

Studies on the roles of GlnK and GlnB in regulating *Klebsiella pneumoniae* NifL-dependent nitrogen control

Tania Arcondéguy^a, Wally C. van Heeswijk^{b,1}, Mike Merrick^{a,*}

^a Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, UK

^b Free University (Vrije Universiteit), Faculty of Biology, Department of Molecular Cell Physiology, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

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Abstract

In *Klebsiella pneumoniae*, nitrogen fixation (*nif*) genes are regulated in response to fixed nitrogen and oxygen. The activity of the *nif*-specific transcriptional activator NifA is modulated by NifL, which mediates both oxygen and nitrogen control. The signal transduction protein GlnK is required to relieve the inhibitory effect of NifL on NifA that occurs when the intracellular N status is high and in a wild-type cell, the action of GlnK cannot be substituted by the structurally related protein PII. We have studied the modulation of NifA activity by NifL in an heterologous system in which the host organism is *Escherichia coli*. Using a $\Delta glnB, \Delta glnK$ mutant, we have shown that the modulation of NifA activity by NifL is dependent on the concentration of GlnK in the cell and that when overproduced, PII can substitute for GlnK. Furthermore, our data suggest that PII can counteract the positive action of GlnK in relieving NifL-dependent inhibition of NifA activity. This negative effect of PII may be physiologically important in establishing repression of *nif* gene expression when the intracellular nitrogen status rises. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

In *Klebsiella pneumoniae*, transcription of the nitrogen fixation (*nif*) genes is dependent on the activator protein NifA. The *nifA* gene is co-transcribed

with *nifL* whose product modulates NifA activity in response to oxygen and fixed nitrogen. Expression of *nifL* and *nifA* is coupled at the translational level and NifL and NifA are expressed stoichiometrically. The two proteins form a protein complex in vitro and in vivo and stoichiometric levels of the two proteins are required for effective modulation of NifA activity [11,14,23].

Studies in *K. pneumoniae* have shown that the response of NifL to fixed nitrogen levels is independent of the signal transduction protein PII (encoded by *glnB*) [16] but is dependent on the structurally similar protein GlnK [18]. The GlnK protein is encoded in a

* Corresponding author. Tel.: +44 (1603) 452571; Fax: +44 (1603) 454970; E-mail: mike.merrick@bbsrc.ac.uk

¹ Present address: Dept. of Plant and Microbial Biology, University of California, Berkeley, CA 94720-3102, USA.

NtrC-dependent operon, *glnK amtB*, in which *amtB* encodes a proposed high-affinity ammonium transporter [29]. In both *K. pneumoniae* and *Escherichia coli*, *glnB* is apparently constitutively expressed whereas *glnK* expression is controlled by the two-component regulatory system NtrB/NtrC [2,18,29]. In response to nitrogen availability, the activities of both PII and GlnK are regulated by the uridylyl-transferase/uridylyl-removing enzyme and by the binding of small effectors molecules [3,29]. However, Edwards and Merrick [9] showed that whilst uridylyl-transferase was required for derepression of *ntr*-regulated promoters in *K. pneumoniae*, it was not involved in the *nif*-specific response to changes in nitrogen status mediated by NifL [9]. The involvement of GlnK and the independence of uridylylation was also shown by studying the inhibition of NifA activity by NifL in an heterologous system using *E. coli* as the host organism [13].

Alignment of amino acid sequences of both PII and GlnK indicates that the two proteins are approximately 70% identical and are characterised by differences at just five of their 112 residues [18]. In addition, GlnK, like PII, is a homotrimer and both these proteins share a common crystal structure [5,31]. Indeed, PII and GlnK are capable of substituting for each other with respect to some of their targets and can even form heterotrimers under some circumstances [10] (van Heeswijk, unpublished results). It has previously been shown that in the absence of PII, GlnK can regulate the adenylation of glutamine synthetase (GS) but cannot substitute for PII in regulation of *glnA* expression [2,29]. Experiments with PII have indicated that residues at the apex of the T-loop (residues 37–54) are essential for the interaction of PII with different protein receptors [19,21] and two residues in this region (52 and 54) have been identified as characteristically distinguishing PII and GlnK [18].

Given the potential interplay between PII and GlnK and the fact that *glnK* expression is regulated by NtrC and hence by PII [2,18,29], we considered that it was important to study the role of each paralogue when constitutively expressed in a background lacking both PII and GlnK. Using such a system, we have examined the effects on NifL/NifA interaction of altering the concentration of GlnK in the cell and the potential for substitution of

GlnK by PII. Our data indicate that GlnK concentration is important for the modulation of NifA activity and that PII might play a role in this modulation.

