity (bar graph) of cells containing a single copy of the *glnK*pϕ were plotted as a function of time for cultures grown with 0.4% glutamine as the sole nitrogen source. Overnight cultures were diluted 1:100 into identical media (nitrogen-rich before OD of 0.2, nitrogen poor thereafter) and grown for 10 h. t_0 = time of subculture after 12 h of nitrogen starvation. Wild type, circles and solid bars; K_3 (*glnK*$^-$*amtB*$^+$), squares and hollow bars; K_2 (*glnK*$^-$*amtB*$^+$), triangles and hatched bars.

observed previously, the plate containing strain K_3 (*glnK*$^-$ *amtB*$^+$) has two distinct populations of colonies: a number of pinpoint colonies that approximates the number of colonies on the other plates and a few large colonies. We refer to the large colonies as 'fugitives' because we showed previously that they are not mutants, but have somehow escaped nitrogen starvation (Blauwkamp and Ninfa, 2002a). When resubjected to nitrogen starvation, the 'fugitives' grow very poorly in liquid and on solid media, like the original strain (Blauwkamp and Ninfa, 2002a).

Additional experiments showed that the upstream *mdl* gene, used in some of the genetic manipulations, did not play a role in the GlnK and AmtB phenotypes. We constructed two additional strains; strain MK_3 [*glnK*pϕ] contains the in frame *glnK* deletion of strain K_3 plus an insertion mutation in *mdl*, and strain K_4 contains the in frame deletion of strain K_3 plus an insertion mutation in *amtB*. In ammonia run-out experiments similar to those described above, strain K_4 (*mdl*$^+$*glnK*$^-$*amtB*$^-$) behaved exactly like strain K_2 (*mdl*$^-$*glnK*$^-$*amtB*$^-$) (Fig. 3), whereas strain MK_3 [*glnK*pϕ] (*mdl*$^-$*glnK*$^-$*amtB*$^+$) behaved exactly like strain K_3 (*mdl*$^+$*glnK*$^-$*amtB*$^+$) (data not shown).

Previous results have shown that cells lacking GlnK have a viability defect during nitrogen starvation (Blauwkamp and Ninfa, 2002a). It is evident from Figs 1 and 2 that the presence of AmtB has very little effect on the role of GlnK in limiting Ntr gene expression during nitrogen starvation, so we probed the role of AmtB in maintaining viability under these conditions. We tested the viability of wild-type, K_2 (*glnK*$^-$*amtB*$^-$), K_3 (*glnK*$^-$*amtB*$^+$) and *amtB* strains after 35 h of nitrogen starvation. We found no difference in the viability of strains K_2 (*glnK*$^-$*amtB*$^-$) and K_3 (*glnK*$^-$*amtB*$^+$), both of these strains having ≈ 200-fold fewer colony-forming units (cfu) than wild-type and *amtB* strains, despite the similar optical densities of all four strains (data not shown).

AmtB influences Ntr gene expression

As AmtB prevented deinduction of the Ntr system after nitrogen starvation in cells lacking GlnK, we tested whether AmtB caused elevated Ntr gene expression when ex-pressed from a constitutive promoter under nitrogen-rich conditions. The entire coding region of *amtB* from 57 nucleotides upstream of the start codon to 83 nucleotides downstream of the stop codon was cloned into the leaky IPTG-inducible vector, p*Trc*99A, to yield plasmid pAmtB1 (*Experimental procedures*). Similar to reported results using different expression systems (Blakely *et al*., 2002; Soupene *et al*., 2002b), IPTG induction of AmtB expression from pAmtB1 was toxic to *E. coli*. This toxicity had nothing to do with the expression of Ntr genes, as it was also observed in cells lacking NRI (data not shown). However, the level of AmtB expressed from the leaky *Trc*

Table 2. Effects of AmtB on *glnK* promoter firing rate during glutamine exhaustion.

	glnK promoter firing rate (Δβ-galactosidase units h^{-1} 10^{-9} cells)[a]		Doubling time (min)	
Strain	Preshift	Post-shift	Preshift	Post-shift
WT	>5	905	85	136
K_3 (*glnK*$^-$*amtB*$^+$)	1116	996	146	220
K_2 (*glnK*$^-$*amtB*$^-$)	>5	1411	82	127

a. Values shown were calculated using the data in Fig. 2.

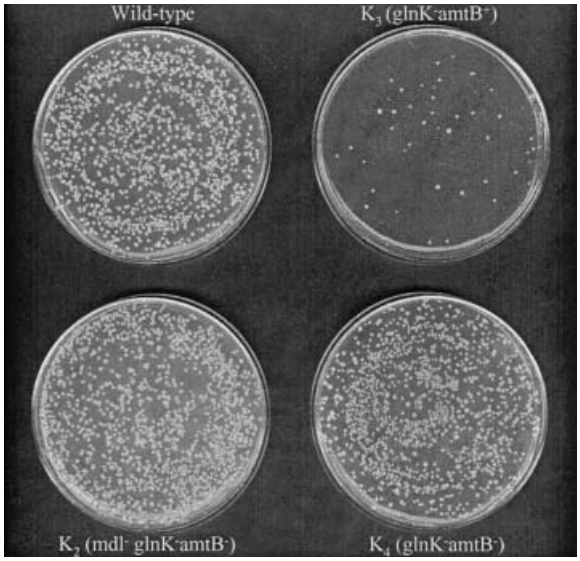

Fig. 3. Memory of nitrogen starvation. Cells of the indicated genotype were grown in media containing 0.005% ammonium sulphate as the sole nitrogen source, held in the nitrogen-starved state for ≈ 12 h, diluted with sterile water and grown on solid media containing 0.2% ammonium sulphate as the sole nitrogen source for 40 h at 37°C.

