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Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*

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Summary

Two structurally similar but functionally distinct PII-like proteins, PII and GlnK, regulate nitrogen assimilation in *Escherichia coli*. Studies with cells indicated that both PII (the *glnB* product) and GlnK (the *glnK* product) acted through the kinase/phosphatase NRII [NtrB, the *glnL* (*ntrB*) product] to reduce transcription initiation from Ntr promoters, apparently by regulating the phosphorylation state of the transcriptional activator NRI∼P (NtrC∼P, the phosphorylated form of the *glnG* (*ntrC*) product). Both GlnK and PII also acted through adenylyltransferase (ATase, the *glnE* product) to regulate the adenylylation state of glutamine synthetase (GS). The activity of both GlnK and PII was regulated by the signal-transducing uridylyltransferase/uridylyl-removing enzyme (UTase/UR, *glnD* product). Our experiments indicate that either PII or GlnK could effectively regulate ATase, but that PII was required for the efficient regulation of NRII required to prevent expression of *glnA*, which encodes GS. Yet, GlnK also participated in regulation of NRII. Although cells that lack either PII or GlnK grew well, cells lacking both of these proteins were defective for growth on nitrogen-rich minimal media. This defect was alleviated by the loss of NRII, and was apparently due to unregulated expression of the Ntr regulon. Also, mutations in *glnK*, designated *glnK, were obtained as suppressors of the Ntr$^-$ phenotype of a double mutant lacking PII and the UTase/UR. These suppressors appeared to reduce, but not eliminate, the ability of GlnK to prevent Ntr gene expression by acting through NRII. We hypothesize that one role of GlnK is to regulate the expression of the level of NRI∼P during conditions of severe nitrogen starvation, and by so doing to contribute to the regulation of certain Ntr genes.**

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Introduction

Nitrogen regulation of gene transcription has been intensively studied in *Escherichia coli* and related bacteria, and a resulting working model for this regulation and the regulation of glutamine synthetase adenylylation is shown in Fig. 1 (reviewed in Rhee *et al*., 1985a; 1989; Kamberov *et al*., 1994; Merrick and Edwards, 1995; Ninfa *et al*., 1995; Magasanik, 1996; Reitzer, 1996a,b). The key sensory components are the uridylyltransferase/uridylyl-removing enzyme (UTase/UR, *glnD* product), which is controlled allosterically by glutamine (Jiang *et al*., 1998), and the PII signal transduction protein (*glnB* product), which is reversibly uridylylated in response to the absence or presence of this signal. PII and PII-UMP, which are allosterically regulated by 2-ketoglutarate, interact with adenylyltransferase (ATase, *glnE* product) to regulate adenylylation (inactivation) and deadenylylation (activation) of glutamine synthetase (GS, *glnA* gene product; Fig. 1, right). In addition, PII interacts with NRII [*glnL (ntrB)* product] to bring about the dephosphorylation of the transcription factor NRI∼P [NRI is product of *glnG (ntrC)*]. This prevents transcription of *glnA* and other genes in response to nitrogen limitation (Ninfa and Magasanik, 1986; Fig. 1, left). PII-UMP does not interact with NRII (Atkinson *et al*., 1994; Kamberov *et al*., 1995; E. S. Kamberov and A. J. Ninfa, unpublished).

There are two known routes by which NRI becomes phosphorylated (Fig. 1, left). NRII binds ATP and phosphorylates itself on a histidine, and these phosphoryl groups are transferred to NRI; phosphoryl groups are also directly transferred from acetyl phosphate to NRI (Feng *et al*., 1992). Either of these routes provides sufficient phosphorylation of NRI to permit full expression of *glnA*, but both routes of NRI phosphorylation are required for growth under certain nitrogen starvation conditions (Feng *et al*., 1992). NRII and NRI constitute a bacterial two-component system, with numerous homologues found in prokaryotes and eukaryotes (for reviews see Parkinson and Kofoid, 1992; Kamberov *et al*., 1994; Hoch and Silhavy, 1995; Ninfa *et al*., 1995; Ninfa, 1996). In enteric bacteria, many such two-component systems are functioning simultaneously to regulate different cellular processes, and for several of these systems regulation by acetyl phosphate has been observed (Deretic *et al*., 1992; Lukat *et al*., 1992; Wanner *et al*., 1992; McCleary and Stock, 1994; Pruss and Wolfe, 1994; McCleary, 1996; reviewed in

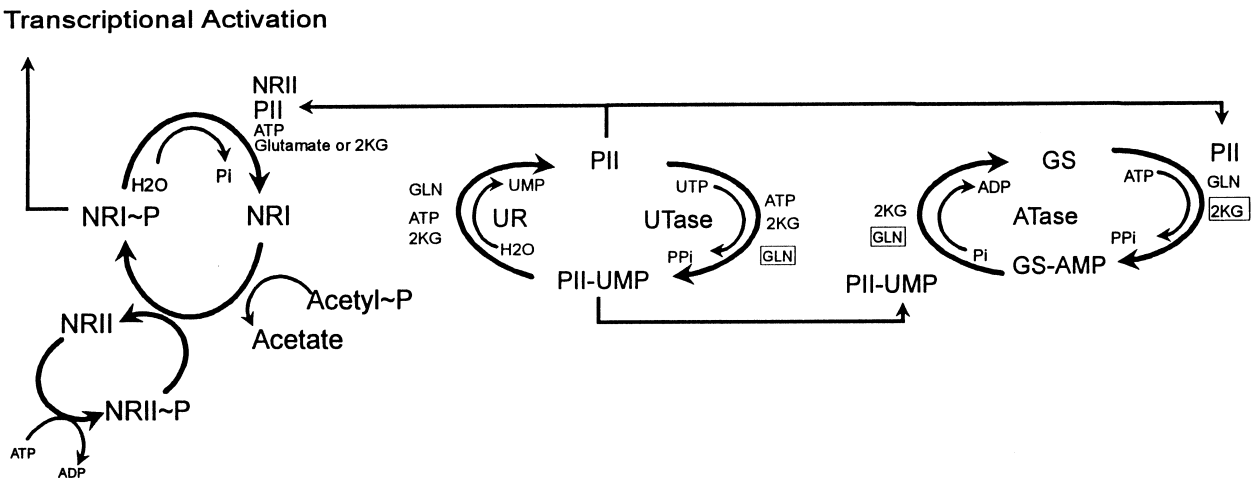

Fig. 1. Signal transduction system regulating GS adenylylation and the expression of the Ntr regulon. Proteins depicted are: NRI, nitrogen regulator I (*glnG(ntrC)* product); NRII, nitrogen regulator II (*glnL(ntrB)* product); PII, *glnB* product; UTase/UR, uridylyltransferase/uridylyl-removing enzyme (activities depicted separately) (*glnD* product); ATase, adenylyltransferase (*glnE* product); GS, glutamine synthetase (*glnA* product). Small molecule effectors: GLN, glutamine; 2KG, 2-ketoglutarate. Boxed effectors are inhibitors of the indicated reaction, and unboxed effectors are activators of the indicated reaction. The figure is similar to Fig. 1 of Jiang *et al*. (1997).

McCleary *et al*., 1993). Indeed, NRII seems to be an important regulator of the intracellular acetyl phosphate concentration (Pruss and Wolfe, 1994), presumably because the PII-activated dephosphorylation of NRI~P by NRII acts as a sink for the phosphoryl groups. There is no known regulation of the phosphorylation of NRI by NRII. Thus, the expression of *glnA* and the Ntr regulon, as well as the intracellular concentration of acetyl phosphate, appear to be regulated by the PII-dependent dephosphorylation of NRI~P by NRII.

Previous studies have suggested that transcription of *glnA* is activated by a very low intracellular concentration of NRI~P, whereas transcription of certain other nitrogen-regulated genes, such as those permitting use of arginine or histidine as a nitrogen source, requires a higher intracellular concentration of NRI~P (Pahel *et al*., 1982; Reitzer and Magasanik, 1986). Indeed, the expression of *glnA* is diminished *in vivo* and *in vitro* at very high concentrations of NRI~P (Shiau *et al*., 1992; Feng *et al*., 1995). The NRI~P concentration dependence for transcriptional activation of various Ntr promoters may be due to the quantity, quality and location of enhancer and silencer sequences bound by NRI~P, the extent of occupancy of the promoter sequences by polymerase and by other factors such as the stability of the open transcription complexes formed at the various promoters (Feng *et al*., 1995). In essence, each Ntr promoter is designed to be activated only when the concentration of NRI~P is within a certain range.

Very serious defects of the model shown in Fig. 1 have been known for over a decade (Bueno *et al*., 1985). The model predicts that, as the UTase/UR acts through PII, in strains lacking PII the *glnD* genotype should be irrelevant. However, this is not found.

The *glnD*-encoded UTase/UR is required for the use of arginine as a nitrogen source (Ntr^+ phenotype), as predicted by the model. Cells lacking only PII are able to use arginine as a nitrogen source and have elevated levels of *glnA* expression under all conditions, as predicted by the model (Bueno *et al*., 1985, Fig. 1). However, a *glnB glnD* double mutant (strain BD), lacking both PII and UTase/UR, has elevated expression of *glnA* but is Ntr^-. The Ntr^- phenotype of the doubly mutant BD strain suggested that this strain had a high enough intracellular concentration of NRI~P to activate *glnA*, but could not generate a high enough concentration of NRI~P to activate the genes required for the use of arginine as a nitrogen source. This Ntr^- phenotype was due to a direct requirement for the *glnD* product, UTase/UR, as transformation of strain BD with a plasmid containing only $glnD^+$ and vector sequences restored the Ntr^+ phenotype (unpublished observations). Thus, the UTase/UR clearly had a role in the regulation of Ntr gene expression in the absence of PII.