2. Materials and methods

2.1. Bacterial strains and media

The strains, plasmids and phage used are listed in Table 1. Strains were grown in Luria broth or M9 media [24] supplemented with 0.4% glucose as carbon source, thiamine ($0.4 \mu\text{g ml}^{-1}$) and antibiotics as required: carbenicillin ($100 \mu\text{g ml}^{-1}$), chloramphenicol ($15 \mu\text{g ml}^{-1}$), gentamicin ($25 \mu\text{g ml}^{-1}$), kanamycin ($30 \mu\text{g ml}^{-1}$). The nitrogen source was varied according to the nitrogen status required (see β -galactosidase assays).

A *glnK* in-frame deletion was constructed using the 3-kb *Bam*HI-*Pst*I fragment from pWVH141 cloned into *Bam*HI/*Pst*I-digested pUC18-Not to give pWVH152. The 214-bp *Cla*I-*Bst*EII fragment within *glnK* was then replaced by a linker containing a *Bam*HI site (gtgacGGATCC/CCTAGGgc), resulting in an in-frame deletion within *glnK* (pWVH153) that was confirmed by sequence analysis. A *Sma*I gentamicin Ω cassette [26] was inserted at the *Ssp*I site 171 bp upstream of *glnK* in pWVH153 to generate pWVH159. The *Not*I fragment from pWVH159 was then recloned into the temperature-sensitive plasmid pWVH115 generating pWVH163. pWVH115 is a derivative of pFC13 [8] in which a linker, containing the restriction sites *Sfi*I, *Not*I and *Bss*HI (5'-GATCCGCGCGCGGCCCTAGGC-CG-3'), was inserted at the *Bam*HI site. pWVH163 was then transformed into the *E. coli* wild-type strain YMC10 and allelic exchange was carried out as described by Hamilton et al. [12]. Nalidixic acid ($2 \mu\text{g ml}^{-1}$) was used to facilitate the curing of the plasmid and gentamicin resistant (Gm^{R}) and Cm^{S} colonies were selected. The structure of the resulting strain WCH30 (Fig. 1) was further confirmed by Southern blotting and PCR. The double mutant ΔglnBK was made by transducing the mutation from strain WCH30 into strain RB9060 using phage P1 [22], resulting in strain UNF3435. Gm^{R} clones were tested by PCR to verify the presence of the *glnK* in-frame

deletion. To check the presence of the *glnB* deletion in UNF3435 (and RB9060), PCR was performed with various primer sets. The size of the deletion in Δ *glnB2306* was estimated as 350 bp and the restriction sites for *Bst*EII, *Bgl*II, *Sac*II and *Dra*I, all of which are within the *glnB* gene, are deleted. The location of the 5' end of Δ *glnB2306* is estimated as 50 bp from the start codon [30].

A derivative of the *glnK* plasmid pWVH149 carrying a *glnK* Y51F mutation was constructed by replacement of the 430-bp *Bst*EII-*Sac*I fragment (encoding most of *glnK* and the 5' end of *amtB*) with a PCR fragment generated as follows. Oligonucleotides K5 and K6 (5'-GATCTATGAAGCTGGTG-3' and 5'-ATTGACGCTGAATTCCGCCC-3', respectively) were used to generate a 160-bp fragment covering the 5' end of *glnK* and oligonucleotides K7 and M_{13–20} (5'-CGGAATTCAGCGTCAAT-3' and 5'-GTAAACGACGGCCAGT-3', respectively) to generate a 460-bp fragment covering the 3' end of *glnK* and the 5' end of *amtB*. Oligonucleotides K6 and K7 incorporate a codon change TAC to TTC converting Y51 to F51 and generating a novel *Eco*RI site. The two PCR fragments then served as templates for a single PCR reaction with *glnK5* and M_{13–20} to generate the mutant fragment carrying the new *glnK* allele, *glnK51*, which was confirmed by sequencing.

The translational fusion *pnifH-lacZ* was constructed by cloning a 400-bp *Eco*RI-*Bam*HI fragment from pRT22 into pRS552 resulting in pTA34. Conversion of the multicopy fusion to single-copy chromosomal fusion, by in vivo recombination between pTA34 and λ RS45, was performed as described [27]. The recombinant phage carrying *pnifH-lacZ* fusion was then inserted in different genetic backgrounds (YMC10, WCH30 and UNF3435).

