

The Signal Transduction Protein GlnK Is Required for NifL-Dependent Nitrogen Control of *nif* Gene Expression in *Klebsiella pneumoniae*

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In *Klebsiella pneumoniae*, transcription of the nitrogen fixation (*nif*) genes is regulated in response to molecular oxygen or availability of fixed nitrogen by the coordinated activities of the *nifA* and *nifL* gene products. NifA is a *nif*-specific transcriptional activator, the activity of which is inhibited by interaction with NifL. Nitrogen control of NifL occurs at two levels: transcription of the *nifLA* operon is regulated by the global *ntr* system, and the inhibitory activity of NifL is controlled in response to fixed nitrogen by an unknown factor. *K. pneumoniae* synthesizes two P_{II}-like signal transduction proteins, GlnB, which we have previously shown not to be involved in the response of NifL to fixed nitrogen, and the recently identified protein GlnK. We have now cloned the *K. pneumoniae* *glnK* gene, studied its expression, and shown that a null mutation in *glnK* prevents NifL from responding to the absence of fixed nitrogen, i.e., from relieving the inhibition of NifA activity. Hence, GlnK appears to be involved, directly or indirectly, in NifL-dependent regulation of *nif* gene expression in *K. pneumoniae*. Comparison of the GlnB and GlnK amino acid sequences from six species of proteobacteria identifies five residues (residues 3, 5, 52, 54, and 64) which serve to distinguish the GlnB and GlnK proteins.

In diazotrophic proteobacteria, transcription of the nitrogen fixation (*nif*) genes is dependent on the *nif*-specific activator protein NifA, which is a member of the σ^N -dependent family of bacterial activators (18, 28, 36). In most organisms, the activity of NifA is controlled in response to two major environmental factors, namely, the availability of oxygen and of fixed nitrogen, but the mechanism of this control differs from one organism to another (20, 36). In *Klebsiella pneumoniae* and *Azotobacter vinelandii*, *nifA* is coordinately transcribed with a second gene, *nifL*, the product of which antagonizes NifA activity in response to both oxygen and fixed nitrogen (6, 23, 34, 46). In *K. pneumoniae*, *nifLA* expression is itself regulated in response to the cellular N status, whereas in *A. vinelandii*, it is constitutive (6, 16, 54). The response of NifL to oxygen is at least partly understood in that NifL in both organisms is a flavoprotein with flavin adenine dinucleotide as a prosthetic group and the oxidized form of NifL inhibits NifA activity (23, 48, 49). However, the mechanism by which NifL senses and responds to the cellular nitrogen status is unknown.

In enteric bacteria, global responses to changes in nitrogen status are mediated by the nitrogen regulation (*ntr*) system, which is considered to comprise four proteins. Two of these proteins, a uridylyltransferase (encoded by *glnD*) and P_{II} (a small, trimeric protein encoded by *glnB*), comprise a sensory transduction system whereby GlnD uridylylates GlnB under N-limiting conditions and deuridylylates GlnB-UMP in N excess. Hence, the uridylylation status of GlnB signals the intracellular nitrogen status, which is then communicated to the NtrB-NtrC two-component regulatory system. In N limitation, NtrB is autophosphorylated on residue His139 and these phos-

phoryl groups are subsequently transferred to NtrC with the concomitant activation of NtrC-P-dependent genes. In N excess, GlnB stimulates the NtrB-dependent dephosphorylation of NtrC and inactivation of *ntr*-dependent operons (37, 40).

The potential roles of one or more of the *ntr* genes in NifL-dependent *nif* regulation has been investigated in both *K. pneumoniae* and *A. vinelandii*. In *K. pneumoniae*, NifL-dependent nitrogen control occurs in the absence of GlnB or GlnD, suggesting that neither protein is essential for the sensing of fixed nitrogen by NifL (19, 24). However, it should be noted that experiments with the *glnD* null mutant utilized a strain with a secondary *ntrB* mutation that allowed constitutive expression of NtrC-dependent genes, including *nifLA*. In *A. vinelandii*, a mutation in *nfrX* (a *glnD* homologue) produces a Nif⁻ phenotype that can be suppressed by a secondary mutation in *nifL*, suggesting that in this organism, the absence of uridylyltransferase results in permanent inhibition of NifA activity by NifL (11).

Our understanding of the global Ntr system in enteric bacteria was recently complicated by the recognition of a second P_{II}-like protein (encoded by *glnK*) in *Escherichia coli* (56). *E. coli* *glnK* appears to be cotranscribed with a downstream gene (*amtB*), the product of which has been proposed to be an ammonium transporter (31, 41, 56), and expression of the operon is NtrC dependent (51). The amino acid sequence of GlnK is 67% identical to that of GlnB, and GlnK can also be uridylylated in N limitation, presumably at the conserved Tyr51 residue (56). The presence of two P_{II}-like proteins has since been reported in *Rhizobium etli*, *Azospirillum brasilense*, *Azorhizobium caulinodans*, *Herbaspirillum seropedicae*, *Azoarcus* sp. strain BH72, *Rhodobacter sphaeroides*, and *Acetobacter diazotrophicus* (5, 13, 14, 32, 38, 42, 43, 53).