Another defect in the model, noted earlier (van Heeswijk *et al*., 1995; 1996), is that cells lacking only PII have normal regulation of the GS adenylylation state in response to nitrogen availability. This finding is also discordant with results with purified components, which indicated that the deadenylylation of GS-AMP by the ATase absolutely required PII-UMP *in vitro* (Stadtman *et al*., 1970). In the doubly mutant *glnB glnD* strain (strain BD), GS is over-adenylylated under nitrogen-limiting conditions. Thus, the UTase/UR was required for regulation of GS adenylylation even in the absence of PII (reviewed in Ninfa *et al*., 1995).

The data discussed above suggested the existence of at least one additional gene product that is capable of regulating Ntr gene expression and GS adenylylation state,

and also suggested that the activity of the unidentified gene product(s) was regulated by the UTase/UR (reviewed in Ninfa *et al*., 1995). Recent studies have revealed that *E. coli* contains a gene, designated *glnK*, that encodes a homologue of the PII protein (Allikmets *et al*., 1993; van Heeswijk *et al*., 1995; 1996). The *glnK* product was observed to be uridylylated in cell extracts by the UTase/UR (van Heeswijk *et al*., 1996). These experiments suggested that the *glnK* gene product may be part of the nitrogen regulatory system and may account for the requirement for the UTase/UR in the strain deleted for *glnB*. Purified GlnK was observed to activate the adenylylation of GS by ATase *in vitro* (van Heeswijk *et al*., 1996). Unlike the PII protein, which is essentially present constitutively, immunoblotting experiments indicated that the expression of *glnK* was itself nitrogen regulated (van Heeswijk *et al*., 1993; 1996). Furthermore, sequences corresponding to an Ntr promoter were identified upstream from *glnK* (van Heeswijk *et al*., 1996). Thus, it could be anticipated that GlnK and PII are likely to have functionally distinct roles.

van Heeswijk *et al.* (1996) proposed a model in which the role of GlnK was to slow down the transcriptional response when nitrogen-starved cells encounter ammonia. They hypothesized that the redundancy of GlnK and PII results in a slow (GlnK) and fast (PII) signal transduction system. They proposed that in nitrogen-starved cells, NRI, NRII and GlnK are at high concentration, and a rapid response to an increase in ammonia could be inefficient if this increase is transitory. They hypothesized that the internal concentrations of NRI, NRII and GlnK are only lowered after prolonged exposure to ammonia, and that NRI, NRII and GlnK serve as 'memory proteins' that buffer the signal transduction system against transitory changes in nitrogen availability.

In the absence of a *glnK* mutant, the role of this gene in nitrogen regulation remained speculative. As a further complication, the prior immunoblotting studies suggested that the *glnK* product was not detectable in a strain isogenic with strain BD (van Heeswijk *et al*., 1996). Thus, it was unclear whether *glnK* or yet another gene was responsible for the Ntr^- phenotype of the strain BD.

We took three approaches to determine whether *glnK* was involved in nitrogen regulation, and more specifically, if $glnK^+$ was responsible for the Ntr^- phenotype of the *glnB glnD* double mutant (strain BD). First, we used the known sequence of *glnK* to construct a null mutation in *glnK in vitro*, and recombined this mutation onto the *E. coli* chromosome. The effect of this null mutation was then assessed in isolation, and in combination with mutations in other regulatory genes. Second, we constructed a *lacZ* transcriptional fusion to the *glnK* promoter, placed this fusion at another location on the chromosome and examined its regulation in various genetic backgrounds. The results of these two studies indicated that *glnK* acted through *glnL* to regulate certain Ntr promoters, including the *glnK* promoter, but did not have an important role in the regulation of the *glnA* promoter. During the course of these studies, we also observed that glutamine synthetase activity appears to regulate, directly or indirectly, the level of acetyl phosphate. Finally, we isolated spontaneous mutations that permitted the BD strain to use arginine as a nitrogen source, and characterized these mutations. Among the spontaneous mutants, we identified 10 mutations that mapped in *glnK*. Most of these were located in a part of *glnK* predicted to encode the T loop, based on the PII structure (Carr *et al*., 1996). The T loop of PII is known to be the part that interacts with the PII receptors, and receptor specificity mutations altering PII map to this part of the protein (Jiang *et al*., 1997). Thus, the suppressor analysis directly indicated that GlnK is responsible for the Ntr^- phenotype of strain BD, and that GlnK has a role in negative regulation of the Ntr regulon.

Results

Physiological characterization of the role of glnK *in nitrogen regulation*

To assess the role of *glnK* in regulation of *glnA*, the GS activity and the adenylylation state were measured in adapted cultures using the γ-glutamyl transferase assay (reviewed in Rhee *et al*., 1985b). To assess the expression of the Ntr regulon, two tests were used. First, the ability of cells to grow on arginine as the sole nitrogen source was tested. Cells must be able to generate a high intracellular concentration of NRI∼P to use this nitrogen source (reviewed in Reitzer *et al*., 1996b). Second, the expression of the nitrogen-regulated *glnK* promoter was assessed by measuring β-galactosidase activity in cells containing a *glnK*::*lacZ* fusion that was present in single copy in the *trp* locus (*Experimental procedures*).

The regulation of GS in the 'wild-type' strain YMC10 containing the *glnK*::*lacZ* fusion (YMC10φ) was the same as previously observed in YMC10 lacking the fusion (Tables 1 and 2, Atkinson and Ninfa, 1992). That is, the level of the enzyme was elevated about sevenfold by nitrogen starvation, with the level of unadenylylated GS subunits elevated about 32-fold (Table 1). In contrast, the expression of the *glnK*::*lacZ* fusion was regulated about 2400-fold under the same conditions (Table 1). This great degree of regulation was largely due to the near absence of fusion expression under nitrogen-rich conditions (Table 1). The wild-type strain grew well on arginine as the sole nitrogen source.

In strain Bφ, deleted for *glnB* (and thus lacking PII) and containing the *glnK*::*lacZ* fusion, the level of total GS was high regardless of nitrogen availability, as noted previously for strain B (Bueno *et al*., 1985, Tables 2 and 3). However, as already noted, the regulation of GS adenylylation state

Table 1. Glutamine synthetase and β-galactosidase expression in adapted cultures and Ntr phenotype.

	Glutamine synthetase[a] (n[b])		β-Galactosidase		
Strain	Ggln[d]	GNgln	Ggln	GNgln	Ntr[c]
YMC10ϕ	1198 (2.8)	173 (10.0)	4866	2	+++
Bϕ	1535 (3.7)	1565 (11.4)	3111	556	+++
Lϕ	569 (6.4)	508 (11.2)	444	200	+++[e]
Dϕ	293 (9.3)	92 (9.4)	< 2	< 2	–
Kϕ	957 (3.3)	234 (10.4)	1978	44	+++
Pϕ	886 (2.4)	129 (10.4)	5444	< 2	+/–[e]
BDϕ	1557 (10.7)	1530 (11.5)	431	391	–
BLϕ	720 (8.0)	607 (11.3)	233	153	+++[e]
KLϕ	618 (2.4)	622 (11.3)	524	164	+++[e]
LEϕ	884 (1.3)	125 (4.0)	1851	44	+++[e]
LPϕ	38 (ND)	41 (ND)	4	< 2	–
LEPϕ	40 (ND)	22 (ND)	< 2	< 2	–
BKLϕ	2281 (11.5)	906 (11.6)	1471	222	+++[e]
BLEK	966 (2.1)	178 (4.3)	ND	ND	++[e]
BLEKP	80 (ND)	25 (ND)	ND	ND	–
BDMϕ	1600 (11.1)	1852 (11.7)	467	540	–
BDMϕ *glnK8*	2235 (11.4)	1998 (11.5)	3956	2267	+++
BDMϕ *glnK9*	2610 (11.5)	2000 (11.6)	3356	1207	+++

a. GS transferase activity. Values are the average of two to six cultures. Results from duplicate cultures generally differed by < 15%. Cultures were grown overnight in the indicated medium, diluted to $OD_{600} = 0.02$ and grown at 30°C to OD = 0.5.
b. Adenylylation state, determined by performing the transferase assay in the presence of Mn^{2+} (total activity) and Mg^{2+} (deadenylylated activity only), expressed as average number of adenylylated subunits/GS dodecamer.
c. Growth on solid glucose–arginine medium supplemented with tryptophan (0.04% w/v). Growth was assessed after 60 h at 37°C. +++ signifies vigorous, wild-type growth; ++ signifies less vigorous growth; +/– signifies pinpoint colonies; and – signifies the inability to grow.
d. Media used were Ggln, glucose–glutamine; GNgln, glucose–ammonia–glutamine. In all cases, glucose was present at 0.4% (w/v) and the nitrogen sources were each present at 0.2% (w/v).
e. Strains lacking *glnL* or the ability to form acetyl phosphate are unable to grow on liquid glucose–arginine medium, a more demanding test of Ntr gene expression (Feng *et al*., 1992). ND, not determined.

occurred in this strain despite the absence of PII. In the experiments shown, the level of unadenylylated GS subunits was regulated about 14-fold in response to nitrogen availability (Table 1). Similar results were obtained for strain B, lacking the *glnK*::*lacZ* fusion (Tables 2 and 3). In strain Bϕ, the *glnK*::*lacZ* fusion was regulated about sixfold by nitrogen availability, with the induced level slightly less than that seen in the wild-type background and the uninduced level ≈275-fold higher than in the wild type. As noted for strain B, strain Bϕ grew like the wild type on arginine as the sole nitrogen source.