2.2. β -Galactosidase assays

Cultures were grown for 24 h in Luria broth before subculture in M9 medium supplemented with either 0.5 mM glutamine for N limitation or 20 mM (NH₄)₂SO₄ for N sufficiency anaerobically. β -Galactosidase assays were performed as described [22]. The results in Table 2 are the means of at least three independent experiments for each strain in which the S.D.s were not greater than $\pm 10\%$.

2.3. Western blot analysis

Five μ g of total protein extract was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the gel was blotted onto a HybondC membrane (Amersham), probed with an anti-serum directed against *E. coli* PII (which also cross-reacts with GlnK) and detected using the ECL system (Amersham). Protein concentrations of cellular extracts were determined using Pierce Coomassie Plus Protein Assay Reagent.

3. Results and discussion

3.1. Construction of Δ *glnK*, Δ *glnBK* and the heterologous system

We first constructed an in-frame deletion, Δ *glnK1*, within the *glnK* coding sequence, therefore minimising polar effects on the downstream *amtB* gene. An Ω gentamicin cassette was inserted upstream of *pglnK* and downstream of the *mdl* gene to facilitate subsequent transfer of the Δ *glnK1* allele by transduction. To avoid any potential read-through, the gentamicin gene was also cloned in the opposite orientation to the *glnK amtB* operon. The Δ *glnK1* mutation in strain WCH30 has no polar effect on the *amtB* gene as judged by methylamine transport activity (Gavin Thomas, personal communication). The double mutant UNF3435 (Δ *glnB2306* Δ *glnK1*) grew aerobically at about half the rate of the wild-type strain YMC10 in liquid minimal medium supplemented with 20 mM (NH₄)₂SO₄ ($\mu = 0.33$ h⁻¹ vs. $\mu = 0.6$ h⁻¹) or 0.5 mM glutamine ($\mu = 0.33$ h⁻¹ vs. $\mu = 0.65$ h⁻¹).

Despite its reduced growth rate, the phenotype of this strain is quite distinct from that of the Δ *glnBK* strain (BK) described by Atkinson and Ninfa [2]. That strain displays a severe growth defect on solid defined media, such that it does not form single colonies, and an even more severe defect in liquid minimal media. In strain BK, a region including a large part of the 3' end of the *mdl* gene upstream of *glnK*, the *mdl-glnK* intergenic region and part of *glnK* is replaced by a kanamycin resistance gene cassette. This insertion may be polar on the expression of *amtB*. Nevertheless, Atkinson and Ninfa report [2]

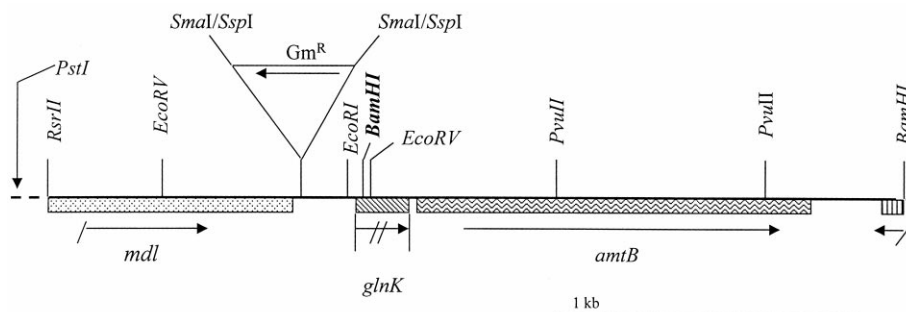


Fig. 1. Genetic organisation of the $\Delta glnK$ mutant. A gentamicin cassette was inserted at the *SspI* site upstream of *glnK* and a linker was used to construct the in-frame deletion and coincidentally to introduce a *BamHI* site.

that the phenotype is completely suppressed by the introduction of multicopy *glnB* or *glnK*, suggesting that neither *mdl* nor *amtB* affect the phenotype.