The precise role of GlnK in nitrogen metabolism remains to be elucidated (2), but recent experiments which examined the activities of *K. pneumoniae* NifL and NifA in a heterologous *E. coli* or *Salmonella typhimurium* background found that the release of NifA from NifL-dependent inhibition does not occur

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Relevant phenotype	Source or reference
<i>K. pneumoniae</i> strains			
UNF122	<i>hisD2 Δlac-2002</i>	Wild type	25
UNF923	$\Delta(\textit{his-nifH})2639 \textit{nifH}::\textit{MudAplac} \Delta\textit{lac-2002} \textit{sbl-300}::\textit{Tn10}$	Nif	35
UNF1537	<i>hisD2 Δlac-2002 glnB502 glnB21::Tn5</i>	GlnB	25
UNF2792	<i>hisD2 Δlac-2002 sbl-300::Tn10 recA56 rpoN71::kan</i>	RpoN	12
UNF3440	$\Delta(\textit{his-nifH})2639 \textit{nifH}::\textit{MudAplac} \Delta\textit{lac-2002} \textit{sbl-300}::\textit{Tn10} \textit{glnB502} \textit{glnB21}::\textit{Tn5}$	Nif GlnB	This work
UNF3432	<i>hisD2 Δlac-2002 glnK1::K1XX</i>	GlnK	This work
UNF3433	$\Delta(\textit{his-nifH})2639 \textit{nifH}::\textit{MudAplac} \Delta\textit{lac-2002} \textit{sbl-300}::\textit{Tn10} \textit{glnK1}::\textit{K1XX}$	Nif GlnK	This work
<i>E. coli</i> strains			
YMC10	<i>ΔlacU169 endA1 thi-1 hsdR17 supE44</i>	Wild type	4
YMC26	<i>ΔlacU169 endA1 thi-1 hsdR17 supE44 glnD99::Tn10</i>	GlnD	44
RB9060	<i>ΔlacU169 endA1 thi-1 hsdR17 supE44 ΔglnB2306</i>	GlnB	7
RB9066	<i>ΔlacU169 endA1 thi-1 hsdR17 supE44 ntrC10::Tn5</i>	NtrC	7
TH1	<i>ΔlacU169 endA1 thi-1 hsdR17 supE44 ΔrpoN2518</i>	RpoN	T. Hunt
Plasmids			
pCC46	<i>K. pneumoniae nifLA</i> expressed from <i>placZ</i> in pHSG575	NifL ⁺ NifA ⁺	10
pCC47	<i>K. pneumoniae nifA</i> expressed from <i>placZ</i> in pHSG575	NifA ⁺	14
pNM480	pBR322-based <i>lacZ</i> fusion vector	Cb ^r	39
pRJ5	PCR fragment from <i>K. pneumoniae glnK</i> cloned in pBluescript KS+	'GlnK' Cb ^r	This work
pRJ16	<i>BamHI-HindIII</i> fragment containing <i>glnK</i> expressed from <i>pglnK</i> in pTZ18	GlnK ⁺ Cb ^r	This work
pRJ32	<i>PvuII</i> fragment from pRJ16 containing promoter region and start of <i>glnK</i> in pNM480	pGlnK::LacZ	This work
pRJ44	<i>K. pneumoniae glnK</i> expressed from <i>pcat</i> in Sp ^r (Ω) derivative of pACYC184	GlnK ⁺ Sp ^r	This work

in an *ntrC* mutant (22). It was concluded that NtrC activates transcription of a gene the product of which functions to relieve NifL inhibition of NifA under N-limiting conditions. One candidate for such a gene is *glnK*.

We have now cloned and sequenced the *glnK* gene of *K. pneumoniae*. We have studied the control of *glnK* expression and investigated the effect of a *glnK* null mutation. We found that in the absence of GlnK, NifA activity is inhibited in an NifL-dependent manner, even under N-limiting conditions, suggesting that GlnK is involved, directly or indirectly, in the sensing of cellular nitrogen status by NifL.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used in this work are listed in Table 1. All strains were grown on Luria medium (Luria broth or Luria agar [LA]). The nitrogen-free medium used (NFD) was as described by Dixon et al. (15). When used for growth of *K. pneumoniae* strains, NFD was supplemented with 25-μg/ml L-histidine, and for *E. coli*, it was supplemented with 1-μg/ml thiamine. Ammonium sulfate was added to NFD as a nitrogen source at a final concentration of 1 mg/ml. The antibiotics used for *E. coli* were 100-μg/ml carbenicillin, 15-μg/ml kanamycin, 15-μg/ml chloramphenicol, and 30-μg/ml streptomycin; those used for *K. pneumoniae* were 200-μg/ml both carbenicillin and ampicillin together, 30-μg/ml kanamycin, 40-μg/ml chloramphenicol, and 200-μg/ml spectinomycin.