promoter in the absence of IPTG was not toxic to wild-type cells and significantly affected Ntr gene expression. In wild-type cells, uninduced (leaky) expression of *amtB* from pAmtB1 caused full induction of the *glnA* promoter and partial induction of the *glnK* promoter on nitrogen-rich media containing ammonia and glutamine, as measured by transcriptional fusions of *lacZYA* to both promoters (Table 3). In strain K_2 (*glnK*$^-$*amtB*$^-$), pAmtB1 fully induced *glnA* promoter expression and raised *glnK* promoter transcription at least 400-fold relative to the vector alone, an effect 10 times greater than that observed in the wild-type strain (Table 3). pAmtB1 also caused a growth defect in strain K_2 (*glnK*$^-$*amtB*$^-$) that was not observed in the wild type (Table 3).

Previous results have shown that strong constitutive Ntr gene expression caused a growth defect resulting from Nac-mediated repression of *serA* that could be relieved by deletion of *nac* or inclusion of glycine in the growth media (Blauwkamp and Ninfa, 2002b). Either inclusion of glycine in the growth media (Table 3) or deletion of Nac (Table 4) relieved the growth defect of strain K_2 (*glnK*$^-$ *amtB*$^-$) harbouring pAmtB1, suggesting that AmtB raised Ntr gene expression to the point at which Nac significantly inhibited *serA* expression. A strain identical to K_2 (*glnK*$^-$ *amtB*$^-$), except containing an in frame deletion within *glnL*, was transformed with pAmtB1, and this strain did not show elevated Ntr gene transcription or the growth defect, consistent with the above hypothesis (Table 4). Thus, AmtB directly or indirectly influenced the activity of NRII.

To determine whether AmtB influenced NRII activity by limiting the availability of nitrogen, such as by facilitating the exit of ammonia/ammonium from the cell, the effect of AmtB expression from pAmtB1 was monitored in strain K_2D (*glnK*$^-$*amtB*$^-$ *glnD*$^-$). This strain lacks UTase/UR and cannot transmit a low nitrogen signal to NRII/NRI via PII uridylylation (Bueno *et al*., 1985; Atkinson and Ninfa, 1998). Expression of AmtB in K_2D caused the same growth defects and elevated Ntr gene expression as it did in strain K_2 (Table 3). These data suggest that the elevated Ntr gene expression was not a result of limited intracellular nitrogen availability. These data also show that AmtB did not affect Ntr gene expression by altering the activity of UTase/UR towards PII.

AmtB influenced Ntr gene expression regardless of ammonia presence

Reports of nitrogen-sensing roles for AmtB in *R. capsulatus* and *MEP2* in *S. cerevisiae* prompted us to explore the possible dependence of AmtB phenotypes on the presence of ammonia (Lorenz and Heitman, 1998; Yakunin and Hallenbeck, 2002). We found that AmtB raised the

Table 3. Effects of AmtB on growth and Ntr-regulated gene expression.

	Doubling time (min)		β-Galactosidase activity (Miller units)	
Strain	GNg	GNg ± Gly	GNg	GNg ± Gly
WT-*glnAp*ϕ [ptrc99a]	120	67	79	71
K_2-*glnAp*ϕ [ptrc99a]	128	72	63	53
K_2D-*glnAp*ϕ [ptrc99a]	141	74	33	32
WT-*glnAp*ϕ [pAmtB1]	110	75	2406	2032
K_2-*glnAp*ϕ [pAmtB1]	216	83	2825	2918
K_2D-*glnAp*ϕ [pAmtB1]	233	88	3087	3366
WT-*glnKp*ϕ [ptrc99a]	124	72	0	0
K_2-*glnKp*ϕ [ptrc99a]	125	73	0	0
K_2D-*glnKp*ϕ [ptrc99a]	135	77	0	0
WT-*glnKp*ϕ [pAmtB1]	101	71	252	102
K_2-*glnKp*ϕ [pAmtB1]	226	81	3050	2207
K_2D-*glnKp*ϕ [pAmtB1]	231	78	2807	1991

Table 4. AmtB signals upstream of NRI.