For this study, the *nifLA* operon was constitutively expressed from the *lac* promoter (pCC46) so that nitrogen regulation of *pnifH* is dependent only on the interaction of NifL with NifA. To maintain *nifLA* expression at a low level characteristic of its normal expression in *K. pneumoniae*, *plac* was not induced by addition of IPTG. We also confirmed, by monitoring β -galactosidase expression from a wild-type *lacZ* gene carried on the same plasmid

vector as pCC46, that *plac* expression was not significantly altered either by the different growth media (–N, +N) or by the different host strain genotypes (data not shown). In some experiments, a derivative of pCC46 (pCC47) containing an in-frame deletion in *nifL* was also used. Finally, to avoid possible titration of transcription factors and to simplify complementation studies, a *K. pneumoniae pnifH-lacZ* translational fusion was inserted in the *E. coli* chromosome.

NifL inhibition of NifA activity was then studied in different *E. coli* backgrounds (wild-type, $\Delta glnB$,

Table 1
Strains and plasmids

Strains and plasmids	Genotype	Relevant phenotype	References
<i>E. coli</i> strains			
YMC10	$\Delta lacU169$ <i>endA1 thi-1 hsdR17 supE44 hutC_K</i>	Wild-type	[6]
RB9060	$\Delta lacU169$ <i>endA1 thi-1 hsdR17 supE44 hutC_K ΔglnB2306</i>	GlnB [–]	[4]
WCH30	$\Delta lacU169$ <i>endA1 thi-1 hsdR17 supE44 hutC_K ΩGm^r ΔglnK1</i>	GlnK [–]	This work
UNF3435	$\Delta lacU169$ <i>endA1 thi-1 hsdR17 supE44 hutC_K ΔglnB2306 ΩGm^r ΔglnK1</i>	GlnB [–] GlnK [–]	This work
Plasmids			
pAH5	<i>E. coli glnB</i> expressed from its own promoter in pUC18	GlnB ⁺	[17]
pCC46	<i>K. pneumoniae nifLA</i> expressed from <i>placZ</i> in pHSG575	NifL ⁺ NifA ⁺	[7]
pCC47	<i>K. pneumoniae nifA</i> expressed from <i>placZ</i> in pHSG575	NifL [–] NifA ⁺	[7]
pRS552	Protein fusion vector		[27]
pRT22	<i>pnifH-lacZ</i> in pACYC184		[28]
pTA34	<i>E. coli lacZ</i> expressed from <i>pnifH</i> in pRS552		This work
pTA48	<i>E. coli glnK51</i> expressed from <i>placZ</i> in pBluescript-II SK+	GlnK ⁺	This work
pUC18Not	pUC18 derivative		[15]
pWVH115	Temperature-sensitive plasmid		This work
pWVH141	<i>E. coli glnK-amtB</i> operon in pBluescript-II SK+	GlnK ⁺ AmtB ⁺	[29]
pWVH149	<i>E. coli glnK</i> expressed from <i>placZ</i> in pBluescript-II SK+	GlnK ⁺	[29]
pWVH152	<i>BamHI-PstI</i> pWVH141 fragment in pUC18Not	GlnK ⁺ AmtB ⁺	This work
pWVH153	pWVH151 containing <i>glnK</i> in-frame deletion	GlnK [–] AmtB ⁺	This work
pWVH159	Insertion of an Ω cassette into pWVH153	GlnK [–] AmtB ⁺	This work
pWVH163	<i>NotI</i> fragment from pWVH159 into pWVH115	GlnK [–] AmtB ⁺	This work