DNA methods. Plasmids were isolated by using a plasmid miniprep kit (Qiagen) in accordance with the manufacturer's instructions. Restriction enzyme digests, blunting of DNA, ligation, and Southern hybridization reactions were carried out as described by Sambrook et al. (45). Plasmid pRJ32 was constructed by cloning the *PvuII* fragment carrying the 3' end of *mdl*, the *glnK* promoter region, and the 5' end of *glnK* into *lacZ* translational fusion plasmid pNM480. Plasmid pRJ44 was constructed by cloning an *EcoRI-DraIII* fragment (which extends from 21 bp upstream of the *glnK* ATG to 15 bp downstream of the termination codon) in place of the *DraI* fragment internal to the *cat* gene of pACYC184. In order to change the selective marker on this plasmid from tetracycline resistance to streptomycin resistance, an Ω cassette was then cloned into the *BamHI* site.

Isolation of fragment containing *glnK*. An internal fragment of the *K. pneumoniae glnK* gene was isolated from genomic DNA by PCR using primers based on conserved regions of GlnB (5' CTGCGAATTCGATHATHAAACCHTTC AARCTGGA 3' and 5' ACGCGATCCTCRCCGGTRCGRATRCGAATSC CSCG 3', where H is A, C, or T, R is A or G, and S is C or G). The resultant fragment was cloned into pBluescript KS+ by using *BamHI* and *EcoRI* sites included in the primers, giving pRJ5, and sequenced to confirm its likely identity.

Chromosomal DNA was isolated from wild-type *K. pneumoniae* UNF122 by the method of Ausubel et al. (3). Isolated DNA (10 μg) was subjected to either single or double digestion with *EcoRI*, *BamHI*, *HindIII*, *SspI*, and *SphI* and separated on 0.8% agarose. Fragments containing *glnK* were identified by Southern hybridization using the *BamHI-EcoRI* fragment from pRJ5 as a probe labelled with [³²P]dCTP using the Rediprime labelling kit (Amersham). DNA was redigested with *BamHI* and *HindIII*, and fragments of 1.5 kb were isolated from agarose and ligated into *BamHI-HindIII*-digested pTZ18 to form a minilibrary. Ligated DNA was used to transform *E. coli* 71-18. White, ampicillin-resistant colonies were patched onto nitrocellulose filters, laid onto LA-carbenicillin plates, and grown for 3 h. Filters were then transferred onto LA-chloramphenicol plates and incubated overnight. Clones containing *glnK* were identified by colony hybridization (45) using the [³²P]dCTP-labelled fragment described above. Sequencing of one hybridizing plasmid (pRJ16) was performed by using the dideoxy sequencing kit and protocol from Pharmacia.

Primer extension analysis of total RNA. Cells were grown overnight in the same media as used for β-galactosidase assays and subcultured into fresh media, and total RNA was isolated from exponentially growing cells by using the RNeasy kit (Qiagen) in accordance with the manufacturer's instructions. Primer extension analysis was performed by using 10 μg of total RNA and 0.2 pmol of a primer end labelled with [γ-³²P]dATP as described by Sawers and Böck (47). The oligonucleotide used (5' GAATGGTTTGATTACCACGG 3') hybridizes to bases 33 to 14 of *glnK*. Primer extension reactions were carried out as described by Sawers and Böck (47). Products were separated on a 6% polyacrylamide sequencing gel alongside a sequence produced by using the same ³²P-labelled primer and pRJ16 as the template.

Construction of *glnK::K1XX* mutation. The kanamycin resistance-encoding K1XX cassette from pUC4K1XX was cloned into the *EagI* site blunted 5'→3' by using mung bean nuclease (New England Biolabs), creating pRJ17, in which the *kan* gene is transcribed in the direction opposite to that of *glnK*. The *BamHI-PvuI* fragment of pRJ17, containing all of the cloned region, was then blunted 3'→5' by using large-fragment Klenow polymerase (Pharmacia), ligated into the *SmaI* site of *sacB*-containing vector pSG335 (21) to give pRJ25, and transformed into UNF122. Recombinant strains were identified by the ability to grow on LA supplemented with 5% sucrose and resistance to kanamycin. The *glnK::K1XX* mutation was then transduced, by using the P1 phage, into *K. pneumoniae* UNF923 to create UNF3433. The correct insertion of *glnK::K1XX* into both chromosomes was checked by PCR. Reactions were carried out in 100-μl volumes using *Taq* DNA polymerase (Boehringer) and primers homologous to the sequence of the *glnK* PCR fragment (sense, 5' TTCAAGCTGGAAGATGTG 3'; antisense, 5' CCTTCTGACGGCGGAAC 3') at a concentration of 20 pmol/reaction mixture.