Strain	Doubling time (min)	β-Galactosidase activity (Miller units)
WT-*glnA*pϕ [ptrc99a]	97	57
WT-*glnA*pϕ [pAmtB1]	93	2281
LG-*glnA*pϕ [ptrc99a]	91	24
LG-*glnA*pϕ [pAmtB1]	93	27
L-*glnA*pϕ [ptrc99a]	87	38
L-*glnA*pϕ [pAmtB1]	91	32
K_2N_1 [ptrc99a]	91	ND
K_2N_1 [pAmtB1]	94	ND

expression of the *glnA* promoter equally well in LB media with and without the addition of ammonia, and in defined media containing glutamine or both glutamine and ammonia as nitrogen sources (Table 5). However, as shown in Fig. 2 and reported previously (Atkinson *et al*., 2002a), media containing 0.04% glutamine as the sole nitrogen source do not become nitrogen limiting until an approximate OD_{600} of 0.2, consistent with the possibility that these media may contain a small amount of ammonia that is consumed as the cells grow. Therefore, to ensure that the cells were growing in the absence of ammonia, the effect of AmtB on Ntr gene expression was monitored in cells grown on 0.04% glutamine well before ('preshift') and after ('post-shift') the optical density at which Ntr gene expression was induced in wild-type cells. As cells grown with glutamine as the sole nitrogen source ('post-shift') have elevated Ntr gene transcription regardless of AmtB presence, we examined the effects of AmtB in cells lacking *glnD*, in which Ntr gene expression is low. As shown in Table 5, pAmtB1 raised the expression of *glnA*pϕ in cells lacking UTase/UR, even in the absence of ammonia. Thus, the AmtB activity detected in our physiology experiments was not regulated by ammonia.

PII and GlnK antagonize AmtB similarly

The results presented thus far suggest that AmtB antagonizes both PII and GlnK. To date, all the different functions of PII and GlnK in *E. coli* have been found to result from differences in their timing of expression and level of accumulation (Atkinson *et al*., 2002b; Blauwkamp and Ninfa, 2002a). We explored the relative abilities of PII and GlnK to abrogate AmtB effects on Ntr induction. We described previously the construction of a gene fusion containing PII expressed from the *glnK* promoter and have shown that the timing and level of PII expression was very similar to that of GlnK when both were expressed from the *glnK* promoter (Atkinson *et al*., 2002b). In the present work, the *glnK* promoter-driven PII and the analogous *glnK* promoter-driven GlnK were placed onto the chromosome of strain K_2 (*glnK*$^-$*amtB*$^-$) in single copy, and the expression of a separate single-copy *glnK*p–*lacZYA* fusion was monitored (*Experimental procedures*). The extent to which pAmtB1 raised the steady-state Ntr gene expression level in wild-type cells and cells containing PII or GlnK expressed from the *glnK* promoter is shown in Table 6. Only slightly higher (≈ 2.4-fold) levels of Ntr gene expression were observed when PII was expressed from the *glnK* promoter instead of GlnK. Relative to the >10-fold elevation in Ntr gene expression in cells lacking an inducible PII/GlnK (Table 3), this 2.4-fold difference suggests that PII and GlnK overcame AmtB antagonism similarly.

AmtB slows the deuridylylation of PII~UMP

To explore why *glnK*$^-$*amtB*$^+$ strains fail to lower Ntr gene expression upon addition of ammonia to nitrogen-starved cultures, we investigated the kinetics of PII deuridylylation under these conditions. All strains, regardless of GlnK or AmtB presence, uridylylated PII during the nitrogen starvation phase as shown 10 min before ammonia addition (Fig. 4, t_0 = ammonia addition). After the addition of ammonia, wild-type cells took ≈ 1 h to deuridylylate PII completely. Strain K_3 (*glnK*$^-$*amtB*$^+$) also took ≈ 1 h to deuridylylate PII completely. Strain K_4 (*glnK*$^-$*amtB*$^-$) and strain K_2 (*mdl*$^-$*glnK*$^-$*amtB*$^-$; data not shown) took <1 min to deuridylylate PII. Strain X (*glnK*$^+$*amtB*$^-$) took <15 min to deuridylylate PII. Interestingly, strains lacking AmtB did not completely deuridylylate PII even after 2 h despite their faster initial rate of deuridylylation. These results show that both AmtB and GlnK influence the kinetics of PII deuridylylation, although these differences fail to explain the growth phenotypes of these strains. Wild-type growth differs most significantly from K_3 (*glnK*$^-$*amtB*$^+$) growth, yet the PII deuridylylation kinetics are similar for these strains. Furthermore, wild-type deuridylylation kinet-

Table 5. Ammonia is not required for AmtB signalling.

	β-Galactosidase activity (Miller units)				
Strain	LB	LBN	GNgln	Ggln (preshift)	Ggln (post-shift)
WT-*glnA*pϕ [ptrc99a]	37	69	79	428	4152
WT-*glnA*pϕ [pAmtB1]	1433	1697	2406	2144	4486
D-*glnA*pϕ [ptrc99a]	ND	ND	ND	186	623
D-*glnA*pϕ [pAmtB]	ND	ND	ND	1426	1988

Table 6. Relative effects of GlnK and PII on AmtB function.

Strain	Doubling time (min)	β-Galactosidase activity (Miller units)
WT [ptrc99a]	90	0
TAB11812 (*glnK*pK) [ptrc99a]	99	0
TAB11814 (*glnK*pB) [ptrc99a]	104	0
WT [pAmtB1]	91	204
TAB11812 (*glnK*pK) [pAmtB1]	98	184
TAB11814 (*glnK*pB) [pAmtB1]	102	465

ics differ most significantly from K_2 (*glnK*$^-$*amtB*$^-$), yet these strains show similar growth phenotypes.