In strain Dϕ, containing the *glnD99*::Tn*10* mutation and

Table 2. Effect of *glnK** alleles on the expression of *glnA* and adenylylation state of GS.

		Glutamine synthetase[a] (n[b])	
Strain	Relevant genotype	Ggln[c]	GNgln
YMC10	Wild type	1076 (2.3)	167 (9.3)
B	*ΔglnB*	1253 (2.9)	1126 (11.4)
K	*Δmdl–glnK*::*kan*	1590 (3.0)	177 (10.2)
BK-8	*ΔglnB mdl*::*chl*..*glnK8* (R47→W)	1250 (3.0)	1243 (11.1)
BK-9	*ΔglnB mdl*::*chl*..*glnK9* (R47→L)	1390 (4.4)	1280 (11.2)

a. GS transferase activity. Values are from single cultures for strains YMC10, B and K, and from three cultures for BK-8 and BK-9. Results from triplicate cultures differed by < 15%. Cultures were grown overnight in the indicated medium, diluted to $OD_{600} = 0.02$ and grown at 30°C to OD = 0.5.
b. Adenylylation state, determined by performing the transferase assay in the presence of Mn^{2+} (total activity) and Mg^{2+} (deadenylylated activity only), expressed as average number of adenylylated subunits/GS dodecamer.
c. Media used were Ggln, glucose-glutamine; GNgln, glucose–ammonia–glutamine In all cases, glucose was present at 0.4% (w/v) and the nitrogen sources were each present at 0.2% (w/v).

Table 3. Complementation of strains BK and B with plasmids expressing PII or GlnK.

Strain	Relevant genotype	Glutamine synthetase[a] (n[b]) Ggln[c]	GNgln
B	*ΔglnB*	1253 (3.3)	909 (11.0)
K	*Δmdl–glnK*::*kan*	1203 (2.7)	129 (8.5)
BK/pBUC10	*ΔglnBΔmdl-glnK*::*kan pglnB*$^+$	218 (3.0)	41 (10.4)
BK/pglnK2	*ΔglnBΔmdl-glnK*::*kan pglnK*$^+$	1014 (2.6)	370 (10.6)
B/pBUC10	*ΔglnB/pglnB*$^+$	241 (3.4)	39 (9.5)
B/p*glnK2*	*ΔglnB/pglnK*$^+$	945 (2.9)	326 (10.3)

a. GS transferase activity. Values are the average of duplicate cultures, which differed by <15%. Cultures were grown overnight in the indicated medium, diluted to $OD_{600} = 0.02$, and grown at 30°C to OD = 0.5.
b. Adenylylation state, determined by performing the transferase assay in the presence of Mn^{2+} (total activity) and Mg^{2+} (deadenylylated activity only), expressed as average number of adenylylated subunits/GS dodecamer.
c. Media used were Ggln, glucose-glutamine; GNgln, glucose–ammonia–glutamine. Glucose was present at 0.4% (w/v) and the nitrogen sources were each present at 0.2% (w/v).

the *glnK*::*lacZ* fusion, GS expression was induced to a level only ≈one-quarter to one-fifth that found in the wild-type strain when starved, and GS was highly adenylylated regardless of nitrogen availability (Table 1, Bueno *et al*., 1985). Furthermore, this strain could not use arginine as the sole nitrogen source regardless of whether the medium is solid or liquid, like strain D (Bueno *et al*., 1985; Feng *et al*., 1992). This is as expected, because in the absence of the UTase/UR, PII is unmodified regardless of nitrogen availability. In this strain background the *glnK*::*lacZ* fusion was essentially not expressed. Thus, the *glnD99*::Tn*10* mutation had a much more severe effect on the regulation of the *glnK*::*lacZ* fusion and on the ability of the cells to use arginine as the sole nitrogen source than it did on the expression of *glnA* (Table 1).

Strain BD contains both the deletion of *glnB* and the *glnD99*::Tn*10* mutation. As noted above, the strain contains high levels of GS regardless of nitrogen availability, but cannot grow on arginine as the sole nitrogen source. Strain BDϕ, which contains in addition the *glnK*::*lacZ* fusion, had fully induced levels of GS under both inducing and repressing conditions but, unlike strain B, had little ability to regulate the adenylylation state of GS (Table 1). In strain BDϕ, the fusion was expressed constitutively at ≈one-tenth the induced wild-type level. Thus, the combination of the *glnB* and *glnD* mutations resulted in constitutive expression of GS, but prevented full expression of *glnK*::*lacZ*. Our GS data for this strain are somewhat at variance with previous data (Bueno *et al*., 1985), in that those workers observed a lower level of GS on both Ggln and GNgln media. Nevertheless, both the data in Table 1 and the previous data (Bueno *et al*., 1985) show that *glnD* has a role in nitrogen regulation in the absence of *glnB*.

In strain Lϕ, containing a null mutation in *glnL* and thus lacking NRII, the level of GS expression was no longer regulated by the availability of nitrogen. The expression of GS in this strain is driven by acetyl phosphate (Feng *et al*., 1992). The reason why the GS adenylylation state is somewhat elevated in nitrogen-limiting conditions in this strain is not known. In the Lϕ strain, the *glnK*::*lacZ* fusion was expressed constitutively at about 1/10–1/20 the wild-type induced level (Table 1). Previous results from this group (Feng *et al*., 1992) and another group (Bueno *et al*., 1985) indicated a higher level of GS expression in strain L (lacking the fusion) on Ggln medium than that reported in Table 1 for strain Lϕ. Apparently, subtle differences in strain cultivation may affect the accumulation of acetyl phosphate and account for this difference.

Strain BLϕ contains null mutations in *glnB* and *glnL* and the *glnK*::*lacZ* fusion. The *glnL* mutation was epistatic to the *glnB* mutation (Table 1), as expected as PII is known to act through NRII to regulate gene transcription (Fig. 1).

Effects of a glnK *null mutation*

In strain Kϕ, which is isogenic to YMC10ϕ except containing the *glnK* null mutation (*Experimental procedures*), the expression of GS was similar to the wild type, and GS adenylylation state regulation was normal (Table 1). Similar results were obtained with strain K, which lacks the *glnK*::*lacZ* fusion (Tables 2 and 3). Although *glnA* expression was not greatly altered by the *glnK* mutation, this mutation did affect the regulation of the *glnK*::*lacZ* fusion. The induced level of the fusion expression was less than half that seen in the wild-type background, and the uninduced level was raised 22-fold, resulting in only 45-fold regulation by nitrogen availability under the conditions used (Table 1). Strains containing only the *glnK* mutation grew well on arginine as the sole nitrogen source; indeed, these strains reproducibly displayed an apparent increased rate of growth in liquid glucose–arginine medium. Growth in this nitrogen-limiting medium is a demanding test of Ntr

expression; for example, the *glnL* mutant cannot grow in this medium, nor can strains defective in acetyl phosphate synthesis (Feng *et al*., 1992). When adapted overnight cultures were diluted into fresh glucose–arginine medium, the wild-type strain and strain lacking PII demonstrated a lag phase that was 2–3 h longer than strain K, but the maximal growth rate for all three strains was similar (doubling time of ≈4.5 h), and the final cell yield was similar for all three strains (data not shown). In both the wild type and the *glnK* mutant, GS was elevated and mainly deadenylylated under these conditions (data not shown). Thus, the *glnK* mutation had little effect on *glnA* expression but had discernible effects on the expression of other nitrogen-regulated genes. Nevertheless, these effects were subtle (Table 1).

We examined whether *glnK*$^+$ conferred an advantage to the cell in shifting from a condition of nitrogen limitation to a condition of nitrogen excess. For this experiment, the wild-type YMC10 and strain K were adapted to nitrogen-limiting medium and shifted to medium lacking a nitrogen source until growth stopped completely. Then, excess ammonium sulphate was added, and the growth rate of the cells was followed for several additional hours. The addition of ammonia brought about an immediate increase in the growth rate of both the wild type and strain K (data not shown). As the growth of both strains was nearly identical, apparently *glnK*$^+$ was not required for the shift from nitrogen-limiting to nitrogen excess conditions (data not shown).

Strain KLϕ contains null mutations in *glnK* and *glnL*, as well as the *glnK*::*lacZ* fusion. The expression of *glnA* and *glnK*::*lacZ* in this strain was similar to that observed with Lϕ, but there was less adenylylation of GS in nitrogen-limiting medium than was observed with Lϕ (Table 1).

