

Physiological Role for the GlnK Protein of Enteric Bacteria: Relief of NifL Inhibition under Nitrogen-Limiting Conditions

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In *Klebsiella pneumoniae*, NifA-dependent transcription of nitrogen fixation (*nif*) genes is inhibited by a flavo-protein, NifL, in the presence of molecular oxygen and/or combined nitrogen. We recently demonstrated that the general nitrogen regulator NtrC is required to relieve NifL inhibition under nitrogen (N)-limiting conditions. We provide evidence that the sole basis for the NtrC requirement is its role as an activator of transcription for *glnK*, which encodes a P_{II}-like allosteric effector. Relief of NifL inhibition is a unique physiological function for GlnK in that the structurally related GlnB protein of enteric bacteria—apparently a paralogue of GlnK—cannot substitute. Unexpectedly, although covalent modification of GlnK by uridylylation normally occurs under N-limiting conditions, several lines of evidence indicate that uridylylation is not required for relief of NifL inhibition. When GlnK was synthesized constitutively from non-NtrC-dependent promoters, it was able to relieve NifL inhibition in the absence of uridylyltransferase, the product of the *glnD* gene, and under N excess conditions. Moreover, an altered form of GlnK, GlnK^{Y51N}, which cannot be uridylylated due to the absence of the requisite tyrosine, was still able to relieve NifL inhibition.

In proteobacteria, the NifA protein activates the transcription of genes whose products are required for biological nitrogen fixation (*nif* genes; reviewed in references 18, 25, and 39). Both the expression and the activity of NifA can be regulated in response to the cellular oxygen (O₂) and/or nitrogen (N) status, but the mechanisms for regulation differ with the organism. (O₂ is a signal because it destroys the function of metal cofactors essential for nitrogen fixation.) In members of the γ subdivision of the proteobacteria, e.g., *Klebsiella pneumoniae* and *Azotobacter vinelandii*, NifA activity is inhibited by a second regulatory protein, NifL, in the presence of O₂ and/or combined N (32, 40). Immunological studies imply that NifL inhibits NifA activity by means of a stoichiometric interaction with NifA (30). Prerequisite to such an interaction, NifL and NifA are synthesized from the chromosome in comparable amounts because their synthesis is translationally coupled (26).

The NifL protein of *A. vinelandii* is known to be a flavoprotein with flavin adenine dinucleotide (FAD) as a prosthetic group (31), and the same is true for the NifL protein of *K. pneumoniae* (47). In vitro, reduction of the FAD cofactor of NifL relieves NifL inhibition of NifA activity in a purified transcription system (31). Hence, in vitro NifL appears to act as a redox switch that allows NifA activity under O₂-limiting conditions. In vivo, however, NifL inhibition can be relieved only under both O₂- and N-limiting conditions (32, 40). This leads to the hypothesis that perhaps the FAD cofactor of NifL can be reduced in vivo only when both of the above limitations pertain. Because environmental iron (Fe) is required for relief of NifL inhibition but is not present in NifL itself, we have postulated that an unidentified Fe-containing protein may be the physiological reductant for NifL (47, 48). In addition, we have demonstrated that transcriptional activation by the general ni-

trogen regulatory protein NtrC is required for relief of NifL inhibition (29) and that regulation of the *K. pneumoniae* NifL protein in response to cellular nitrogen or oxygen availability can be studied in the related enteric bacterium *Escherichia coli* (29).

NtrC is required for transcription of the *glnK* gene (51, 52), which encodes a P_{II}-like protein allosteric effector that is known to be involved in the regulation of nitrogen metabolism (5, 52). Therefore, we tested the hypothesis that GlnK is directly required to relieve NifL inhibition. We present evidence that GlnK is the only protein under NtrC control that is needed for relief of NifL inhibition, that the related GlnB protein cannot substitute and hence is a paralogue of GlnK, and that covalent modification of GlnK by uridylylation is not required for relief of NifL inhibition. The latter finding was surprising because GlnK is normally highly uridylylated under N-limiting conditions.

MATERIALS AND METHODS

Strains and strain constructions. The bacterial strains and plasmids used in this work are listed in Table 1. Plasmid transformation was done as described previously (29). Ampicillin, spectinomycin, chloramphenicol, kanamycin, and tetracycline were generally used at 100, 50, 25, 25, and 10 μ g/ml, respectively. For selection of pZC320 (a mini-F-based plasmid), the concentration of antibiotics was reduced to half of the normal value.