Discussion

Refining the roles of PII, GlnK and AmtB in E. coli

In this work, we have explored the roles of PII, GlnK and AmtB in nitrogen regulation. We have found that GlnK is required to regulate Ntr gene expression during nitrogen starvation for two reasons. GlnK was required, regardless of AmtB, to prevent rapid loss of viability and the associated 'run-away' expression of Ntr genes during nitrogen starvation (Figs 1 and 2; Blauwkamp and Ninfa, 2002a). Additionally, GlnK was required to prevent a debilitating memory of nitrogen starvation in cells containing intact *amtB* (Figs 1–3; Blauwkamp and Ninfa, 2002a). We identified the molecular mechanism imparting the debilitating memory of starvation as the accumulation of disproportionate levels of AmtB relative to GlnK. Accumulated AmtB, in the absence of proportional levels of GlnK, prevented PII from lowering Ntr gene expression when a good nitrogen source became available (Tables 1 and 2), probably by titrating PII (see below). This led to constitutive expression of Ntr genes regardless of nitrogen availability and a growth defect caused by Nac repression of *serA* (Tables 1–4; Blauwkamp and Ninfa, 2002b). This may explain why nearly every prokaryote containing an AmtB homologue also contains a GlnK homologue under similar transcriptional control (Thomas *et al*., 2000). These results show that GlnK is required for proper regulation of NRII during nitrogen starvation and for proper regulation of AmtB during the transition from nitrogen starvation to nitrogen-rich growth.

The role of PII was also expanded by this work in that the fixed level of PII was fully capable of regulating the elevated NRII levels associated with nitrogen starvation upon transition from nitrogen starvation to nitrogen-rich growth, as long as the levels of GlnK and AmtB were proportional (or completely absent) (Figs 1 and 2, strain K_2). Although not expected, this result is not that surprising as the PII-activated phosphatase activity of NRII is stronger than the kinase activity, making only a small amount of PII relative to NRII necessary to dephosphorylate NRI~P. Additionally, the concentration of NRII rises only to a certain point (Atkinson and Ninfa, 1993), even in the absence of GlnK, because of NRI~P-mediated gov-

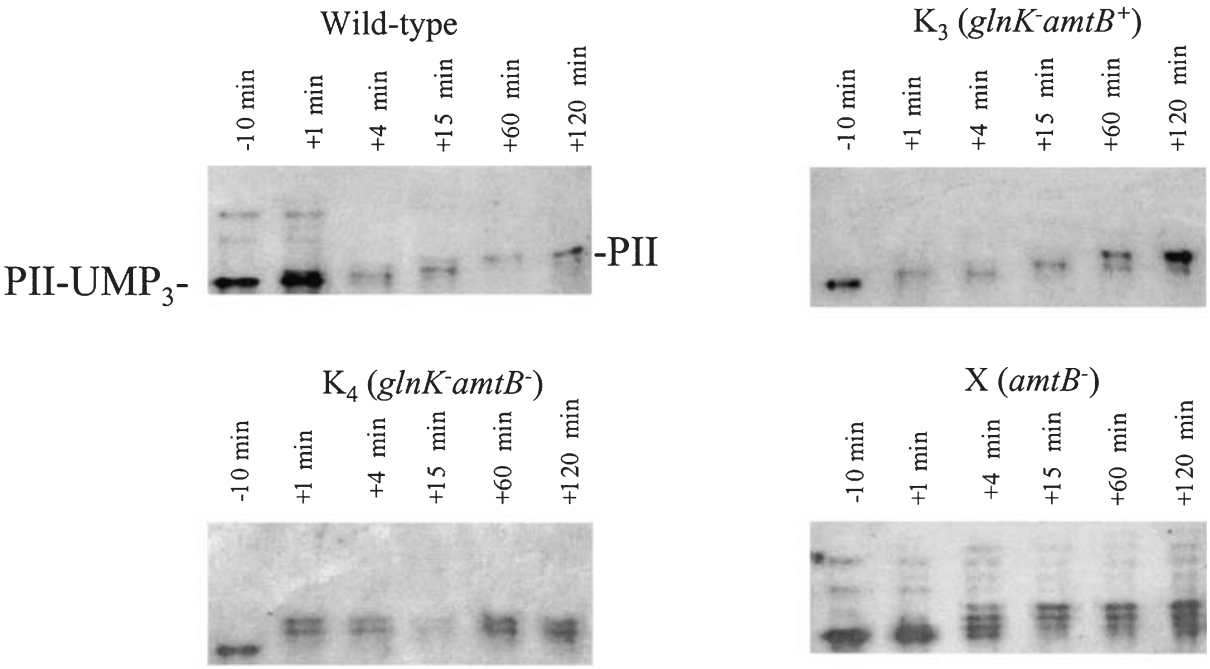

Fig. 4. Kinetics of PII~UMP deuridylylation in GlnK and AmtB mutants. The uridylylation state of PII was determined before and at various times after the addition of ammonium sulphate (0.1% final concentration) to 12 h nitrogen-starved cells (see *Experimental procedures*). Time 0 corresponds to the addition of ammonium sulphate. PII was observed by Western blotting with polyclonal antisera against PII.

ernment of *glnALG* transcription (Atkinson *et al*., 2002c; Blauwkamp and Ninfa, 2002a). Apparently, the combination of transcriptional governing of the *glnALG* operon and strong PII-activated phosphatase activity prevents the NRII concentration from exceeding the amount that a fixed level of PII can control.