The doubly mutant strain BK displays a severe growth defect on nitrogen-rich minimal medium

We constructed strain BK, containing null mutations in *glnB* and *glnK*, by our usual methods, which involve selection for drug-resistance markers on rich LB medium after P1-mediated generalized transduction (*Experimental procedures*). The doubly mutant BK strain grew well on rich LB medium, like the rest of the strains, but displayed a severe growth defect on solid defined media. Best growth was observed on the nitrogen-limiting glucose–arginine medium. There was some variability in the growth of strain BK on nitrogen-rich defined medium at the position where a heavy innoculum was placed onto the media, but in all cases single-colony isolates were not obtained. We attempted to measure GS expression and adenylylation in the double mutant, but the growth defect in liquid minimal media was even more severe than that observed with solid medium. In one experiment, duplicate cultures grew very slowly, and never reached the cell density usually used in the experiment (OD_{600} 0.5), these were harvested at OD 0.1. In another experiment, duplicate cultures grew slowly to ≈OD 0.1, and then shot up to OD 0.5. It seems likely that additional mutation(s) suppressing the growth defect were accumulated in these cultures during the experiments. Nevertheless, these cultures always contained elevated GS, which appeared to be almost completely adenylylated (data not shown). In two other attempts to repeat this experiment, strain BK failed to grow at all in liquid minimal medium. Thus, it is clear that the combination of *glnB* and *glnK* mutations in an otherwise wild-type background resulted in a severe growth defect in minimal medium.

The combination of the glnB *and* glnK *mutations was epistatic to* glnD

Although strain BK grew poorly even on the severely nitrogen-limiting glucose–arginine medium compared with the wild type, it grew considerably better than strain BD, which essentially did not grow at all. We introduced the *glnK* null mutation into strain BD; the resulting strain BDK had growth properties similar to strain BK, some ability to grow on minimal medium with arginine as the nitrogen source and a severe growth defect on nitrogen-rich minimal media. Thus, by reconstruction, in strain BD the *glnK*$^+$ gene product was at least partially responsible for preventing growth on glucose–arginine medium, and was necessary to permit good growth on the other defined media tested.

A null mutation in glnL *was epistatic to the combination of* glnB *and* glnK *mutations for the growth defect on minimal medium and for expression of Ntr genes but was not epistatic for the defect in GS adenylylation control*

The severe growth defect of the BK strain was ameliorated upon introduction of a null mutation in *glnL*. The triply mutant BKL strain (*glnK ΔglnB ΔglnL2001*) had a growth phenotype similar to the singly mutant *glnL* strain, indicating that for this phenotype *glnL* is epistatic to loss of both *glnB* and *glnK*. Strain BKLϕ contains null mutations in *glnB*, *glnK* and *glnL*, as well as the *glnK*::*lacZ* fusion. In this strain, GS expression was elevated on both nitrogen-rich and nitrogen-limiting medium, and GS was very highly adenylylated regardless of nitrogen availability. Also, the *glnK*::*lacZ* fusion was induced to one-third of the wild-type level under inducing conditions and was expressed about 11-fold higher than in the wild type under repressing conditions, resulting in approximately sevenfold regulation by nitrogen availability. Thus, in strain BKLϕ, the *glnL* null mutation was epistatic for the growth defect and for the regulation of *glnA* and *glnK* but was not epistatic for the

GS adenylylation state defect resulting from the combination *ΔglnB glnK*, as expected. However, it is curious that the expression of both *glnA* and *glnK*::*lacZ* were considerably elevated over the levels observed with strains Lϕ, BLϕ and KLϕ. As this strain grew well, the presence of a high intracellular concentration of GS-AMP was apparently not toxic.

The elevated expression of *glnA* and *glnK*::*lacZ* in strain BKLϕ was in part due to the presence of the wild-type *glnE* gene, encoding ATase. Strain BLEK contains null mutations in *glnB*, *glnL*, *glnE* and *glnK*. (An analogous strain with the *glnK*::*lacZ* fusion could not be constructed due to a lack of unique drug resistance selectable markers.) In strain BLEK, GS expression more closely resembles that seen in strain YMC10, and the GS is mostly unadenylylated (Table 1). The *glnE* mutation that we used should be null (*Experimental procedures*). The appearance of residual adenylylation in this strain may be due to a small difference in the activity of unadenylylated GS in the Mn^{2+} and Mg^{2+} assays, or may indicate the presence of an unknown enzyme that has ATase activity. To test whether the expression of GS was due to acetyl phosphate in this strain, we constructed strain BLEKP, which also lacks the capacity to form acetyl phosphate because of deletion of the *pta–ackA* portion of the chromosome. Strain BLEKP had very low levels of GS in both nitrogen-limiting and nitrogen excess conditions, as expected (Table 1). Thus, by comparing our results for strains BLK, BLEK and BLEKP, it is apparent that *glnE* has a role in this background in regulating the level of acetyl phosphate. This possibility was examined further by comparing strains Lϕ, LPϕ, Pϕ and LEϕ (Table 1). Comparison of Lϕ and LPϕ indicated that the expression of *glnA* and the *glnK*::*lacZ* fusion in Lϕ were due to acetyl phosphate (Table 1). In LEϕ, *glnA* and *glnK*::*lacZ* expression were clearly nitrogen regulated and higher than in strain Lϕ (Table 1). Thus, under certain circumstances, *glnE* was a regulator of acetyl phosphate accumulation.

The growth defect of strain BK seemed to be due to overexpression of one or more Ntr *genes*

As the *glnL* null mutation suppressed the growth defect associated with the combination of *glnB* and *glnK* null mutations; the growth defect of strain BK may be due to the unregulated action of the *glnL* product, NRII, driving expression of the Ntr regulon. We observed that the growth defect of strain BK was eliminated when a null mutation in *glnA* was introduced, as long as glutamine was then provided. The *glnA*::Tn*5* mutation not only eliminates GS activity but also results in a decreased intracellular concentration of NRI and NRII because of polarity (Reitzer and Magasanik, 1986). Although the regulation of *glnA in trans* is normal in such a strain, the cells are unable to increase the concentration of NRI by activation of the *glnAp2* promoter (Pahel *et al*., 1982) and thus cannot accumulate NRI∼P to the level necessary for Ntr gene expression. Our result with the *glnA*::Tn*5* mutation thus did not distinguish whether the growth defect was due to *glnA* or another nitrogen-regulated gene, but did indicate that by preventing expression of both *glnA* and the Ntr regulon the ability to grow on minimal medium was restored.

We also examined the effect of introducing the *glnG*::Tn*5* mutation into strain BK. This mutation, eliminating NRI, renders the cell Ntr^-, but does not cause glutamine auxotrophy unless combined with *glnB* (Bancroft *et al.*, 1978; Bueno *et al*., 1985). The *glnG*::Tn*5* mutation reduces the expression of *glnA* greatly, but does not eliminate it. However, in cells containing a low amount of GS, over-adenylylation of GS such as in the *glnB glnG* strain renders the cells glutamine auxotrophs, and suppressing mutation mapping in *glnE* (encoding ATase) may compensate for this defect (Bancroft *et al*., 1978). Studies with the purified ATase indicated that PII was not required for the adenylylation reaction, but PII∼UMP was required for the deadenylylation of GS∼AMP (reviewed in Rhee *et al*., 1985). That is, in the absence of regulation by PII and PII∼UMP, the ATase has a bias to adenylylate GS. As the expression of *glnK* is nitrogen regulated and dependent on *glnG* (van Heeswijk *et al*., 1996), we may reinterpret the previous observation of GS overadenylylation in the *glnB glnG* strain and the resulting glutamine auxotrophy as a result of the simultaneous absence of GlnK and PII. Strain GBK, although also phenotypically a glutamine auxotroph, grew considerably better than strain BK on minimal media containing glutamine. This indicates that expression of the Ntr regulon in strain BK is at least partly responsible for the growth defect on nitrogen-replete minimal media.

As a further test of whether unregulated Ntr gene expression was responsible for the growth defect of strain BK, we introduced the pBR322-based $glnG^+$ plasmid pgln31 into strain GBK (Backman *et al*., 1983). This plasmid, lacking an obvious promoter for $glnG^+$, results in a constitutive very low level of NRI. This concentration of NRI is sufficient for normal regulation of *glnA*, but is not sufficient for full expression of Ntr genes. The resulting strain GBK/pgln31 grew well on nitrogen-rich minimal medium. Thus, limiting the intracellular NRI∼P concentration suppressed the growth defect resulting from the combination of *glnB* and *glnK* null mutations.

The growth defect of strain BK can be ameliorated by addition of amino acids to minimal medium

As several Ntr genes are involved in the catabolism of amino acids as nitrogen sources, one possible explanation for the growth defect of the doubly mutant BK strain is that constitutive expression of the Ntr system results in an amino

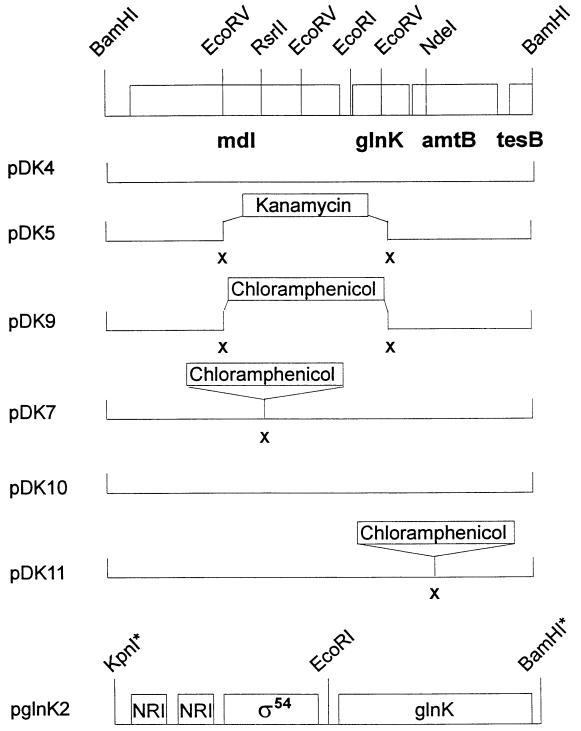

Fig. 2. Construction of *glnK*, *mdl* and *amtB* null mutations *in vitro*. The 10.1 min region of the *E. coli* chromosome is depicted at the top, along with the positions of the restriction cleavage sites used in this study. × indicates restriction sites destroyed as part of the cloning process. An asterisk is used to indicate sites introduced by our manipulations. At the bottom of the figure, the structure of the insert in pglnK2 is shown.

acid requirement. We observed that no single amino acid, when added to minimal medium, could restore to strain BK the ability to grow well. Also, the combination of arginine, proline and histidine, which are degraded by the *hut*, *put* and *aut* Ntr genes present in these cells, could not restore to BK the ability to grow on minimal medium. However, strain BK grew well on minimal medium supplemented with a complex mixture of amino acids, casein hydrolysate, to 0.1% (w/v). This suggests that catabolism of several amino acids in strain BK may be the cause of the growth defect.