Construction of a *glnK::Spe*^r null mutation. Strain NCM1971 was obtained by insertion of a spectinomycin resistance cassette (Ω -Spc) into the *glnK* gene of strain NCM1529 (29), as achieved in the following steps. (i) A 1.3-kb *NdeI-EcoNI* fragment from plasmid p149B6 (2) (also known as [aka] pJES999), which carries the *mdl-glnK-antB-tesB'* region of *E. coli*, was subcloned into the *SmaI* site of pUC19 to yield pJES1006. (ii) A 2-kb *SmaI* fragment carrying the spectinomycin resistance cassette from pUT-Sm/Spc (15) (aka pJES857) was inserted into pJES1006, which had been cleaved with *BstEII* and made blunt ended by the Klenow fragment of DNA polymerase I, to yield pJES1007. (iii) A 3.3-kb *SacI-SalI* fragment carrying *glnK::Spe*^r was ligated into the suicide vector pCVD442 (21) (aka pJES1034), which had been digested with *SacI* and *SalI*, and the resulting plasmid, pJES1073, which contains the *sacB* gene of *Bacillus subtilis*, was then transferred by electroporation into *E. coli* SM10 λ pir. (iv) Plasmid pJES1073 was transformed into NCM1529, and ampicillin-resistant transformants were chosen for further analysis. Because pJES1073 cannot replicate in NCM1529 (there is no π protein available), ampicillin-resistant transformants should be the ones in which pJES1073 has been integrated into the chromosome.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or characteristic(s)	Reference or description
Strains		
NCM1529	<i>araD139</i> Δ (<i>argF-lacU</i>)169 <i>flhD5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1 trpDC700::putPA1303::[Kan^r-Φ(<i>nifH'</i>-'<i>lacZ</i>)] (wild type)</i>	29
NCM1528 ^a	NCM1529/pNH3	29
NCM1527	NCM1529/pJES851	29
NCM1686	<i>glnD99::Tn10</i> [Kan ^r - Φ (<i>nifH'</i> -' <i>lacZ</i>)]	29
NCM1687 ^b	NCM1686/pNH3	29
NCM1851 ^c	<i>ntrC10::Tn5</i> [Kan ^r - Φ (<i>nifH'</i> -' <i>lacZ</i>)]/pNH3	29
NCM1971	<i>glnK::Spc^r</i> [Kan ^r - Φ (<i>nifH'</i> -' <i>lacZ</i>)]	See Materials and Methods
NCM1974 ^d	NCM1971/pNH3	See Materials and Methods
NCM1975	NCM1971/pJES851	See Materials and Methods
NCM1799	[<i>araD139</i>] Δ (<i>argF-lacU</i>)205 <i>flhD5301 fruA25 rpsL150 (strR) rha-10 deoC1</i> Δ [(<i>ntrC?</i> - <i>ntrB-glnA</i>)229] <i>trpDC700::putPA1303::[Kan^r-Φ(<i>nifH'</i>-'<i>lacZ</i>)]/pNH3</i>	29
Plasmids		
p149B6	6-kb <i>Bam</i> HI fragment of <i>E. coli</i> <i>mdl-glnK-antB-tesB'</i> region in pBluescriptII KS	2
pDK601	<i>E. coli</i> ' <i>yfjA-glnB</i> ' region in pUC18	53
pNH3	<i>K. pneumoniae</i> <i>nifLA</i> controlled by <i>tac</i> promoter	30
pJES851	<i>K. pneumoniae</i> <i>nifA</i> controlled by <i>tac</i> promoter	48
pUT-Sm/Spc	Spectinomycin resistance cassette (Ω -Spc)	15
pCVD442	Suicide vector	21
pZC320	Mini-F vector	49
pJES1006	' <i>mdl-glnK-antB</i> ' in pUC19	See Materials and Methods
pJES1007	' <i>mdl-glnK::Spc^r-antB</i> ' in pUC19	See Materials and Methods
pJES1073	' <i>mdl-glnK::Spc^r-antB</i> ' in pCVD442	See Materials and Methods
pJES1086	<i>glnK</i> at <i>Eco</i> RI site of pACYC184 (<i>Pcat-glnK⁺</i>)	See Materials and Methods
pJES1104	<i>glnK</i> at <i>Sal</i> I site of pACYC184 (<i>Ptet-glnK⁺</i>)	See Materials and Methods
pJES1106	<i>glnK5</i> at <i>Sal</i> I site of pACYC184 [<i>Ptet-glnK5</i> (GlnK ^{Y51N})]	See Materials and Methods
pJES1111	<i>glnB</i> in pACYC184	See Materials and Methods
pJES1118	' <i>mdl-glnK-cat</i> ' in pUC19 (<i>PglnK-glnK⁺</i>)	See Materials and Methods
pJES1119	' <i>mdl-glnK5-cat</i> ' in pUC19 [<i>PglnK-glnK5</i> (GlnK ^{Y51N})]	See Materials and Methods
pJES1160	<i>glnB</i> in pZC320	See Materials and Methods
pJES1161	' <i>mdl-glnK-cat</i> ' in pZC320 (<i>PglnK-glnK⁺</i>)	See Materials and Methods
pJES1162	' <i>mdl-glnK5-cat</i> ' in pZC320 [<i>Ptet-glnK5</i> (GlnK ^{Y51N})]	See Materials and Methods
pJES1163	<i>cat</i> in pZC320	See Materials and Methods