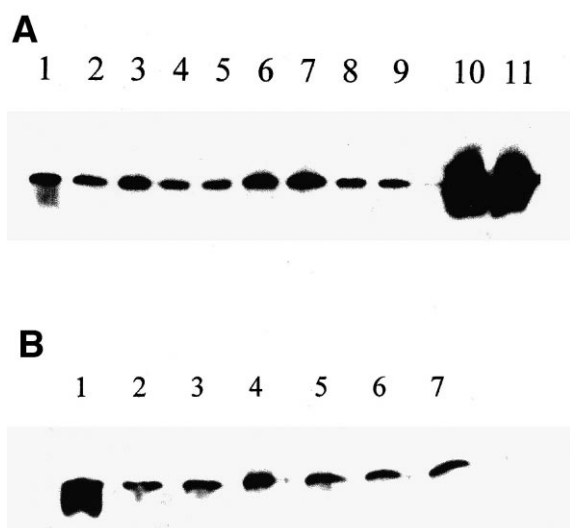


Fig. 2. Western blot analysis of PII and GlnK levels in different backgrounds. (A) Lane 1, purified GlnK (1.8 pmol); lanes 2 and 3, wild-type; lanes 4 and 5, $\Delta glnB$; lanes 6 and 7, $\Delta glnBK$ (pWVH149); lanes 8 and 9, $\Delta glnK$; lanes 10 and 11, $\Delta glnBK$ (pAH5). Lanes 3, 5, 7, 9 and 11, cells grown in $-N$; lanes 2, 4, 6, 8 and 10, cells grown in $+N$. (B) Lane 1, purified GlnK (3.6 pmol); lanes 2 and 3, wild-type; lanes 4 and 5, $\Delta glnBK$ (pWVH149); lanes 6 and 7, $\Delta glnBK$ (pTA48). Lanes 3, 5 and 7, cells grown in $-N$; lanes 2, 4 and 6, cells grown in $+N$.

$\Delta glnBK$ and $\Delta glnK$) under anaerobiosis in minimal media plus 20 mM $(NH_4)_2SO_4$ as nitrogen excess conditions or plus 0.5 mM glutamine as nitrogen-limiting conditions. Constitutive overexpression of *glnK*, *glnK51* or *glnB* was achieved by introduction of plasmids pWVH149, pTA48 or pAH5, respectively. It has been reported by Atkinson and Ninfa [3] that pWVH149 should have the potential to synthesise a LacZ'-GlnK fusion protein and that when

grown with IPTG induction of *plac*, such a protein is observed. In all our experiments, no IPTG induction was used and we could only detect synthesis of a native GlnK polypeptide (see Fig. 2 and [29]). Western blot analysis indicated that pWVH149 causes a 2–3-fold increase in cellular GlnK levels, whereas pAH5 causes a dramatic increase (estimated to be around 90-fold) in PII levels (Fig. 2A). The presence of the *glnK51* mutation (GlnKY51F) did not affect the level of expression of the protein compared to wild-type GlnK (compare pWVH149 and pTA48, Fig. 2B).

3.2. Modulation of *NifA* activity is dependent on the GlnK concentration

There was negligible β -galactosidase activity driven from *pnifH* in the absence of active *NifA*. When *NifL* and *NifA* were present, induction of *pnifH* expression occurred in a wild-type or $\Delta glnB$ strain grown under N-limiting conditions but no induction occurred in a $\Delta glnK$ or $\Delta glnBK$ background (Table 2). These data confirm a role for GlnK in relieving the inhibitory effect of *NifL* on *NifA* as previously observed by He et al. [13] and Jack et al. [18]. When *glnK* was expressed from *plac* under N-limiting conditions, an increase in β -galactosidase activity of 2–4-fold was observed compared to the level in a wild-type strain without pWVH149 (Table 2). This activity was correlated with an increased intracellular level of GlnK (Fig. 2, compare lanes 5 and 7). The highest levels of *pnifH* expression observed with increased GlnK were comparable to the lower values seen when *NifA* was expressed in the absence of *NifL* (see pCC47, Table 2). These data suggest that

Table 2
Effects of GlnK and GlnB on *pnifH-lacZ*^a expression in different backgrounds

Strain	Plasmids											
	–		pCC46 <i>nifL</i> ⁺ <i>nifA</i> ⁺		pCC47 <i>nifA</i> ⁺		pCC46, pWVH149 <i>nifL</i> ⁺ <i>nifA</i> ⁺ <i>glnK</i> ⁺		pCC46, pTA48 <i>nifL</i> ⁺ <i>nifA</i> ⁺ <i>glnK51</i>		pCC46, pAH5 <i>nifL</i> ⁺ <i>nifA</i> ⁺ <i>glnB</i> ⁺	
	–N	+N	–N	+N	–N	+N	–N	+N	–N	+N	–N	+N
YMC10 (wild-type)	10	5	1180	25	4150	4300	3500	810	930	35	1050	140
RB9060 ($\Delta glnB$)	30	15	2200	15	8500	5990	nd	nd	2750	350	nd	nd
WCH30 ($\Delta glnK$)	60	25	60	50	6660	6370	1955	420	290	25	800	95
UNF3435 ($\Delta glnBK$)	60	55	50	60	7600	8000	4420	890	2235	360	1180	185

–N: nitrogen-limiting medium, +N: Nitrogen sufficient medium, nd = not done.

^a β -Galactosidase activity (Miller units).

under the standard derepressing conditions used here, the wild-type strain does not realise the maximum potential level of *pnifH* expression.