β-Galactosidase assays. For *K. pneumoniae* strains, β-galactosidase assays were carried out as described by Holtel and Merrick (24) for N-limitation, the nitrogen source was 100-μg/ml glutamine (see Table 2 experiments) or 100-μg/ml serine (see Table 3 experiments), and for N sufficiency, it was 100-μg/ml glutamine plus 1-mg/ml (NH₄)₂SO₄ (see Table 2) or 1-mg/ml (NH₄)₂SO₄ (see

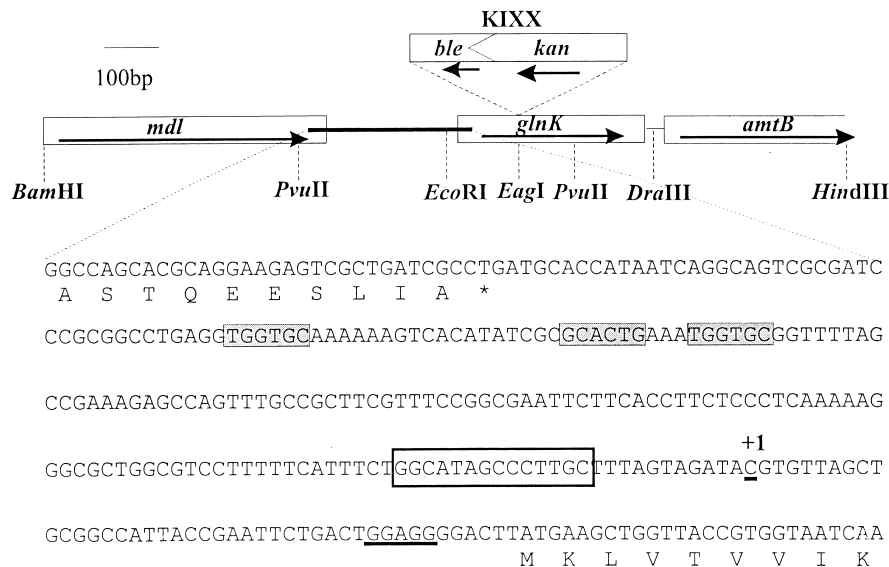


FIG. 1. Map of the cloned *Bam*HI-*Hind*III fragment (pRJ16) showing restriction sites used for genetic manipulations and the site of insertion of the KIXX cassette in *glnK*. The sequence of a region of the cloned fragment (300 bp, bp 481 to 781 of the sequence under accession no. AJ006531) comprising the 3' end of *mdl*, the *glnK* promoter region, and the start of *glnK* is shown. The proposed NtrC binding sites (filled boxes), σ^{54} -dependent promoter (clear box), and ribosome-binding site (underlined) are highlighted, and the transcriptional start site (+1) is marked.

Table 3). For *E. coli* strains, cultures were grown for 24 h in Luria broth before subculture in NFD medium supplemented with 0.02% Casamino Acids rather than glutamine. The data in Tables 2 and 3 are the means of at least three experiments in which the standard deviations were not greater than $\pm 10\%$.

[14 C]methylamine transport assays. Cells were grown overnight in the same way as for β -galactosidase assays. Cultures were then washed two times in saline phosphate and resuspended in 4.8 ml of NFD medium. The optical density at 650 nm was measured to determine the protein concentration. At zero time, 20 μ l of 14 CH₃NH₃⁺ (826 Ci/mol) was added to give a final concentration of 10 μ M 14 CH₃NH₃⁺. Samples of 500 μ l were taken at 0, 2, 4, 6, 10, and 20 min, and uptake was terminated by filtration through nitrocellulose filters (Millipore type HA; 0.45 μ m pore size) under a constant vacuum. Filters were then washed five times with NFD medium and exposed for 1 h to a PhosphorImager plate from which counts were determined. Data were calibrated by using internal standards spotted on filters and counted in the same experiment.

Nucleotide sequence accession number. The sequence of the *Bam*HI-*Hind*III fragment carried on pRJ16 (Fig. 1) has been assigned EMBL accession no. AJ006531.

RESULTS

Cloning of *glnK*. A 300-bp fragment encoding part of *K. pneumoniae glnK* was amplified by PCR using degenerate primers based on the conserved regions of the *E. coli* and *K. pneumoniae glnB* sequences. This fragment was cloned into Bluescript KS+ by using *Eco*RI and *Bam*HI restriction sites incorporated into the primers. The sequence of the cloned fragment confirmed that it encoded a polypeptide with 93% identity to the Ile8-to-Ala94 region of *E. coli* GlnK. This cloned fragment was then used as a probe to isolate a 1.5-kb hybridizing fragment from a minigene bank of *Bam*HI-*Hind*III-digested chromosomal *K. pneumoniae* DNA cloned in pTZ18. The resultant plasmid was designated pRJ16.