^a Strains NCM2086 and NCM2089 carry plasmids pJES1104 and pJES1106, respectively, in NCM1528.

^b Strains NCM2093, NCM2094, NCM2080, and NCM2082 carry plasmids pJES1104, pJES1106, pJES1161, and pJES1162, respectively, in NCM1687.

^c Strains NCM2088 and NCM2091 carry plasmids pJES1104 and pJES1106, respectively, in NCM1851.

^d Strains NCM1982, NCM2087, NCM2090, NCM2048, NCM2068, NCM2069, NCM2081, NCM2070, and NCM1990 carry plasmids pJES1086, pJES1104, pJES1106, pJES1111, pJES1160, pJES1161, pJES1162, pJES1163, and pACYC184, respectively, in NCM1974.

(v) After overnight growth in LB medium (0.5% NaCl, 1% tryptone, 0.5% yeast extract) without ampicillin, transformants were plated on 7% sucrose-LB medium and grown at 30°C. Among the sucrose-resistant colonies should be those that have achieved allelic exchange at *glnK* (Ap^r *glnK::Spc^r*). The presence of the *glnK::Spc^r* insertion mutation was confirmed by Southern blot analysis (data not shown).

Construction of plasmids. To construct a plasmid carrying *Pcat-glnK⁺*, the small *Eco*RI-*Bsg*I (blunt ended by the Klenow fragment) fragment of pJES1006, which carries the intact *glnK* region (including the ribosomal binding site for *glnK*) and the first 50 bp of *antB*, was ligated to the large *Eco*RI-*Scal*I fragment of pACYC184 to yield plasmid pJES1086.

Because pJES1086 encodes tetracycline resistance as its only drug resistance, it cannot be selectively maintained in *glnD99::Tn10* mutant strains. Therefore, we constructed plasmid pJES1104, in which the 0.4-kb *Xho*I-*Sal*I *glnK* fragment from pKOP2 (3), which carries the intact *glnK* coding region from the first codon (ATG) and a strong ribosomal binding site of *atpE* (46), was inserted into the *Sal*I site of pACYC184 and thereby expressed from the *tet* promoter. Plasmid pJES1104 confers chloramphenicol resistance. The orientation of the insertion was checked by digestion with appropriate restriction enzymes. Plasmid pJES1106 was constructed similarly to pJES1104, except that it carries *glnK5* (GlnK^{Y51N}) (5) from pKOPY51N (3).

To express *glnK* and the *glnK5* allele from the native *glnK* promoter in a very low-copy-number vector, we made use of the vector pZC320 (aka pJES1120), which is a mini-F vector (49). To do so, we first inserted a chloramphenicol resistance gene into pJES1006 (*PglnK-glnK⁺*) by replacing the small *Bst*EII-*Bsg*I (blunt ended by the Klenow fragment) fragment of pJES1006 with the large *Bst*EII-*Bst*Z171 fragment of pJES1104 (or pJES1106) to yield pJES1118 (or pJES1119); the large *Ecl*136II-*Hind*III fragment of pJES1118 (or pJES1119) was then ligated to the large *Scal*I-*Hind*III fragment of pZC320 to yield pJES1161 (or pJES1162). Thus, plasmid pJES1161 (or pJES1162) carries the 3' portion of the *mdl* gene and the *glnK* promoter and coding regions (or *PglnK-glnK5* [GlnK^{Y51N}]). To construct a control plasmid, pJES1163, the *Eco*RV-*Hind*III

fragment of pJES1118, which carries only the chloramphenicol resistance gene, was inserted into the large *Scal*I-*Hind*III fragment of pZC320.