AmtB antagonizes PII and GlnK signalling through NRII

Evidence from this study suggests that AmtB affects Ntr induction in the absence of GlnK by sequestering PII. Expression of PII from the *glnK* promoter abrogated the effect of AmtB expression nearly as well as GlnK, suggesting that additional expression of either PII-like protein is sufficient to overcome PII titration by AmtB. Also, using a plasmid that expressed AmtB from the leaky *Trc* promoter, we observed that AmtB expression raised Ntr gene expression during nitrogen-rich growth as well. However, AmtB did not affect Ntr gene expression in cells lacking NRII, indicating that AmtB signalled through NRII to increase the concentration of NRI~P. Expression of AmtB in strains lacking intact UTase/UR, and therefore unable to signal nitrogen starvation to PII or GlnK via uridylylation, resulted in elevated Ntr gene expression. This latter result indicates that, although AmtB may still affect intracellular nitrogen availability because of its function as an ammonia permease, changes in the uridylylation state of PII and GlnK were not the cause of AmtB-mediated Ntr induction. Taken together, these results show that AmtB acts upstream of NRII and downstream of UTase/UR in the signalling pathway, implying that its target is either PII or NRII.

Formally, hypotheses regarding the mechanism of AmtB-mediated Ntr gene activation can be divided into those that make PII less available for interaction with its receptors (such as changes in α-ketoglutarate concentration or titration of free PII by AmtB) and those that do not affect PII activity (such as AmtB activation of NRII kinase activity or competition for a binding site on NRII with PII). We attempted to distinguish the PII-dependent mechanisms from PII-independent mechanisms by looking for AmtB-mediated effects in cells lacking PII and GlnK. Although AmtB expression did not produce any significant change in growth rate or Ntr gene expression in cells lacking PII and GlnK, the effects may be masked by the already strong (probably maximal) expression of Ntr genes in cells lacking PII and GlnK (Blauwkamp and Ninfa, 2002b; data not shown). In support of a PII-dependent mechanism, AmtB also disrupts the interaction of PII~UMP with UTase/UR as shown in Fig. 4.

Our results are consistent with and extend the recent results of Coutts *et al*. (2002), who observed an AmtB-dependent association of PII and GlnK with the membrane fraction of *E. coli* cell lysates, suggesting that both interact directly with AmtB (Coutts *et al*., 2002). These workers also reported that GlnK regulated the methylammonium transport activity of AmtB twofold; however, our results suggest an alternative interpretation of these data. As shown in Fig. 2, the presence of the non-polar Δ*glnK1* mutation (strain K_3) resulted in approximately twofold higher levels of *glnK* promoter expression on media containing glutamine as the sole nitrogen source. As this probably corresponds to a twofold increase in AmtB expression from the *glnK* promoter, this could account for the reported twofold GlnK-dependent increase in methylammonium transport activity in the strains lacking GlnK (Coutts *et al*., 2002). Future experiments should be conducted with purified components to examine whether GlnK and PII directly regulate the AmtB permease activity.

If AmtB is a PII/GlnK receptor, then its ability to sequester PII upon accumulation to non-physiological levels is consistent with the behaviour of the known PII/GlnK receptors. We will show elsewhere that expression of UTase/UR or glutamine synthetase adenyltransferase (ATase) from multicopy plasmids in cells lacking GlnK also elevates Ntr gene expression to the point at which these cells behave like cells lacking PII and GlnK (unpublished data). Even low expression of a UTase/UR mutant (D105N) with very little enzymatic activity caused full induction of GS expression (A. J. Ninfa and M. R. Atkinson, unpublished data). Furthermore, overexpression of NRII also raised Ntr gene expression under nitrogen-rich conditions (unpublished data). Wild-type cells avoid these complications by controlling the expression of all known PII receptors in a way that prevents them from potentially titrating PII. Expression of *glnALG* is turned off when NRI levels accumulate to a certain point, such that the NRII concentration does not increase to levels more than 10- to 12-fold higher than those observed during nitrogen-rich growth (Atkinson and Ninfa, 1993; Atkinson *et al*., 2002c, Blauwkamp and Ninfa, 2002a). The *glnD* and *glnE* genes are not nitrogen regulated and are expressed at a constant low level (van Heeswijk *et al*., 1993). Co-expression of AmtB and GlnK at proportional levels ensures enough free PII to regulate all its receptors. Clearly, the levels of PII and its receptors are fine-tuned, and large changes in the relative levels of PII and its receptors are avoided.

Although PII-independent mechanisms for AmtB signalling have not been rigorously excluded, our data are best explained at this time by a model in which proportional amounts of GlnK and AmtB are required to prevent AmtB from titrating PII and thereby preventing its interaction with its other receptors such as NRII and UTase/UR.