Sequencing. Sequencing of the fragment in pRJ16 identified three open reading frames, all of which were transcribed in the same direction, from *Bam*HI to *Hind*III, and which, by homology to the equivalent *E. coli* genes, encode the 3' end of *mdl*, all of *glnK*, and the 5' end of *amtB* (Fig. 1). Within the 240-bp *glnK* promoter region (Fig. 1), a consensus σ^N -dependent -24, -12 promoter sequence is located 60 bp upstream of the *glnK* translation start and two potential NtrC-binding sites are present; one complete site is 100 bp upstream of the proposed

promoter, and a half site is 30 bp upstream of that. There are two potential initiating methionine codons for *amtB*, of which we consider the second the most likely to be correct, based on homology with *E. coli amtB* and the presence of an appropriate ribosome-binding site. In this case, the *K. pneumoniae amtB* gene is separated by 35 bp from the 3' end of *glnK*, a spacing almost identical to that found in *E. coli* (56). No obvious termination or promoter sequences are apparent between *glnK* and *amtB*, suggesting that the two genes comprise a single operon, as proposed for the homologous genes in *E. coli*, *R. etli*, and *A. vinelandii* (33, 50, 53). The *K. pneumoniae glnK* gene encodes a polypeptide that is 94% identical to *E. coli* GlnK but only 69% identical to *E. coli* GlnB.

Comparison of GlnB and GlnK. Database searching for homologues of *K. pneumoniae* GlnK identified 21 gene products in the α and γ subdivisions of the class *Proteobacteria*. These fall into two distinct groups, which are homologous to *E. coli* GlnB and GlnK, respectively. In six organisms, both GlnB and GlnK have been identified, and alignment of their amino acid sequences using ClustalW indicates that the two proteins are characterized by differences at just 5 of the 112 residues (residues 3, 5, 52, 54, and 64) (Fig. 2). In GlnB proteins, residue 3 is lysine, residue 5 is glutamate or aspartate, residue 52 is methionine or valine, residue 54 is aspartate, and residue 64 is valine. By contrast, in GlnK, residue 3 is leucine or isoleucine, residue 5 is threonine, methionine, or isoleucine, residue 52 is serine or alanine, residue 54 is serine or asparagine, and residue 64 is alanine (with the exception of *R. sphaeroides* GlnK, in which residue 64 is valine).

This subdivision on the basis of polypeptide homology is consistent with the genetic organization of the P₁₁ structural genes within the α and γ subdivisions of the class *Proteobacteria*. The *glnB* genes are either monocistronic, as in *E. coli* and *K. pneumoniae* (25, 30), or linked to *glnA*, as in *Rhizobium* or *Rhodospirillum* spp. (9, 26), whereas genes encoding GlnK proteins are almost always linked to *amtB* homologues. One exception is the *A. brasilense glnZ* gene, which encodes a GlnK-



FIG. 2. Alignment of GlnB and GlnK polypeptide sequences. Residues that distinguish GlnK and GlnB (residues 3, 5, 52, 54, and 64) are in boldface type.

like protein on the basis of the predicted amino acid sequence but which is not linked to *amtB* (55).

In vivo transcript analysis. Primer extension analysis was carried out on RNA from wild-type *K. pneumoniae* (UNF122), an *rpoN* derivative (UNF2792), and the wild-type strain carrying a *glnK-lacZ* fusion plasmid (pRJ32). One specific signal was identified as the major transcript with a number of minor signals which appeared to be nonspecific in that they were present in all samples and did not correspond to any obvious motifs in the nontranslated sequence. Transcription was shown to begin 44 bp upstream of the translational start and at the appropriate distance from the proposed σ^N -binding site (Fig. 3). Transcription from this site was greatly reduced when cells were grown under nitrogen-rich conditions and was undetectable in an *rpoN* mutant which does not contain σ^N .

In vivo analysis of *glnK* expression. To analyze the expression of the *glnK amtB* operon in vivo, a translational *glnK-lacZ* fusion (pRJ32) was constructed and β -galactosidase activity was assayed in a variety of genetic backgrounds under nitrogen-limiting and nitrogen-sufficient conditions (Table 2). The pattern of expression of *glnK* was largely as predicted from the promoter elements identified in the DNA sequence. Expression was elevated under nitrogen-limiting compared to nitrogen-sufficient conditions and was essentially eliminated in the absence of σ^N (*rpoN*), NtrC, or GlnD. Expression from *pglnK* was constitutive in the absence of GlnB, whereas it was essentially wild type in the absence of GlnK. In both *K. pneumoniae* and *E. coli*, the level of expression in nitrogen sufficiency was considerably greater than in an *rpoN* or *ntrC* mutant strain.