To construct *PglnB-glnB⁺*, a 1.5-kb *Sal*I-*Bgl*III fragment of pDK601 (53) was ligated into the *Sal*I and *Bam*HI sites of pACYC184 to yield plasmid pJES1111, which carries the 3' part of *yfjA* and the intact *glnB* gene of *E. coli*. Plasmid pJES1160 carries *PglnB-glnB⁺* in the pZC320 vector; it was constructed by ligating the large *Hind*III-*Bst*Z171 fragment of pJES1111 to the *Hind*III and *Pml*I sites of pZC320.

Growth conditions and β -galactosidase assay. We recently constructed *E. coli* strains that carry a single copy of a *K. pneumoniae* Φ (*nifH'*-'*lacZ*) (hereafter designated simply *nifH'*-'*lacZ*) translational fusion at the *trp* locus and a plasmid that carries either *Ptac-nifLA* (pNH3) or *Ptac-nifA* (pJES851) of *K. pneumoniae* (29). *NifA*-mediated expression from the *nifH* promoter was monitored by measuring the differential rate of β -galactosidase synthesis during exponential growth. Inhibitory effects of NifL on NifA were assessed by virtue of a decrease in *nifH* expression. Transcription of *nifA* or *nifLA* was induced from the *tac* promoter with 10 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) unless specified otherwise. Cells were grown in modified K medium at 30°C as described previously (29). Samples of the growing cultures were taken every 2 to 3 h to determine the optical density at 600 nm (OD₆₀₀) and β -galactosidase activity [units/milliliter = 1,000 (OD₄₂₀ - 1.75 \times OD₅₅₀)/($\Delta t \times v$)] (41). The differential rates (35) of β -galactosidase synthesis reported here were obtained by determining the slopes of plots of β -galactosidase activity versus the OD of the culture (units/milliliter/OD₆₀₀).

Western blotting. Western blotting was performed as reported previously (29). At an OD₆₀₀ of 0.8 to 1.0, cells were harvested from 1 ml of culture and concentrated 20-fold into sodium dodecyl sulfate (SDS) gel-loading buffer (44). (The differential rate of β -galactosidase synthesis was also measured in these cultures.) The NifL and NifA proteins were separated by SDS-10% polyacrylamide gel electrophoresis (PAGE) using a Tris-glycine system (44) and were detected with rabbit polyclonal antisera against the proteins from *K. pneumoniae*. To compare the amounts of these proteins in different strains or under different

TABLE 2. Effects of a *glnK* null allele on activity of the *K. pneumoniae* NifL protein

Strain ^a	Relevant genotype	Expression of <i>nifH</i> '-' <i>lacZ</i> ^b (U/ml/OD ₆₀₀)	
		With IPTG ^c	Without IPTG
NCM1528	Wild type/ <i>Ptac-nifLA</i>	5,780	2,060
NCM1974	<i>glnK/Ptac-nifLA</i>	<10	<10
NCM1975	<i>glnK/Ptac-nifA</i>	6,310	ND ^d
NCM1990	<i>glnK/Ptac-nifLA/pACYC184</i>	<10	ND
NCM1982	<i>glnK/Ptac-nifLA Pcat-glnK</i> ⁺	ND	1,180
NCM2069	<i>glnK/Ptac-nifLA PglN-glnK</i> ⁺	5,160	1,950

^a All strains carry a single chromosomal copy of a *nifH*'-'*lacZ* fusion at the *trp* locus.

^b Specific activity is the slope of a plot of β-galactosidase activity versus the OD₆₀₀ of the culture. Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine as the sole nitrogen source.

^c 10 μM.