Our results contrast with those of He et al. [13], who compared the effects of *glnK* expressed from a high or low copy number plasmid (pACYC184-based or miniF-based, respectively) and reported similar levels of expression from *pnifH-lacZ* in each case. It is difficult to compare these experiments directly with ours as there are a number of differences, notably, the *pnifH-lacZ* fusion itself or its chromosomal location, the chromosomal genotype of the host strains and also that He et al. expressed *nifLA* from *ptac* induced with 10 μ IPTG. All of these factors may be important. In particular, we recognise that the relative levels of expression of all the relevant proteins may have significant effects.

Comparison of the data derived from *glnB*⁺ strains as compared to *glnB*⁻ strains reveals a negative effect of the presence of PII on the levels of derepression from *pnifH* (compare YMC10(pCC46) and RB9060(pCC46) or WCH30(pCC46, pWVH149) and UNF3435(pCC46, pWVH149), Table 2). This effect is most pronounced when the GlnK protein is locked in the non-uridylylated form, GlnKY51F (compare WCH30(pCC46, pTA48) and UNF3435(pCC46, pTA48), Table 2). The levels of *pnifH-lacZ* expression are consistently lower with GlnKY51F compared with wild-type GlnK. These data suggest that PII can antagonise the effect of GlnK in relieving NifA inhibition by NifL and that this effect is much more marked when GlnK is in a non-uridylylated form.

3.3. Overexpressed PII can substitute for GlnK

In contrast to a GlnK⁻ mutant, a PII⁻ mutant has no effect on NifL inhibition of NifA activity (Table 2). This difference is quite striking given the similarity between the two proteins but reflects other specificity differences already recognised between PII and GlnK and described above [2,29]. In order to determine whether PII has any ability at all to substitute for GlnK, we significantly overexpressed *glnB* (using pAH5) in the Δ *glnBK* background (Table 2). In this case, the *EcoRI-SalI* fragment containing the *glnB* gene cloned into *EcoRI-SalI* sites of pUC18 allows for the expression of *glnB* from its own pro-

moter and also from *plac*. When PII was overexpressed (Fig. 2, lanes 10 and 11), we observed relief of the NifL-mediated inhibition to levels comparable to those seen in the wild-type strain, indicating that PII is capable of substituting for GlnK. However, in this case, the PII concentration was estimated as some 90 times higher than in the Δ *glnK* mutant (Fig. 2, lanes 9 and 11). We conclude that the binding coefficient of PII in its presumed interaction with one or more components of the NifLA complex is significantly lower than that of GlnK but that this is overcome when the protein concentration is raised to such levels. Whilst this situation is clearly non-physiological, such a phenomenon has been observed previously in that PII has also been shown to activate the deadenylation of GS-AMP in vitro when present in a relative high concentration [20].

3.4. The nitrogen response

In a wild-type strain, *glnK* expression is very low or completely absent under nitrogen excess (+N) conditions [2] and the expression of *pnifH* is around 1–2% of the induced (-N) level, i.e. hardly above background (Table 2). By contrast, when GlnK is overexpressed in YMC10 or UNF3435, the level of expression in +N increases very significantly, to 20% of the -N level. It would therefore appear that the presence of elevated levels of GlnK even under nitrogen excess conditions is sufficient to allow for relief of NifA inhibition. Nitrogen-limiting conditions induce a further 5-fold increase in *pnifH* expression with no apparent increase in GlnK levels (see Fig. 2, lanes 6 and 7). A similar effect is seen when PII is overexpressed in nitrogen sufficiency. *pnifH* expression is elevated 4–7-fold compared to the wild-type strain but these activities increase by a further 6–10-fold when nitrogen is limiting (Table 2).

4. Conclusions

In the heterologous system that we have used to analyse the effects of GlnK and PII on the relief of NifL-mediated inhibition of NifA activity, we find that the 'effective' NifA activity can be increased by an increase in the cellular concentration of GlnK. This suggests that under normal derepressing

conditions in a wild-type cell, a significant proportion of NifA may still be complexed with NifL and therefore inactive. Such a hypothesis is supported by the fact that the induced level of *pnifH* expression in the wild-type is significantly elevated when *nifL* is inactivated both in *E. coli* (this work) and in *K. pneumoniae* [1]. These data also suggest that in wild-type *K. pneumoniae*, maximal nitrogenase expression might not be achieved under normal nitrogen limitation. This is consistent with the phenomenon of hyperinduction where elevated nitrogenase activity is observed when *K. pneumoniae* is grown under argon rather than atmospheric nitrogen [25].