Polarity of *glnK::KIXX* mutation *glnK1*. The introduction of a *glnK::KIXX* mutation into the *glnK amtB* operon might be expected to have a polar effect on expression of *amtB*. This was analyzed by using [¹⁴C]methylamine transport assays to measure the activity of AmtB. [¹⁴C]methylamine transport was assessed in three strains: wild-type UNF122, *glnK::KIXX* mu-

tant UNF3432, and UNF3432 carrying a plasmid-borne, constitutively expressed *glnK* gene [UNF3432(pRJ44)]. In N-limited wild-type cells, methylamine uptake was linear for 20 min at a rate of 140 pmol/mg of dry weight/min, and this uptake was reduced to less than 20 pmol/mg of dry weight/min in ammonia-grown cells. Methylamine transport was less than 5 pmol/mg of dry weight/min in *glnK::KIXX* mutant strain UNF3433 and was not complemented by the reintroduction of a plasmid-borne copy of *glnK* (pRJ44). Expression of *glnK*

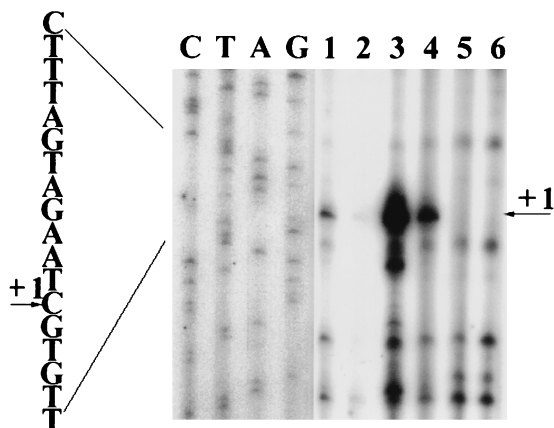


FIG. 3. Transcriptional start site of the *glnK amtB* operon determined by primer extension analysis of RNA from wild-type *K. pneumoniae* UNF122 (lanes 1 and 2), UNF122 with *glnK-lacZ* fusion plasmid pRJ32 (lanes 3 and 4), and UNF2792 *rpoN* (lanes 5 and 6). The RNAs in lanes 1, 3, and 5 were from strains grown under nitrogen-limiting conditions [NFD containing histidine (25 μ g/ml), and glutamine (100 μ g/ml)], and those in lanes 2, 4, and 6 were from strains grown under nitrogen-sufficient conditions [NFD containing histidine (25 μ g/ml) and (NH₄)₂SO₄ (1 mg/ml)]. Reaction mixtures run alongside the sequence were generated by using the same primer.

TABLE 2. Regulation of *pglnK-lacZ* in response to nitrogen

Strain	Relevant genotype	β -Galactosidase activity ^a	
		-N ^b	+N ^c
<i>Escherichia coli</i>			
YMC10(pRJ32)	Wild type	4,395	1,104
YMC26(pRJ32)	<i>glnD</i>	129	132
RB9060(pRJ32)	<i>glnB</i>	3,427	4,734
RB9060(pRJ32, pRJ44)	<i>glnB (glnK⁺)</i>	3,807	535
RB9066(pRJ32)	<i>ntrC</i>	189	137
TH1(pRJ32)	<i>rpoN</i>	92	97
<i>Klebsiella pneumoniae</i>			
UNF122(pRJ32)	Wild type	4,623	473
UNF1537(pRJ32)	<i>glnB</i>	5,367	4,921
UNF2792(pRJ32)	<i>rpoN</i>	248	167
UNF3432(pRJ32)	<i>glnK</i>	4,705	634

^a From pRJ32 (in Miller units).

^b -N, nitrogen-limiting medium.

^c +N, nitrogen-sufficient medium.

from pRJ44 was shown to be constitutive by introducing the plasmid into a *glnB glnK* double mutant of *E. coli* and analyzing GlnK levels in N limitation and N sufficiency by Western blotting with an antibody raised against an oligopeptide equivalent to conserved residues 31 to 50 of GlnB (14) (data not shown). These data confirm the polarity of the *glnK1* mutation on *amtB* and support the proposal that the two genes constitute an operon.

Phenotypic characterization of a *glnK::K1XX* mutation. The effect of the *glnK::K1XX* mutation on the activity of the *nifLA* gene products was assessed by using, as a genetic background, *K. pneumoniae* UNF923, which carries a chromosomal *pnifH-lacZ* fusion and has all of the *nif* genes, from *nifD* through to *nifQ* and into the *his* operon, deleted. The *nifLA* genes were cloned on low-copy-number plasmid pCC46 so that their expression from *plac* on this replicon was independent of the cellular nitrogen status. This independence of expression was confirmed by measuring *lacZ* expression from the same promoter on the same replicon in N limitation and N sufficiency (data not shown). When pCC46 is introduced into UNF923, expression from *pnifH* is normally regulated in response to the cellular nitrogen status (Table 3). Hence, in this strain, where *nifLA* expression is uncoupled from *ntr*-dependent regulation, the response of *pnifH* to nitrogen status reflects the activity of the NifA protein as regulated by its interaction with NifL. As

a control plasmid, pCC47, which expresses NifA alone from the same promoter as that in pCC46, was used.