The mdl *and* amtB *genes play no obvious role in nitrogen regulation*

As the *glnK* mutation in strain BK in also *mdl*, and may be polar on expression of *amtB*, it is unclear from the results described above whether the effects attributed to *glnK* were due to the absence the *glnK* product, *amtB* product, *mdl* product or all of these gene products. We conducted several tests to see whether *mdl* or *amtB* had a role in nitrogen regulation and whether these genes had a role in the growth defect observed when the *glnK* null mutation was combined with *ΔglnB*. We compared nitrogen regulation of *glnA* expression in the *mdl* and *amtB* strains with the wild type and several control strains (*glnB*, *glnD*, *glnL*). These experiments indicated that the expression of GS in the *mdl* and *amtB* strains seemed to be wild type (data not shown). We also constructed strains that were *ΔglnB* and null for either *amtB* or *mdl* but were *glnK*$^+$ (strain BX and strain BM respectively). These strains grew as well as the *ΔglnB* strain on all media tested, indicating that the combination of *ΔglnB* with either *amtB* or *mdl* did not result in a growth defect. Finally, we transformed strain BK with multicopy plasmids encoding either *glnB*$^+$ (pBUC10; Jiang *et al*., 1997), *glnK*$^+$ (pglnK2; Fig. 2), or *glnK*$^+$ *amtB*$^+$ (pDK10; Fig. 2). In all three cases, the transformants were able to grow well on nitrogen-rich minimal media, suggesting that *amtB* and *mdl* had little role, if any, in the growth defect. This experiment also indicated that the presence of either PII or GlnK was sufficient to permit growth on nitrogen-rich minimal medium. In addition, we transformed strain BK with plasmid pBB180, which is identical to pBUC10 but contains a point mutation resulting in the alteration A49P in PII. This mutation in *glnB* was previously selected as defective in the negative regulation of *glnA* expression, and work with the purified protein indicated that it was uridylylated normally and interacted with the ATase well but could not interact with NRII (Jiang *et al*., 1997). Transformation of strain BK with pBB180 resulted in poor complementation for growth on nitrogen-rich defined solid media. This result suggests that NRII must be properly controlled for growth on these media, and thus that GlnK must be involved in regulation of NRII in cells lacking PII.

Either glnB *or* glnK *can regulate GS adenylylation state and* glnA *expression*

The regulation of *glnA* expression and the GS adenylylation state were examined in transformants of strains BK and B containing pglnB$^+$(pBUC10) and pglnK2 (Table 3). The presence of pBUC10 in either of these strains resulted in the inability to induce fully *glnA* in nitrogen-limiting medium, as previously described (Jiang *et al*., 1997), but the regulation of GS adenylylation was essentially normal. The pBUC10 plasmid caused the massive overexpression of PII (Jiang *et al*., 1997). In contrast, the presence of pglnK2 resulted in the normal regulation of GS adenylylation, as well as good regulation of *glnA* expression (Table 3). Although the pglnK2 plasmid did not vastly hyperexpress GlnK, as deduced by examining crude extracts on gels (data not shown), it is likely that the level of GlnK in these transformants is higher than typically found in wild-type cells. These results therefore suggest that, at least under these conditions, GlnK could interact with the ATase to bring about regulation of GS adenylylation state

and could interact with NRII to bring about regulation of *glnA* expression. The fact that *glnA* expression was regulated in response to ammonia when GlnK was present suggests that signalling occurred via the reversible covalent modification of GlnK by the *glnD*-encoded UTase/UR, in agreement with earlier observations (van Heeswijk *et al*., 1996).

The results shown in Tables 1 and 3 suggested that either GlnK or PII was sufficient for the regulation of GS adenylylation state. As the cultures used in these experiments were adapted to the growth medium, no information is provided about the kinetics of the regulation of GS adenylylation. We examined the kinetics of GS adenylylation in response to ammonia shock in strains lacking either GlnK or PII. For these experiments, cultures were adapted to nitrogen-limited conditions and had a low level of GS adenylylation. Upon shock with ammonia, both strain B and strain K rapidly adenylylated GS, with the same rate of adenylylation as the wild-type strain (data not shown). This result is consistent with the hypothesis that either GlnK or PII is sufficient for proper adenylylation state regulation, and furthermore suggests that the reversible modification of both proteins *in vivo* is rapid.

The amtB *gene does not seem to be involved in ammonium transport*

The *amtB* gene shares some homology with the *amtB* gene of yeast, which is thought to be involved in ammonia transport. Thus, it was proposed that *amtB* may have a similar role in bacteria (van Heeswijk *et al*., 1996). We examined whether *amtB* and/or *glnK* had a role in facilitating growth on ammonia at low concentration by streaking out the wild-type strain, strain K (lacking *glnK* and probably also *amtB* because of polarity) and the strain lacking only *amtB* on a series of plates containing decreasing concentrations of ammonia. The result of this experiment was that no discernible difference in growth was observed, suggesting no obvious role for *glnK* and/or *amtB* in ammonia transport.

Spontaneous supressor mutations allowing strain BD to use arginine as the sole nitrogen source map in glnK *and result in GlnK proteins with altered function*

Previous experiments have indicated that spontaneous supressor mutations permitting strain D (*glnD99*::Tn*10*) to use arginine as nitrogen source can occur in *glnL (ntrB)* (McNeil *et al*., 1982a,b; Atkinson and Ninfa, 1992), and some of these affect the interaction of NRII with PII (Atkinson and Ninfa, 1992). Mutations in *glnG* (*ntrC*) encoding altered NRI proteins that are better transcription activators or that activate transcription in the absence of phosphorylation can also suppress the Ntr^- phenotype of strain D (McNeil *et al*., 1982b; Weglenski *et al*., 1989; Flashner *et al*., 1995). Although strain BD could not use arginine as a nitrogen source, spontaneous mutations that restore this ability could be readily selected. Transductional mapping of these supressor mutations revealed that most of them were linked to the *glnALG* operon; that is, when the suppressor strains were used as the donor to introduce the *glnALG* region into a wild-type background, elevated *glnA* expression in the presence of ammonia was observed (data not shown). These suppressor mutations probably map to *glnL* and/or *glnG*, and correspond to the classes of suppressing mutations observed previously. To avoid these *glnA*-linked supressors, we transformed strain BD with either pgln2, which contains the entire wild-type *glnALG* operon in pBR322 and modestly overexpresses all three gene products (Backman *et al*., 1981), or pgln62, which contains only $glnL^+$ in pBR322 (Ueno-Nishio *et al*., 1984) and modestly overexpresses NRII (Atkinson and Ninfa, 1993). The BD transformants containing these plasmids were observed to be unable to use arginine as a nitrogen source and to throw off variants that were able to use arginine as a nitrogen source, like their parent. To determine if these variants contained extragenic suppressors in *glnK*, we crossed them with a donor phage grown on a strain containing *mdl*::*kan*, with selection for kanamycin resistance. As *mdl* is tightly linked to *glnK* in transductional crosses, introduction of the *mdl* marker should simultaneously result in the introduction of $glnK^+$, and this should eliminate the suppression if the suppression were due to mutations in *glnK*. The results of these experiments were that 5 out of 10 suppressed strains examined contained suppressing mutations closely linked to *mdl*. The DNA sequence of the *glnK* gene from the five strains containing putative *glnK* mutations was examined by direct sequencing from the bacterial chromosome after PCR amplification (*Experimental procedures*). Each of these five strains was observed to contain a point mutation in the *glnK* coding sequence altering a single amino acid, as indicated in Table 4. Interestingly, three of the five *glnK* mutations resulted in three different alterations of residue arginine-47 of GlnK. Thus, consistent with the results already presented, $glnK^+$ appears to be responsible for the inability of strain BD to use arginine as a nitrogen source.

We used another method to simplify the identification of extragenic suppressors of strain BD that map in *glnK*, and to simplify the transfer of these mutations to other strains. We constructed strain BDM by crossing *mdl*::*chl* into strain BD. As expected, mutation of the *mdl* gene had no significant effect on the phenotype of strain BD (Table 1 and data not shown). Spontaneous extragenic suppressors permitting strain BDM to grow on arginine as a sole nitrogen source were then isolated by direct selection, and transducing phage lysates were prepared on these strains. The phage were then used to backcross the *mdl* marker,

Table 4. *glnK* point mutants.