Revised view of the transitions between nitrogen-excess growth and nitrogen starvation

In view of the recent advances in our understanding of the

roles of PII, GlnK and AmtB, we present the following model of Ntr regulation during the transitions between various states of nitrogen availability. PII is almost entirely responsible for regulating the expression of Ntr genes during nitrogen-rich growth (Blauwkamp and Ninfa, 2002a; this work). Upon nitrogen limitation, PII is uridylylated, and *glnALG* expression is induced, resulting in a positive feedback loop that rapidly amplifies the level of NRII and NRI(~P). NRII and NRI~P concentrations are only increased ≈ 10- to 12-fold because high concentrations of NRI~P mediate governing of the *glnA* promoter (Atkinson *et al*., 2002c). This high concentration of NRI~P also induces transcription of less sensitive Ntr genes, including *glnKamtB* and *nac*. Uridylylation of PII appears to be complete during starvation as expression of GlnK is required to prevent runaway expression of Ntr genes (other than *glnALG*) under these conditions (Blauwkamp and Ninfa, 2002a; this work). GlnK acts through NRII to shut off Ntr gene expression in nitrogen-starved cells such that only a short burst of Ntr gene expression occurs after the onset of nitrogen starvation (Fig. 1). The mechanism by which GlnK turns off Ntr gene expression after a short burst remains unclear. One possible explanation is that the uridylylation of GlnK is incomplete and, upon sufficient accumulation of GlnK, the unmodified fraction reaches a concentration able to activate the phosphatase activity of NRII and thereby turn off Ntr gene expression. Failure to limit Ntr gene expression during nitrogen starvation in cells lacking GlnK is correlated with a much faster loss of viability under these conditions (Blauwkamp and Ninfa, 2002a). Neither the unchecked Ntr gene expression nor the viability defects of strains lacking GlnK is influenced by AmtB expression (Figs 1 and 2; data not shown).

When nitrogen-starved cells are provided with a good nitrogen source, PII and GlnK are deuridylylated and activate phosphatase activity of NRII, ensuring that Ntr genes are not expressed. The fixed level of PII is fully capable of ensuring that Ntr genes are not expressed when a good nitrogen source is available; however, the expression of AmtB requires that proportional levels of GlnK be present to prevent AmtB from titrating PII (Blauwkamp and Ninfa, 2002a; this work).

Multiple roles of AmtB and GlnK

It appears that the presence of the *glnKamtB* operon in many bacterial species serves multiple purposes. First, it allows cells to assimilate very low concentrations of ammonia during nitrogen starvation (Soupene *et al*., 2002a) without preventing PII signalling. Secondly, it provides the cell with additional PII-like protein required for optimal cell viability maintenance during nitrogen starvation (Blauwkamp and Ninfa, 2002a). Thirdly, given the likely ability of AmtB to bind PII and GlnK, it may also play a role in regulating nitrogen assimilation. The sequestering of GlnK by AmtB in the presence of ammonia has been proposed to account for one aspect of nitrogen assimilation in *Klebsiella pneumoniae*. Unuridylylated GlnK prevents NifL inhibition of NifA-mediated transcriptional activation of *nif* genes (He *et al*., 1998). Upon addition of ammonia to nitrogen-limited cultures, AmtB binding of unuridylylated GlnK has been proposed to sequester GlnK, thereby freeing NifL to inhibit NifA (Coutts *et al*., 2002). The selective binding of unuridylylated PII and GlnK by AmtB reported by Coutts *et al*. (2002) should also modulate GlnK and PII interactions with the other PII receptors (UTase/UR, NRII and ATase in *E. coli*). Furthermore, if ammonia regulated the potential of AmtB to antagonize PII and GlnK signalling, AmtB would also provide an additional sensory element to the Ntr system. We did not observe an effect of ammonia on the ability of AmtB to antagonize PII signalling in our experiments. However, in our experiments, the effects of AmtB were detected in cells modestly overexpressing AmtB from either its own promoter (*glnKp*) or from a multicopy plasmid. This level of expression caused dramatic phenotypes, but may have obscured regulation of the AmtB–PII/GlnK interaction by ammonia that may occur at physiological levels of AmtB.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are shown in Table 7. Defined media was W-salts (Pahel *et al*., 1978) supplemented with 0.005% thiamine hydrochloride and carbon and nitrogen sources as indicated in the text. Ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (20 μg ml^{-1}) and gentamicin (10 μg ml^{-1} on W-salts, 50 μg ml^{-1} on LB) were included when appropriate. Preparation of LB media and plasmid transformation were performed as described previously (Maniatis *et al*., 1982). Plasmid DNA was prepared with the Concert Rapid Plasmid preparation kits (Marligen Bioscience). Generalized transduction was performed as described using P1vir (Silhavy *et al*., 1984) and used to move the antibiotic marker and linked mutations between strains. All experiments were performed at 37°C.