Our data also suggest that the relief of NifL-mediated inhibition may involve both changes in the intracellular level of GlnK and changes in its activity. It is known that nitrogen limitation leads to a marked elevation of *glnK* expression (both in *E. coli* and in *K. pneumoniae*) and our data indicate that the consequent increase in the intracellular GlnK concentration is alone sufficient to promote relief of inhibition. Both our data and previous reports [9,13] demonstrate that this effect is independent of the uridylylation of GlnK. Consequently, relief of inhibition is also seen when GlnK is expressed in nitrogen sufficiency. However, it is also clear that the levels of relief achieved are significantly greater in nitrogen limitation than nitrogen sufficiency. With either GlnK or GlnKY51F, activation of *pnifH* expression increases 5–8-fold in nitrogen limitation, indicating that a second component (which could be some other modification of GlnK or a change in the level of a critical metabolite) is required to achieve full induction.

Finally, when we uncouple transcriptional control of *glnK* from the Ntr system, we observe a marked antagonistic effect of PII on GlnK that is more pronounced when GlnK is not uridylylated. This effect could be direct or indirect but could conceivably occur by the formation of heterotrimeric complexes between PII and GlnK with a consequent inactivation of GlnK. It has been reported that, unlike PII, GlnK is not rapidly deuridylylated by the uridylyl-removing activity of uridylyltransferase [3]. Hence, given that both the uridylylated and deuridylylated forms of GlnK are active in relieving NifL-mediated inhibition, the question arises as to how NifL inhibition of NifA activity might be rapidly restored when am-

monium is added back to the medium [13]. Given that our data suggest a potential interaction between PII and GlnK, one possibility is that inactivation of GlnK is actually mediated through PII. Such an effect could occur via heterotrimer formation between PII and GlnK, if GlnK-UMP has a greater affinity for PII than for PII-UMP.

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References

- [1] Arnott, M., Sidoti, C., Hill, S. and Merrick, M. (1989) Deletion analysis of the nitrogen fixation regulatory gene *nifL* of *Klebsiella pneumoniae*. Arch. Microbiol. 151, 180–182.
- [2] Atkinson, M. and Ninfa, A.J. (1998) Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. Mol. Microbiol. 29, 431–447.
- [3] Atkinson, M. and Ninfa, A.J. (1999) Characterization of the GlnK protein of *Escherichia coli*. Mol. Microbiol. 32, 301–313.
- [4] Bueno, R., Pahel, G. and Magasanik, B. (1985) Role of *glnB* and *glnD* gene products in regulation of the *glnALG* operon of *Escherichia coli*. J. Bacteriol. 164, 816–822.
- [5] Carr, P.D., Cheah, E., Suffolk, P.M., Vasudevan, S.G., Dixon, N.E. and Ollis, D.L. (1996) X-ray structure of the signal transduction protein P_{II} from *Escherichia coli* at 1.9 Å. Acta Cryst. D52, 93–104.
- [6] Chen, Y.M., Backman, K. and Magasanik, B. (1982) Characterisation of a gene, *glnL*, the product of which is involved in the regulation of nitrogen utilisation in *Escherichia coli*. J. Bacteriol. 150, 214–220.
- [7] Contreras, A. and Drummond, M. (1991) Cys¹⁸⁴ and Cys¹⁸⁷ of NifL protein of *Klebsiella pneumoniae* are not absolutely required for inhibition of NifA activity. Gene 103, 83–86.
- [8] Cornet, F., Mortier, I., Patte, J. and Louarn, J.M. (1994) Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. J. Bacteriol. 176, 3188–3195.
- [9] Edwards, R. and Merrick, M. (1995) The role of uridylyltrans-