Strain	Allele	Alteration in GlnK	DNA sequence change
Selection: BD/p62 → glucose-arginine +			
OB10	*glnK1*	R47 → Q	CGG → CAG
OB14	*glnK2*	R47 → L	CGG → CTG
OB22	*glnK3*	E44 → G	GAC → GGG
OB25	*glnK4*	R47 → W	CGG → TGG
Selection: BD/pgln2 → glucose-arginine +			
OB113	*glnK5*	Y51 → N	TAC → AAC
Selection: BDM → glucose-arginine +			
KM4	*glnK6*	Y46 → C	TAC → TGC
KM14	*glnK7*	F11 → I	TTC → ATC
KM25	*glnK8*	R47 → W	CGG → TGG
KM28	*glnK9*	R47 → L	CGG → CTG
KM31	*glnK10*	Y46 → C	TAC → TGC

and tightly linked *glnK* gene, into strain BD, and the ability of the transductants to grow on glucose–arginine was assessed. Of 28 suppressed strains examined in this way, five contained suppressor mutations linked to *mdl*. DNA sequencing of the five *mdl*-linked suppressors indicated that in all cases the *glnK* gene was altered, as shown in Table 4. Interestingly, two of the mutations resulted in the same GlnK alterations (R47 → W, R47 → L) obtained from the selection performed in strain BD/pgln62.

To determine if the *glnK* point mutations obtained as suppressors of strain BDM were null, we examined whether these strains exhibited the severe growth defect on minimal medium that was observed with strain BK and strain BDK. The BDM-based strains containing *glnK6* (which is identical to *glnK10*) had a growth defect, and strains containing *glnK7* had a very severe growth defect, reminiscent of strain BDK and BK. Thus, it is possible that *glnK7* is a null allele and *glnK6* encodes a product with low activity. In contrast, the strains containing *glnK8* and *glnK9* displayed no apparent growth defect on nitrogen-rich minimal medium. This phenotype suggests that these *glnK** alleles are not null, but rather encode products that interact with NRII less well than does wild-type GlnK.

To assess the effects of the *glnK** mutations on the expression of the *glnK*::*lacZ* fusion, we constructed strains BDMK*ϕ containing the *glnK8* and *glnK9* alleles. In all cases, the expression of *glnA* was similar to strain BDM but the expression of the *glnK*::*lacZ* fusion was greatly elevated under both nitrogen-limiting and nitrogen excess conditions (Table 1). This result indicated that in strain BDMϕ, GlnK plays a role in negative regulation of the *glnK* promoter.

To assess the phenotype resulting from the *glnK** mutations in the presence of *glnD*$^+$, we constructed BK* strains containing the *glnK8*, and *glnK9* alleles. Strains lacking PII and containing *glnK8* and *glnK9* alleles grew well, again indicating that these *glnK* alleles are not null. The normal regulation of GS adenylylation state in these strains indicated that these GlnK proteins are able to productively interact with the ATase and UTase/UR (Table 2).

Discussion

Our work indicates that *glnK* has a role in nitrogen regulation, and that at least part of this role is to bring about the repression of Ntr gene expression by acting through the *glnL* product, NRII to control the level of NRI~P. Although much remains to be learned, it is clear from our results and previous results that *glnK* and *glnB* have only partially overlapping functions. For example, in adapted liquid cultures the absence of GlnK had only a minor effect on *glnA* expression, whereas the absence of PII resulted in the loss of negative regulation by ammonia, as noted earlier (Bueno *et al*., 1985). Thus, GlnK and PII are not interchangeable. On the other hand, the regulation of the adenylylation of GS was essentially normal in strains lacking either PII or GlnK (van Heeswijk *et al*., 1996, this work), suggesting that either protein may suffice for this regulation.

Several lines of evidence indicated that GlnK acts through NRII to control the level of NRI~P. In the aggregate, the data suggested that GlnK was less effective than was PII in reducing the level of NRI~P, and thus had little effect on expression from the *glnAp2* promoter, which is extraordinarily sensitive to NRI~P. However, GlnK plays an important role in the regulation of Ntr promoters that require a higher concentration of NRI~P for activation. One such promoter is the *glnK* promoter, which apparently requires a higher intracellular concentration of NRI~P for activation than does the *glnAp2* promoter. The *glnD99*::Tn*10* mutation, which is known to be leaky (Atkinson and Ninfa, 1992), had a relatively modest effect on the induction of *glnA* by nitrogen limitation, although this mutation essentially eliminated expression from the *glnK* promoter. Also, *glnK* was not highly expressed in strain BD, whereas *glnA* was highly expressed. Our results indicated that GlnK was responsible for regulation of the *glnK*

promoter in cells lacking PII, and this regulation was reduced by the *glnK8* and *glnK9* mutations.

The role of GlnK in regulating the expression of other Ntr genes is suggested by the properties of the double mutant lacking both PII and GlnK. This strain cannot grow on defined medium, but this defect was ameliorated by further loss of NRII, NRI or by genetic manipulations that limited the intracellular concentration of NRI. Thus, the growth defect of the double mutant is apparently due to unregulated expression of one or more Ntr genes. As many Ntr genes are involved in the catabolism of amino acids, this conclusion is consistent with the observation that the growth defect could be eliminated by supplementation with a complex mixture of amino acids. Because the strain lacking only PII does not display a similar growth defect, it is clear that GlnK has a role in the regulation of Ntr gene expression, and this role is by acting through NRII and NRI.

Previous structural studies of PII have indicated that the protein contains a large loop (the T loop) that contains the site of uridylylation and is probably the site of interaction with NRII and ATase (Carr *et al*., 1996). This hypothesis is consistent with structure/function studies indicating that the T loop is required for interaction with the receptors and that mutations altering PII receptor specificity mapped there (Jiang *et al*., 1997). For example, alteration of residue alanine 49 of PII resulted in an altered protein that interacted normally with the ATase, UTase, and small molecule effectors, but did not interact with NRII. In the current work, we observed that alteration of arginine-47 of GlnK altered the negative regulation of gene expression by GlnK (i.e. its interaction with NRII) while apparently not altering significantly the regulation of GS adenylylation state (indicating interaction with the UTase/UR and ATase). The arginine-47 residue seems to be particularly interesting, as 5 of the first 10 sequenced *glnK** mutations altered this residue. It is likely that good growth under the selective conditions required both the retention of ability to regulate the ATase and a decrease in the interaction with NRII, and that relatively few random mutation satisfy these criteria.

The existence of structurally similar but functionally distinct PII proteins has been previously observed in *Azospirillum brasilense* (de Zamaroczy *et al*., 1996) and may be a common theme in bacteria. What regulatory advantage is obtained by having slightly different versions of the PII protein with the expression of one regulated by the other? One possibility is that the cells contain additional receptors that are specifically controlled by GlnK or GlnK∼UMP, thus forming a regulatory cascade. Although this may be so, our results indicate that GlnK had a role in regulating the level of phosphorylation of NRI. GlnK may act as a 'memory protein' as proposed by van Heeswijk *et al.* (1996). If so, then additional experiments in which cells are shifted between various conditions may reveal an advantage conferred by the presence of GlnK. Our results indicated that GlnK was not required to efficiently shift from a condition of nitrogen limitation to a condition of nitrogen excess. However, whether GlnK confers an advantage to nitrogen-starved cells that encounter transitory increases in ammonia remains to be investigated. Additional possibilities are that GlnK serves to uncouple the regulation of NRII from the regulation of ATase under conditions of nitrogen limitation, that GlnK simply acts as a governor preventing sustained full expression of certain Ntr genes under all conditions or that GlnK is required for the fine control of NRI∼P levels when these are near the upper end of the physiological range. Many other hypotheses are possible, and experiments with purified components are required to reduce the range of possibilities.

Our experiments with strains lacking NRII unexpectedly revealed an inverse correlation between the level of glutamine synthetase activity and the level of acetyl phosphate accumulation under certain conditions. The acetyl phosphate pool may be influenced by the acetyl∼CoA pool, which is in turn influenced by the rate of glycolysis and tricarboxylic acid (TCA) cycle reactions (reviewed by McCleary *et al*., 1993). As one substrate for GS is glutamate, derived from 2-ketoglutarate, high GS activity may drain TCA cycle intermediates and reduce the intracellular acetyl phosphate (McCleary *et al*., 1993). In the absence of GS activity, the accumulation of acetyl phosphate may be favoured. This hypothesis is consistent with our observation that a mutation affecting ATase (*glnE*), which controls GS activity in cells, appears to affect acetyl phosphate accumulation under some circumstances. In strain BKL, GS is always highly adenylylated, and consequently GS activity is low. The overadenylylation of GS in this strain is probably because of the bias of ATase to adenylylate GS in the absence of regulation. The low GS activity in strain BKL is somehow compensated for by an increase in acetyl phosphate, as revealed by very high expression of *glnA*. In contrast, strain BKLE lacks the ability to adenylylate GS and consequently has higher GS activity. This is somehow compensated for by decreased accumulation of acetyl phosphate, as revealed by a reduced activation of *glnA* as compared with strain BKL. If this correlation indicates that GS activity influences acetyl phosphate accumulation, then it may be predicted that in cells lacking NRII and ATase, point mutations in *glnA* reducing GS catalytic activity should result in increased acetyl phosphate and thus increased expression of *glnA*.