The absence of GlnB (UNF3440) had only a minor effect on *pnifH* expression in this assay system, resulting in a slight reduction in the fully derepressed level of activity. By comparison, the *glnK::K1XX* mutation reduced the derepressed level by 75%, indicating that in the absence of GlnK, NifA was unable to escape fully from the inhibitory effects of NifL, even under nitrogen-limiting growth conditions. Normal NifLA-dependent regulation was restored to the *glnK* mutant by introduction of a constitutively expressed *glnK* gene on pRJ44. Neither the *glnB* nor the *glnK* mutation had any effect on activation by NifA alone (pCC47).

DISCUSSION

Sequence comparison of GlnB and GlnK proteins. The occurrence of duplicate copies of genes encoding P_{II}-like proteins now appears to be common among members of the α and γ subdivisions of the class *Proteobacteria*. The tertiary structures of *E. coli* GlnB and GlnK are very similar, the major differences being in the conformations of the T loops and of C-terminal residues 109 to 112 (57). Nevertheless, under some conditions, GlnB and GlnK are simultaneously expressed in the cell, and this raises questions about their respective roles. The fact that in some organisms, specific phenotypes can be assigned to mutations in *glnB* demonstrates that, at least in some respects, the two proteins have discrete functions, but the precise function of GlnK is still unclear (2).

Three of the residues, 3, 5, and 64, which distinguish GlnB and GlnK, cluster closely together in their three-dimensional structures (8, 57). In GlnB, Lys3 and Glu5 form a ring of alternating charged residues in the central cavity of the protein, whereas in GlnK, residues 3 and 5 are uncharged. These differences may have a functional significance in that the charge differences could serve to prevent trimer formation between heterologous polypeptides, i.e., to ensure that only homogeneous GlnB or GlnK trimers are formed at times when both *glnB* and *glnK* are expressed in the same cell. The other two distinguishing residues, 52 and 54, are at the base of the T loop and could affect the structure or mobility of the T loop, which is that part of P_{II} which interacts with other proteins.

Regulation of *glnK* and *amtB*. Expression of *K. pneumoniae glnK* is dependent on σ^N and NtrC, as in *E. coli*, *R. etli*, *A. caulinodans*, and *A. brasilense (glnZ)* (13, 38, 53, 56). The functionality of the proposed σ^N promoter in *K. pneumoniae glnK* was supported by transcript mapping and by analysis of the expression of a *pglnK-lacZ* fusion which showed a signifi-

TABLE 3. Effect of GlnK on the regulation of *K. pneumoniae pnifH*

Strain	Relevant genotype		β -Galactosidase activity ^a	
	Chromosome	Plasmid	-N ^b	+N ^c
UNF923(pCC46)	Wild type	<i>nifL⁺ nifA⁺</i>	618	34
UNF3440(pCC46)	<i>glnB</i>	<i>nifL⁺ nifA⁺</i>	498	31
UNF3433(pCC46)	<i>glnK</i>	<i>nifL⁺ nifA⁺</i>	161	33
UNF3433(pCC46, pRJ44)	<i>glnK</i>	<i>nifL⁺ nifA⁺, glnK⁺</i>	613	109
UNF923(pCC46, pRJ44)	Wild type	<i>nifL⁺ nifA⁺, glnK⁺</i>	609	56
UNF923(pCC47)	Wild type	<i>nifA⁺</i>	1,336	1,124
UNF3440(pCC47)	<i>glnB</i>	<i>nifA⁺</i>	1,075	967
UNF3433(pCC47)	<i>glnK</i>	<i>nifA⁺</i>	1,045	1,233

^a Expressed from the chromosomal *nifH::MudAplac* fusion (in Miller units).

^b -N, nitrogen-limiting medium.

^c +N, nitrogen-sufficient medium.

cant level of expression under nitrogen-sufficient conditions that was still NtrC dependent. Very similar results were reported for the *A. caulinodans glnK-amtB* promoter (38) and for *A. brasilense glnZ* expression (13). These data suggest that *pglnK* could be activated by low levels of NtrC-P present under nitrogen-sufficient growth conditions. However, studies with single-copy chromosomal *lacZ* fusions to the *E. coli glnK* promoter did not show such expression (2, 51). Our observation of *pglnK-lacZ* expression in ammonia may be a consequence of NtrC titration by the fusion plasmid, and indeed, we recognize that regulation in this system may be very sensitive to gene expression levels and copy number. A mutation in *glnK* did not affect *pglnK-lacZ* expression, indicating that GlnK is not autoregulatory.