^d ND, not determined.

conditions of induction, the sample that gave the higher intensity of staining was diluted serially by a factor of 2 until its staining intensity was less than that of the other. The GlnK protein was separated by SDS-16.5% PAGE in a Tris-Tricine system (45) or by SDS-20% PAGE in a 2× Tris-glycine system (12). It was detected with either rabbit polyclonal antiserum against the *E. coli* GlnK protein (42) or the *E. coli* GlnB protein (see figure legends). Purified NifL, NifA, and GlnK and prestained protein molecular size markers (Broad Range; New England Biolabs) were used as standards.

RESULTS

GlnK is required to relieve NifL inhibition of NifA activity.

To determine whether GlnK is required for relief of NifL inhibition, we constructed a *glnK* null mutation in *E. coli* by inserting a spectinomycin cassette into the very beginning of the *glnK* gene (*glnK-amtB* operon) and measured the differential rate of expression of a *nifH*'-'*lacZ* fusion in the resulting strain (see Materials and Methods). In agreement with our previous results, the inhibition of NifA activity by NifL was relieved only under anaerobic and N-limiting (derepressing) conditions (29) (Tables 2 and 3, parent strain NCM1528). However, in the *glnK* null mutant (NCM1974), NifL inhibition apparently persisted under derepressing conditions: expression from the *nifH* promoter was more than 100-fold lower than in the parent strain (NCM1528). In the absence of NifL, NifA was able to activate transcription from the *nifH* promoter in the *glnK* mutant strain (NCM1975), indicating that effects of GlnK were indeed transduced through NifL.

To demonstrate that failure of the *glnK* mutant strain to express *nifH* under derepressing conditions could not be accounted for by a decrease in the amount of NifA, we compared the amounts of the NifL and NifA proteins in the *glnK* mutant strain to those in a congenic wild-type strain by immunological means (see Materials and Methods). Under derepressing conditions and at our usual level of induction with 10 μM IPTG, the *glnK* mutant strain had about one-fourth as much NifL and NifA as the wild-type strain (Fig. 1, compare lanes 3 and 7 to lanes 1 and 5 and the results obtained with diluted samples [see Materials and Methods; data not shown]). However, it is unlikely that this 4-fold decrease in the amounts of the NifL and NifA proteins resulted in a greater-than-100-fold decrease in the level of *nifH* expression. When the amounts of NifL and NifA in the *glnK* mutant strain were adjusted to be greater than or equal to those in the wild-type strain by adding IPTG to the culture of the *glnK* mutant strain but not the wild-type strain (Fig. 1, lanes 3 and 7 versus lanes 2 and 6) or omitting IPTG in both cases (Fig. 1, lanes 4 and 8 versus lanes 2 and 6), the level of *nifH* expression in the *glnK* mutant strain remained >100-

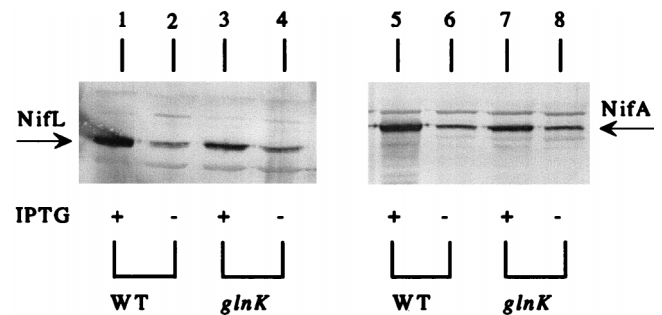


FIG. 1. Amounts of NifL and NifA in wild-type (wt) and *glnK* mutant strains of *E. coli*. Amounts of proteins in crude cell extracts were determined by Western blotting with polyclonal antisera against NifL (lanes 1 to 4) or NifA (lanes 5 to 8). All strains carried plasmid pNH3 (*Ptac-nifLA*). Cultures were grown in K medium under derepressing conditions, and expression of NifL and NifA was induced with 10 μM IPTG (+) or was not induced (-). Lanes: 1, 2, 5, and 6, strain NCM1528 (wild type); 3, 4, 7, and 8, strain NCM1974 (*glnK* mutant).

fold lower than that in the wild-type strain (Table 2, compare NCM1974 with or without IPTG induction to NCM1528 without IPTG induction). These results were very similar to those obtained previously by comparing an *ntrC* mutant strain to a wild-type strain (29).