Measurement of growth rate and glnK promoter expression during nitrogen-rich, nitrogen-starved and post-starvation nitrogen-rich growth conditions

For ammonia exhaustion experiments, overnight cultures grown in W-salts supplemented with 0.4% (w/v) glucose, 0.2% (w/v) ammonium sulphate, 0.005% tryptophan and 50 μg ml^{-1} kanamycin were washed twice and diluted 1:500 into W-salts supplemented as above, except containing 0.005% (w/v) ammonium sulphate. Samples were removed from the cultures at the indicated times, and the optical density and β-galactosidase activity were determined. Optical

Table 7. Strains and plasmids used in this study.

Strain[a]	Relevant genotype[b]	Source or construction
WT (YMC10)	*endA1 thi-1 hsdR17 supE44 ΔlacU169 hutC$_{klebs}$*	Backman *et al.* (1981)
WT[*glnK*pϕ] (YMC10ϕ)	*trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	Atkinson and Ninfa (1998)
K$_2$[*glnK*pϕ] (Kϕ)	*Δmdl-glnK*::chl^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	Atkinson and Ninfa (1998)
K$_3$[*glnK*pϕ]	ΩGm^r…*ΔglnK1 trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	Blauwkamp and Ninfa (2002b)
K$_4$[*glnK*pϕ]	ΩGm^r…*ΔglnK1 amtB*::chl^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	This work, see *Experimental procedures*
MK$_3$[*glnK*pϕ]	ΩGm^r…*ΔglnK1 mdl*::chl^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	K$_3$ϕ × MAM P1 *vir*; Gm$^+$chl$^+$
MAM	*recD mdl*::chl^r	Atkinson and Ninfa (1998)
X	*amtB*::chl^r	Atkinson and Ninfa (1998)
X[*glnK*pϕ]	*amtB*::chl^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	WT[*glnK*pϕ] × X P1 *vir*
WT[*glnAp2*ϕ] (YMC10Apϕ2)	*trpDC700::putPA1303* [kan^r chl^s *glnAp2-lacZYA*]	Atkinson *et al.* (2002a)
K$_2$[*glnAp2*ϕ]	*Δmdl-amtB*::chl^r trpDC700::*putPA1303* [kan^r chl^s *glnAp2-lacZYA*]	WT[*glnAp2*ϕ] × MAKc P1 *vir*
MAKc	*Δmdl-glnK*::chl^r	Atkinson and Ninfa (1998)
K$_2$D[*glnAp2*ϕ]	*Δmdl-amtB*::chl^r *glnD99*::Tn*10 trpDC700::putPA1303* [kan^r chl^s *glnAp2-lacZYA*]	K$_2$[*glnAp2*ϕ] × DE P1 *vir*
K$_2$D[*glnK*pϕ]	*Δmdl-amtB*::chl^r *glnD99*::Tn*10 trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	K$_2$[*glnK*pϕ] × DE P1 *vir*
DE	*glnD99*::Tn*10 glnE*::Tn*5*	RB9040 × EB2717 P1 *vir*
RB9040	*glnD99*::Tn*10*	Bueno *et al.* (1985)
EB2717 (RB9098)	*ΔglnE*::Tn*5 glnL-302*	B. Magasanik
K$_2$N$_1$	*Δmdl-amtB*::chl^r *nac-28*(*kan^r*)	MAKc × EB3365 P1 *vir*
EB3365	*nac-28* (kan^r)	Muse and Bender (1998)
L[*glnAp2*ϕ]	*glnL2001 trpDC700::putPA1303* [kan^r chl^s *glnAp2-lacZYA*]	RB9132 × MAAplac3 P1 *vir*
RB9132	*glnL2001*	Bueno *et al.* (1985)
MAAplac3	*trpDC700::putPA1303* [kan^r chl^s *glnAp2-lacZYA*]	Atkinson *et al.* (2002a)
LG[*glnA*p2ϕ]	*ΔglnLG trpDC700::putPA1303* [kan^r chl^s *glnAp2-lacZYA*]	YMC10LG × MAAplac3 P1 *vir*
YMC10LG	*ΔglnLG*	YMC10 × SN24 P1 *vir*
SN24	*ΔglnLG lacI^q lacL8/λ*gln105	Schneider *et al.* (1991)
D[*glnA*p2ϕ]	*glnD99*::Tn*10*	RB9040 × MAAplac3 P1 *vir*
TAB11812	*Δmdl-amtB*::chl^r Δ*rbs*::*glnK*…gent^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	K$_2$[*glnK*pϕ] × TAB11802 P1 *vir*
TAB11814	*Δmdl-amtB*::chl^r Δ*rbs*::*glnK*p-*glnB*…gent^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	K$_2$[*glnK*pϕ] × TAB11804 P1 *vir*
TAB11802	*recD rbs*::*glnK*…gent^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	K4633 × pGlnK101 DNA
TAB11804	*recD rbs*::*glnK*p-*glnB*…gent^r *trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	K4633 × pGlnKpB101 DNA
K4633	*recD*::Tn*10*	D. Friedman
BK$_2$[*glnK*pϕ]	*ΔglnB ΔglnK-amtB::chl^r trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	Bϕ × MAKc P1 *vir*
Bϕ	*ΔglnB trpDC700::putPA1303* [kan^r chl^s *glnK*p-*lacZYA*]	Atkinson *et al.* (1998)

a. Parentheses bracket strain names as designated in the given reference.
b. All strains were derived from YMC10, except WCH30 and K4633.