Data on the effects of a *glnB* deletion on *glnK* expression are contradictory. We observed constitutive expression of *K. pneumoniae glnK* in *glnB* deletion strain RB9060 and, using the same strain, Atkinson and Ninfa (2) also found very significant levels of *E. coli glnK* expression in ammonium. By contrast, van Heeswijk et al. (56) also used the same *glnB* strain in Western blots and detected no GlnK in the presence of ammonium.

Role of GlnK in nitrogen fixation. The NifL protein of *K. pneumoniae* was first implicated in *nif*-specific nitrogen regulation by Merrick et al. (34), but the means by which NifL senses the nitrogen status of the cell has remained elusive. Our previous studies indicated that neither uridylyltransferase (GlnD) nor the P_{II} protein (GlnB) was directly involved in the regulation of NifL activity in response to nitrogen status. We concluded that another nitrogen-sensing component, possibly an alternative P_{II}-like protein, might be responsible (19, 24), and we have now shown that this is so.

The absence of GlnK severely impairs the ability of NifA to adopt an active form in the presence of NifL when cells are grown in nitrogen limitation. There is still some residual NifL-dependent regulation present in the *glnK* mutant (approximately 25% of that in the wild type), but given the similarities between GlnK and GlnB, we suggest that GlnB may be able to substitute partially for GlnK in this situation. Studies with *E. coli* have shown that in other situations, e.g., regulation of adenylyltransferase, either GlnB or GlnK can mediate regulation (2). Likewise, multicopy *glnK* restores regulation in a *glnB* mutant (Table 2), indicating that, when expressed at high levels in ammonium, GlnK can substitute for GlnB, presumably in promoting NtrB-dependent dephosphorylation of NtrC. Normal regulation was restored to a *glnK* mutant by the presence of a constitutively expressed plasmid-borne copy of *glnK*, establishing that GlnK is only effective in relieving the inhibitory effects of NifL on NifA in nitrogen-limiting medium. Our present results do not distinguish between a direct or an indirect effect of GlnK on NifL activity but are consistent with the observations of He et al. (22), who concluded that the product of an NtrC-dependent gene was required to relieve NifL inhibition of NifA activity.

GlnB or GlnK proteins have now been implicated in the regulation of NifA-dependent gene expression in a number of diazotrophs. In *A. brasilense glnB* mutants, NifA is inactive (29) but *nif* gene expression is restored by deletions in the N-terminal domain of NifA, suggesting that GlnB is required to activate NifA by preventing the inhibitory effect of its N-terminal domain (1). The N-terminal domains of *H. seropedicae* NifA and *A. vinelandii* VnfA and AnfA have also been implicated in nitrogen sensing (17, 52). Furthermore, an *H. seropedicae glnB* mutant is Nif⁻ while *nifA* expression, which is NtrC dependent, is expected to be constitutive in this background (5). Finally the Nif⁻ phenotype of a *glnD* (*nfrX*) mutant of *A. vinelandii* implicates a P_{II}-like protein in the regulation of NifA

activity in that organism, and a candidate *glnK* gene has recently been identified (11, 33).

Our data are consistent with a model in which the nitrogen-responsive inhibition of NifA activity by NifL in *K. pneumoniae* is regulated by an interaction between GlnK and the amino-terminal domain of NifA. Under nitrogen-limiting conditions, a particular form of GlnK could interact with NifA, thereby preventing the inhibitory effects of NifL. In a *glnK* mutant, this would not occur (although GlnB might partially substitute for GlnK) and NifL would inhibit NifA activity even in N limitation. In the wild type, a change to nitrogen sufficiency would alter the activity of GlnK and would also repress *glnK* transcription, thereby potentially allowing NifL to interact with NifA. With the present data, we cannot, however, exclude an alternative model in which the regulatory interaction is between GlnK and NifL.

The binding of 2-ketoglutarate and ATP to GlnB has been shown to activate the protein with respect to its control of adenylyltransferase and NtrB, and it has been proposed that an allosteric alteration of GlnB upon the binding of 2-ketoglutarate activates the uridylyltransferase reaction (27). Our previous data indicated that uridylylation is not required for control of NifL inhibition of NifA activity (19), and it is therefore possible that an allosteric change induced in GlnK by the binding of a low-molecular-weight ligand such as 2-ketoglutarate might be sufficient to control interaction with NifA and/or NifL.

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ADDENDUM IN PROOF

After this article was accepted for publication, He et al. published an article in the *Journal of Bacteriology* (L. He, E. Soupene, A. Ninfa, and S. Kustu, *J. Bacteriol.* **180**:6661–6667, 1998) which also identifies a role for GlnK in relieving inhibition of NifA activity by NifL.

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