- ferase in the control of *Klebsiella pneumoniae nif* gene regulation. Mol. Gen. Genet. 247, 189–198.
- [10] Forchhammer, K., Hedler, A., Strobel, H. and Weiss, V. (1999) Heterotrimerization of PII-like signalling proteins: implications for PII-mediated signal transduction systems. Mol. Microbiol. 33, 338–349.
- [11] Govantes, F., Molina-Lopez, J.A. and Santero, E. (1996) Mechanism of coordinated synthesis of the antagonistic regulatory proteins NifL and NifA of *Klebsiella pneumoniae*. J. Bacteriol. 178, 6817–6823.
- [12] Hamilton, C.M., Aldea, M., Washburn, B.K., Babitzke, P. and Kushner, S.R. (1989) New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. 171, 4617–4622.
- [13] He, L., Soupene, E., Ninfa, A.J. and Kustu, S. (1998) Physiological role for the GlnK protein of enteric bacteria: relief of NifL inhibition under nitrogen-limiting conditions. J. Bacteriol. 180, 6661–6667.
- [14] Henderson, N., Austin, S.A. and Dixon, R.A. (1989) Role of metal ions in negative regulation of nitrogen fixation by the *nifL* gene product from *Klebsiella pneumoniae*. Mol. Gen. Genet. 216, 484–491.
- [15] Herrero, M., Delorenzo, V. and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. J. Bacteriol. 172, 6557–6567.
- [16] Holtel, A. and Merrick, M.J. (1989) The *Klebsiella pneumoniae* P_{II} protein (*glnB* gene product) is not absolutely required for nitrogen regulation and is not involved in NifL-mediated *nif* gene regulation. Mol. Gen. Genet. 217, 474–480.
- [17] Holtel, A.H. and Merrick, M. (1988) Identification of the *Klebsiella pneumoniae glnB* gene: nucleotide sequence of wild-type and mutant alleles. Mol. Gen. Genet. 215, 134–138.
- [18] Jack, R., de Zamaroczy, M. and Merrick, M. (1999) The signal transduction protein GlnK is required for NifL-dependent nitrogen control of *nif* expression in *Klebsiella pneumoniae*. J. Bacteriol. 181, 1156–1162.
- [19] Jaggi, R., Ybarlucea, W., Cheah, E., Carr, P.D., Edwards, K.J., Ollis, D. and Vasudevan, S.G. (1996) The role of the T-loop of the signal transducing protein P_{II} from *Escherichia coli*. FEBS Lett. 391, 223–228.
- [20] Jiang, P., Peliska, J.A. and Ninfa, A.J. (1998) The regulation of *Escherichia coli* glutamine synthetase revisited: role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. Biochemistry 37, 12802–12810.
- [21] Jiang, P., Zucker, P., Atkinson, M.R., Kamberov, E.S., Tirasophon, W., Chandran, P., Scheffe, B.R. and Ninfa, A.J. (1997) Structure/function analysis of the PII signal transduction protein of *Escherichia coli*: genetic separation of interactions with protein receptors. J. Bacteriol. 179, 4342–4353.
- [22] Miller, J.H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, New York.
- [23] Money, T., Jones, T., Dixon, R. and Austin, S. (1999) Isolation and properties of the complex between the enhancer binding protein NIFA and the sensor NIFL. J. Bacteriol. (in press).
- [24] Neidhardt, F.C., Bloch, P.L. and Smith, D.F. (1974) Culture medium for enterobacteria. J. Bacteriol. 119, 736–747.
- [25] Postgate, J.R. and Krishnapillai, V. (1977) Expression of *Klebsiella nif* and *his* genes in *Salmonella typhimurium*. J. Gen. Microbiol. 98, 379–385.
- [26] Schweizer, H.P. (1993) Small broad-host-range gentamicin resistance gene cassettes for site-specific insertion and deletion mutagenesis. Biotechniques 15, 831–833.
- [27] Simons, R.W., Houtman, F. and Kleckner, N. (1987) Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene 53, 85–96.
- [28] Tuli, R. and Merrick, M.J. (1988) Over-production and characterisation of the *nifA* gene product of *Klebsiella pneumoniae* - the transcription activator of *nif* gene expression. J. Gen. Microbiol. 134, 425–432.
- [29] van Heeswijk, W.C., Hoving, S., Molenaar, D., Stegeman, B., Kahn, D. and Westerhoff, H.V. (1996) An alternative P_{II} protein in the regulation of glutamine synthetase in *Escherichia coli*. Mol. Microbiol. 21, 133–146.
- [30] van Heeswijk, W.C., Rabenberg, M., Westerhof, H.V. and Kahn, D. (1993) The genes of the glutamine synthetase adenylylation cascade are not regulated by nitrogen in *Escherichia coli*. Mol. Microbiol. 9, 443–457.
- [31] Xu, Y., Cheah, E., Carr, P.D., van Heeswijk, W.C., Westerhof, H.V., Vasudevan, S.G. and Ollis, D. (1998) GlnK, a P_{II}-homologue: Structure reveals ATP binding site and indicates how the T-loops may be involved in molecular recognition. J. Mol. Biol. 282, 149–165.