density at 600 nm was monitored in a Beckman DU65 spectrophotometer, and β-galactosidase activity was measured by the Miller assay and expressed as Miller units, using SDS and chloroform to disrupt the cells, as described previously (Miller, 1992). Once the cultures stopped growing and Ntr gene transcription was activated, the cells were considered to be nitrogen starved. Cells were fed nitrogen after nitrogen starvation either by subculturing at 1:100 into fresh media containing 0.005% ammonium sulphate as the sole nitrogen source or by plating a diluted aliquot of cells from nitrogen-starved cultures on solid media containing 0.4% glucose, 0.005% tryptophan and either 0.2% ammonia, 0.2% glutamine, 0.2% ammonia plus 0.2% glutamine or 0.2% ammonia plus 0.2% glutamate as the sole nitrogen source(s). In the glutamine exhaustion experiments, cells were grown as described above, except that 0.04% glutamine was used instead of ammonia and the cells were not washed. The *glnK* promoter firing rate (β-galactosidase units ml^{-1} h^{-1} per cell) was determined by dividing the change in β-galactosidase units ml^{-1} expressed from the *glnK* promoter by the time elapsed between measurements and the change in optical density of the cultures at 600 nm between measurements, assuming 10^9 cells ml^{-1} when $OD_{600} = 1$.

The viability of nitrogen-starved cells was determined after 35 h of nitrogen starvation as described previously (Blauwkamp and Ninfa, 2002a).

Construction of pAmtB1

The entire coding region of *amtB* from 57 nucleotides upstream of the start codon to 83 nucleotides downstream of the stop codon was polymerase chain reaction (PCR) amplified from strain N2 using primers AmtB-US (CGGAATTCCGGCACA CAGCAACAGGAACG) and AmtB-DS (CGGGATCCCGT TCAGGAAGGGGTGATGCG). This PCR fragment was cloned into the pTrc99A vector (Amersham Pharmacia Biotech), containing the leaky IPTG-inducible Trc promoter and plasmid-

encoded *lacI*^{q}, at the *Eco*RI and *Bam*HI sites. DNA sequencing at the University of Michigan DNA Sequencing core confirmed the wild-type sequence of the *amtB* gene and 5′ untranslated region.

Construction of glnKp fusions to the glnK *and* glnB *structural genes*

A novel vector, pRBS3a, facilitating recombination of cloned genes linked to gentamicin resistance into a transcriptionally isolated landing pad in the *rbs* operon of *E. coli* was generously provided by M. R. Atkinson and will be described in detail elsewhere. We have described previously the construction of plasmids that contain the promoter region of *glnK* fused to the start codon of either *glnK* (pglnK81) or *glnB* (pglnKpB1) via an engineered *Nde*1 restriction site at the start codon (Atkinson *et al*., 2002b). Klenow end-filled *Bam*HI fragments of pglnK81 and pglnKpB1 containing the promoter–gene fusions were cloned into a Klenow-blunted *Pst*1 site in pRBS3a to yield plasmids pglnK101 and pglnKpB101 respectively. A sample of 0.5 μg of pglnK101 and pglnKpB101 plasmid DNA was linearized by *Not*I digestion and electroporated into strain K4633. Gentamicin-resistant transformants were screened for the correct size insert in the *rbs* operon by PCR using primers that anneal outside the cloned region and confirmed recombination into the *rbs* operon.

Determination of PII deuridylylation kinetics

Overnight cultures of the indicated strains grown in W-salts containing 0.4% glucose, 0.2% ammonium sulphate, 0.005% trp and 50 μg ml^{-1} kanamycin were diluted 1:300 into media identical to that described above except containing 0.008% ammonium sulphate. Cells were grown for 23 h at 37°C to ensure consumption of the ammonia. At the times indicated in the text, aliquots of cells were removed into EDTA (50 mM final concentration) to kill UTase/UR activity, sonicated for 25 s on power 3 (Branson sonifier 250) and frozen in a dry ice–isopropanol bath. The time from removal of the cell sample to freezing was ≈ 1 min. Frozen samples were placed at 65°C for 20 min to destroy any remaining UTase/UR activity without disrupting PII(~UMP) trimers, and the supernate was collected after a 10 min centrifugation at 12 000 *g*. Supernatant protein (7 μg) was resolved by 16% PAGE, and the uridylylation state of PII was determined by immunoblotting as described previously (Atkinson *et al*., 2002b).

Determination of AmtB effects on growth and Ntr gene expression

Cultures (25 ml) of cells of the indicated genotype harbouring either p*Trc*99A (empty vector) or pAmtB1 were grown from single isolated colonies in W-salts containing 0.2% ammonium sulphate, 0.2% glutamine and 0.1% glycine as indicated in the text. Cell growth was monitored in a Beckman DU-65 spectrophotometer, and β-galactosidase activity expressed from *glnA* or *glnK* promoter fusions to *lacZYA* was determined at an OD_{600} of ≈ 0.4 by the Miller assay and expressed as Miller units, using SDS and chloroform to disrupt the cells, as described previously (Miller, 1992). The standard deviation of the β-galactosidase assays was <15% for all experiments.

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