Experimental procedures

Bacteriological techniques

Rich L-broth + glutamine medium and defined media were as described previously (Atkinson and Ninfa, 1992). Generalized transduction was as described using P1vir (Silhavy *et al*., 1984). Transformation with plasmid DNA was performed as

Table 5. Strains and plasmids used in this study.

Strain	Relevant genotype	Source or construction
YMC10	Wild–type	Backman *et al*. (1981)
G	*glnG*::Tn*5*	Backman *et al*. (1981)
B	*ΔglnB*	Bueno *et al*. (1985)
D	*glnD99*::Tn*10*	Bueno *et al*. (1985)
L	*glnL2001*	Bueno *et al*. (1985)
A	*glnA*::Tn*5*	Reitzer and Magasanik (1986)
BA	*ΔglnB glnA*::Tn*5*	Feng *et al*. (1992)
BL	*ΔglnB glnL2001*	Feng *et al*. (1992)
RB9017	*glnE*::Tn*5*	Bueno *et al*. (1985)
BD	*ΔglnB glnD99*::Tn*10*	RB9060×RB9040P1vir
BDL	*ΔglnB glnD99*::Tn*10 glnL2001*	BL×RB9040 P1vir
MW1	*ΔglnB*. . .Tn*5chl*^{r}	Laboratory collection
K4633	*recD*	David Friedman
MAE	*recD glnE*3::*chl*^{r}	K4633×p*glnE*3 DNA
MAK	*recD Δmdl–glnK*::*kan*^{r}	K4633×pDK5 DNA
MAKc	*recD Δmdl–glnK*::*chl*^{r}	K4633×pDK9 DNA
MAM	*recD mdl*::*chl*^{r}	K4633×pDK7 DNA
MAAmtB	*recD amtB*::*chl*^{r}	K4633×pDK11 DNA
K	*Δmdl–glnK*::*kan*^{r}	YMC10×MAK P1vir
BK	*ΔglnB Δmdl–glnK*::*kan*^{r}	RB9060×MAK P1vir
BKc	*ΔglnB Δmdl–glnK*::*chl*^{r}	RB9060×MAKc P1vir
BG	*ΔglnB glnG*::Tn*5*	YMC12×MW1 P1vir
GBK	*ΔglnB Δmdl–glnK*::*chl*^{r}*glnG*::Tn*5*	BKc×YMC12 P1vir
GKc	*glnG*::Tn*5 Δmdl–glnK*::*chl*^{r}	YMC12×MAKc P1vir
BDK	*ΔglnB Δmdl–glnK*::*kan*^{r} *glnD99*::Tn*10*	BD×MAK P1vir
KA	*Δmdl–glnK*::*chl*^{r} *glnA*::Tn*5*	TH16×MAKc P1vir
BKA	*ΔglnB Δmdl–glnK*::*chl*^{r} *glnA*::Tn*5*	BA×MAKc P1vir
BM	*ΔglnB mdl*::*chl*^{r}	B×MAM P1vir
BDM	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r}	BD×MAM P1vir
X	amtB::*chl*^{r}	YMC10×MAAmtB P1vir
BX	*ΔglnB amtB*::*chl*^{r}	RB9060×MA*AmtB*P1vir
LK	*glnL2001 Δmdl–glnK*::*kan*^{r}	RB9132×MAK P1vir
BLK	*ΔglnB glnL2001 Δmdl–glnK*::*kan*^{r}	BL×MAK P1vir
LE	*glnL2001 glnE*3::*chl*^{r}	L×MAE P1vir
BW1311	w.t	B. L. Wanner
BW18500	*ΔglnL2001 Δpta–ackA..zej*::Tn*10*	B. L. Wanner
P	*Δ*pta–*ackA*..zej::Tn*10*	Feng *et al*. (1992)
LP	*ΔglnL2001 Δpta–ackA..zej*::Tn*10*	Feng *et al*. (1992)
LEP	*ΔglnL2001 Δpta–ackA..zej*::Tn*10 glnE*3::*chl*	LE×BW18500 P1vir
BLEK	*ΔglnBΔglnL2001 glnE*3::*chl Δmdl–glnK*::*kan*^{r}	BLK×MAE P1vir
BLEKP	*ΔglnBΔglnL2001 glnE*3::*chl Δmdl–glnK*::*kan*^{r}*Δpta–ackA.zej*::Tn*10*	BLEK×BW18500 P1vir
TE2680	*recD*1903::Tn*10 trpDC700*::*putPA1303*[Kan^{s}Chl^r*lac*]	Elliott (1992)
TEϕ	*recD*1903::Tn*10 trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	TE2680×pglnK3 DNA
Bϕ	*ΔglnB trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	B×TEϕ P1vir
BDϕ	*ΔglnB glnD99*::Tn*10trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	BD×TEϕ P1vir
Dϕ	*glnD99*::Tn*10trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	D×TEϕ P1vir
YMC10ϕ	*trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	YMC10×TEϕ P1vir
Lϕ	*ΔglnL2001trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	L×TEϕ P1vir
BLϕ	*ΔglnBΔglnL2001trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	BL×TEϕ P1vir
LEϕ	*ΔglnL2001glnE*3::*chltrpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	LE×TEϕ P1vir
Pϕ	*Δpta–ackA..zej*::Tn*10trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	P×TEϕ P1vir
LPϕ	*ΔglnL2001Δpta–ackA.zej*::Tn*10 trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	LP×TEϕ P1vir
BLKϕ	*ΔglnBΔglnL2001Δmdl–glnK*::*chl*^{r} *trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	BLϕ×MAKc P1vir
KLϕ	*Δmdl–glnK*::*chl*^{r}*glnL2001trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	Lϕ×MAKc P1vir
Kϕ	*Δmdl–glnK*::*chl*^{r}*trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	YMC10ϕ×MAKc P1vir
LEPϕ	*ΔglnL2001glnE*3::*chl*^{r}*trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*] *Δpta–ackA..zej*::Tn*10*	LEϕ×BW18500 P1vir
BDMϕ	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r} *trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]	BDM×TEϕ
BD/pgln62	*ΔglnB glnD99*::Tn*10*pgln62(*glnL*$^+$)	BD×pgln62
OB10	*ΔglnB glnD99*::Tn*10*pgln62 *glnK1*	BD/pgln62→Garg$^+$
OB14	*ΔglnB glnD99*::Tn*10*pgln62 *glnK2*	BD/pgln62→Garg$^+$
OB22	*ΔglnB glnD99*::Tn*10*pgln62 *glnK3*	BD/pgln62→Garg$^+$
OB25	*ΔglnB glnD99*::Tn*10*pgln62 *glnK4*	BD/pgln62→Garg$^+$
BD/pgln2	*ΔglnB glnD99*::Tn*10*pgln2(*glnALG*$^+$)	BD×pgln2
OB113	*ΔglnB glnD99*::Tn*10*pgln2 *glnK5*	BD/pgln2→Garg$^+$
KM4	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r}*glnK6*	BDM→Garg +
KM14	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r}*glnK7*	BDM→Garg +

Table 5. Continued.

Strain	Relevant genotype	Source or construction
KM25	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r}*glnK8*	BDM → Garg +
KM28	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r}glnK9	BDM → Garg +
KM31	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r}*glnK10*	BDM → Garg +
BK-6	*ΔglnB mdl*::*chl*^{r}*glnK6*	RB9060 KM4 P1vir
BK-7	*ΔglnB mdl*::*chl*^{r}*glnK7*	RB9060 KM14 P1vir
BK-8	*ΔglnB mdl*::*chl*^{r}*glnK8*	RB9060 KM25 P1vir
BK-9	*ΔglnB mdl*::*chl*^{r}*glnK9*	RB9060 KM28 P1vir
BDMϕ*glnK8*	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r} *trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]*glnK8*	KM25×TEϕ P1vir
BDMϕ*glnK9*	*ΔglnB glnD99*::Tn*10 mdl*::*chl*^{r} *trpDC700*::*putPA1303*[Kan^{r}Chl^s*glnKp–lac*]*glnK9*	KM28×TEϕ P1vir
CAG12148	tsx–247::Tn*10* (9.5 min)	Miller (1992), via Rowena Matthews
CAG12154	zbb–3055::Tn*10* (11.5 min)	Miller (1992), via Rowena Matthews
CAG12107	zba–3101::Tn*10 kan* (10.5 min)	Miller (1992), via Rowena Matthews
CAG12017	zba–3054::Tn*10* (10.5 min)	Miller (1992), via Rowena Matthews

Plasmid	Relevant genotype or construction	Source/reference
pUC18	amp^r cloning vector	Maniatis *et al*. (1982)
pUC18N	amp^r, pUC18, but unique NdeI site destroyed by filling in with Klenow and religation	
pBR322	amp^r tet^r cloning vector	Maniatis *et al*. (1982)
pRS551	*lac* promoter probe vector	Simons *et al*. 1987)
pgln2	*glnALG*$^+$ in pBR322	Backman *et al*. (1981)
pgln62	glnL$^+$ in pBR322	Backman *et al*. (1983)
pgln31	*glnG*$^+$ in pBR322 with no obvious promoter	Backman *et al*. (1983)
pBR325	amp^r tet^r chl^r (source of chl^r gene)	Maniatis *et al*. (1982)
pUC4K	amp^r kan^r (source of kan^r gene)	Pharmacia
pDK4	*Bam*HI of Kohara clone 149 into pUC18	(a reconstruction as per Allikmets *et al*. (1993))
pDK5	*Pst*I fragment (kan^r) from pUC4K into *Eco*RV sites of pDK4 (Fig. 2)	
pDK7	*Bst*U1 fragment (chl^r) from pBR325 into blunted *Rsr*II site of pDK4 (Fig. 2)	
pDK9	*Bst*U1 fragment (chl^r) from pBR325 into *Eco*RV sites of pDK4 (Fig. 2)	
pDK10	*Bam*HI from pDK4 in pUC18N (Fig. 2)	

Plasmid	Relevant genotype or construction	Source/reference
pDK11	*Bst*U1 fragment (chl^r) from pBR325 into blunted *Nde*I site of pDK11	
pglnK2	*glnK*$^+$ and promoter region (PCR amplified) into pUC18. (Fig. 2)	
pglnK3	*glnK* promoter (*Eco*RI–*Bam*HI) into pRS551 pWM5 *glnE*$^+$ in pBLUESCRIPT	W. Muse, Ph.D. thesis
p*glnE*2	*Kpn*I–*Bam*HI of pWM5 (blunted) into *Sca*I site of pBR322	
p*glnE*3	*Nsi*I–*Sca*I fragment deleted from p*glnE*2 and replaced with the *Bst*UI fragment (*chl*) from pBR325	
pBUC10	*glnB*$^+$ in pUC18	Jiang *et al*. (1997)
pBB180	*glnB*–A49P in pUC18	Jiang *et al*. (1997)

described (Maniatis *et al*., 1982). Transformation with linear DNA to obtain recombination onto the chromosome, was performed by electroporation of the *recD* strain K4633 as described (Winans *et al*., 1985). For this purpose, ≈0.5 μg of the desired DNA fragment was band purified on low-melting-point agarose gels in 1× TAE buffer as described (Maniatis *et al*., 1982) and electroporated into strain K4633 using a Bio-Rad Gene Pulser apparatus. Transformants were selected on rich LB + glutamine medium supplemented with the appropriate drug. P1vir was then used to transfer the drug resistance marker and associated mutations to various strains in which the effects were assessed.

Assessment of the Ntr phenotype

The ability of cells to grow on arginine as the sole nitrogen source was examined. Previous studies had suggested that cells must be able to generate a high intracellular concentration of NRI~P to use this nitrogen source (reviewed in Reitzer, 1996b).

Construction of a glnK *null mutation*

The DNA sequence of the 10.1 min region of the *E. coli* chromosome shows the presence of the *mdl* gene, which does not have a role in nitrogen regulation, a nitrogen-regulated operon consisting of *glnK* and *amtB* (van Heeswijk *et al*., 1996) and *tesB*. We constructed null mutations in *mdl*, *glnK* and *amtB* by deletion of convenient restriction fragments and insertion of drug resistance markers, as depicted in Fig. 2. Our *glnK* null mutation is also *mdl* and is likely to have a polar effect on *amtB* (Fig. 2). The null mutations were then recombined onto the *E. coli* chromosome (Elliott *et al*., 1992), moved transductionally into a fresh background and mapped relative to known markers in the 10 min region. The *Δmdl–glnK*::*chl*, *Δmdl–glnK*::*kan*, *mdl*::*chl* and *amtB*::*chl* mutations were transductionally mapped relative to the sites of drug resistance mutations in strains CAG12017, CAG12148, CAG12154 and CAG12107 (Table 5), as well as to each other. In addition, the absence of wild-type sequences in the recombinants was confirmed by DNA typing using PCR. DNA typing of putative *glnK* mutants, and amplification of

Table 6. Strain aliases.

Alias	Strain name	Reference
G	YMC12	Backman *et al*. (1981)
B	RB9060	Bueno *et al.* (1985)
D	RB9040	Bueno *et al.* (1985)
L	RB9132	Bueno *et al.* (1985)
A	TH16	Reitzer and Magasanik (1986)
P	WS6002	Feng *et al*. (1992)
LP	WS6005	Feng *et al.* (1992)

glnK alleles for DNA sequencing was performed as described (Saiki *et al*., 1990) using the following primers:

upstream, 5′-GAAGCTTGGTACCGAGGAATCATTGAG-CGCCTG-3′; downstream, 5′-CGGATCCGTCGACTTCCTG-TTGCTGTGTGCCAGAG-3′ The DNA-typing data indicated that the *glnK*, *amtB* and *mdl* mutations formed *in vitro* and crossed onto the *E. coli* chromosome were recombined in single copy into the normal chromosomal context (data not shown).

Construction of a glnK::lacZ *operon fusion, and recombination of this fusion into the* trp *operon*

Using the known DNA sequence as a guide, PCR primers were designed to amplify the *glnK* control region. The *glnK* promoter was amplified using the following primers: (upstream primer) 5′-GGAATTCATTGAGCGCCTGAATAGCGC-3′; and (downstream primer) 5′-GGGATCCGGTAATGGTTCTGCTA-CACG-3′. The amplification product was cleaved with *Eco*RI and *Bam*HI and cloned into pRS551 (Simons *et al*., 1987) to form a transcriptional fusion to *lacZ* bracketed by transcriptional termination sequences with flanking sequence homology to the *trp* genes. The fusion was recombined onto the *E. coli* chromosome into the *trp* operon by digesting the DNA with *Sal*I, followed by electroporation into TE2680 with recombinants selected for kanamycin resistance, as described (Eliott, 1992). The recombinants were confirmed to have a new auxotrophic requirement for tryptophan, indicating correct recombination into the *trp* locus (Simons *et al*., 1987).

Construction of the glnE3 *allele*

The *Kpn*I–*Bam*HI fragment of pWM5 (Wilson Muse, Ph.D. thesis, 1996 University of Michigan Department of Biology) was rendered blunt with T4 DNA polymerase and ligated into the *Sca*I site of pBR322 forming pglnE2. This plasmid was cleaved with *Nsi*I and *Sca*I to release a fragment containing most of *glnE*. The *Bst*UI fragment from pBR325, containing the *chl*^{r} gene, was ligated into the backbone of pglnE2, forming pglnE3. The *glnE3*::*chl* allele was then recombined onto the bacterial chromosome after transformation with linear DNA, with selection for *chl*^{r}. The correct chromosomal location of the mutation was confirmed by DNA typing, using the following primers: (upstream) 5′-GTGAGTGACGAAGCCGGG-3′ and (downstream) 5′-GCGCCGCGGGGAATTCACTGAG-3′. The chromosomal site of insertion of the *glnE3*::*chl* allele was mapped relative to the known *glnE*::Tn*5* allele in strain RB9017 (Bueno *et al*., 1985).

Suppressor analysis

Spontaneous suppressors of the growth defect of strains BDM, BD/pgln2 and BD/pgln62 on glucose–arginine medium were obtained by direct selection on glucose–arginine plates, followed by repurification on this medium.

Strain nomenclature

All strains used in this study are summarized in Table 5. To facilitate the presentation, we use a strain nomenclature in which the strain name contains a description of the relevant genotype. Thus, for example, a strain containing *ΔglnL2001* is called L, a strain containing *ΔglnB* is called B and a strain containing *ΔglnBΔglnL2001 glnE*::*chl Δmdl–glnK*::*kan* is called BLEK. In this nomenclature, the order of the letters has no significance. The names previously used for strains renamed in this work are presented in Table 6.

Molecular biology techniques

DNA manipulations were by standard methods as described (Maniatis *et al*., 1982). Internal primers used for DNA sequencing of *glnK* and *glnK* mutant alleles were as follows: 5′-GGTCATCAGCAATCGCCAC-3′, 5′-CGTCAGTAAGGCGG-CTTACAC-3′ and 5′-GATTATCACGGTCACCAGCTTC-3′. DNA sequencing on 3 μg of plasmid DNA or 2.5 pmol PCR amplification product was performed using a Fidelity DNA sequencing system (Oncor). Plasmid DNA for DNA sequencing was prepared using a Qiagen plasmid midi kit. PCR product was prepared for DNA sequencing using a Qiagen Qiaquick PCR purification kit.

Glutamine synthetase assays

The γ-glutamyl transferase assay was used as described previously (Atkinson and Ninfa, 1992). Results are expressed as nmol of glutamyl-hydroxamate formed $min^{-1} mg^{-1}$ protein. Two to six cultures were used for each determination. Total GS was determined in reactions in which Mn^{2+} was the sole metal ion, whereas unadenylylated GS was determined in reactions in which Mg^{2+} ions were present in large excess over Mn^{2+} ions, as described previously (reviewed in Rhee *et al*., 1985b). In the latter case, only unadenylylated enzyme should be active, whereas in the former case both unadenylylated and adenylylated forms of the enzyme should be equally active. However, our experience with the *glnE3*::*chl* allele suggests that, for an unknown reason, the experiments with excess Mg^{2+} may be under-reporting the activity of unadenylylated GS (see *Results*). Thus, the measurements of GS adenylylation state reported here, especially when the extent of adenylylation was low, may be systematically overestimated. Alternatively, *E. coli* may contain, in addition to the *glnE*-encoded ATase, another enzyme capable of modifying GS.

β-Galactosidase assays

The assay was performed on cell sonicates using the conditions previously described (Silhavy *et al*., 1984). Data were calculated according to Wanner *et al*. (1977), and cell sonicates

were used as recommended by those authors except that cells were sonicated on ice in 50 mM potassium phosphate buffer, pH 7.5. Two cultures were used for each determination, and results are expressed as μmol of ONPG hydrolysed min^{-1} mg^{-1} protein.

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