The *glnK* insertion mutation is expected to have a polar effect on *amtB* (ammonium-methylammonium transport B) because *amtB* lies downstream of *glnK* and is apparently co-transcribed with it (52). To determine whether AmtB plays any role in *nif* regulation, we constructed an *amtB* null mutation (51). Relief of NifL inhibition in the *amtB* mutant strain under derepressing conditions was similar to that in the wild-type strain (data not shown). In addition, when a *glnK*⁺ allele was provided in *trans* to the *glnK* disruption (*Pcat-glnK*⁺, pJES1086; *Ptet-glnK*⁺, pJES1104; *PglN-glnK*⁺, pJES1161), the GlnK protein alone was sufficient to relieve NifL inhibition (Table 2, compare strains NCM1982 [*glnK* mutant strain with pJES1086] and NCM2069 [*glnK* mutant strain with pJES1161] with strain NCM1990 [*glnK* mutant strain with pACYC184 vector], Table

TABLE 3. Effects of GlnK and GlnK^{Y51N} on NifL activity in different backgrounds

Strain ^a	Relevant genotype	Expression of <i>nifH</i> '-' <i>lacZ</i> ^b (U/ml/OD ₆₀₀)	
		-N	+N
NCM1528	Wild type	3,580	<10
NCM1974	<i>glnK</i>	<10	<10
NCM1851	<i>ntrC</i>	<10	<10
NCM1687	<i>glnD</i>	20	<10
NCM2086	Wild type/ <i>Ptet-glnK</i> ⁺	3,090	1,060
NCM2087	<i>glnK/Ptet-glnK</i> ⁺	3,860	1,240
NCM2088	<i>ntrC/Ptet-glnK</i> ⁺	3,510	1,850
NCM2093	<i>glnD/Ptet-glnK</i> ⁺	3,940	1,340
NCM2089	Wild type/ <i>Ptet-glnK5</i> (GlnK ^{Y51N})	1,450	1,330
NCM2090	<i>glnK/Ptet-glnK5</i> (GlnK ^{Y51N})	2,200	910
NCM2091	<i>ntrC/Ptet-glnK5</i> (GlnK ^{Y51N})	2,990	1,450
NCM2094	<i>glnD/Ptet-glnK5</i> (GlnK ^{Y51N})	3,390	2,670

^a All strains carry a single chromosomal copy of a *nifH*'-'*lacZ* fusion at the *trp* locus and plasmid pNH3 (*Ptac-nifLA*).

^b See footnote b to Table 2. Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine (-N) or with 2 mM glutamine plus 4 mM NH₄Cl (+N).

3, compare strain NCM2087 [*glnK* mutant strain with pJES1104] with strain NCM1974 [*glnK* mutant strain without pJES1104]). Results from the two sorts of control experiments—effects of disrupting *amtB* and of complementing *glnK* alleles with *glnK*⁺—indicated that the AmtB protein is not involved in *nif* regulation. Rather, GlnK is essential for relief of NifL inhibition under derepressing conditions.

GlnK is the missing link between NtrC and NifL. We previously demonstrated that transcriptional activation by NtrC is required for relief of NifL inhibition under derepressing conditions (29) (Table 3, NCM1851 versus NCM1528). To determine whether this requirement could be accounted for by the role of NtrC in activating the transcription of *glnK* (51, 52), we expressed *glnK* from promoter *Ptet* (pJES1104) or *Pcat* (pJES1086) in an *ntrC* null mutant strain. Under these circumstances, NtrC was no longer needed to relieve NifL inhibition (Table 3, compare *nifH* expression in strains NCM1851 and NCM2088; data not shown). Similarly, the constitutive expression of *glnK* (pJES1086) suppressed effects of the *ntrB* mutation in NCM1872 (29) and those of the *glnA ntrB ntrC* deletion mutation in NCM1799 (Table 1) to allow relief of NifL inhibition (data not shown). Because the parental strain of NCM1799 is different from that of the other strains used in this study, the requirement for GlnK to relieve NifL inhibition does not appear to be strain specific. Hence, the NtrC requirement for relief of NifL inhibition can be accounted for entirely by the requirement for synthesis of GlnK.

Uridylation of GlnK is not required for relief of NifL inhibition. In agreement with the results of van Heeswijk et al. (52), we confirmed that the GlnK protein is highly uridylylated under N-limiting conditions in a *glnD*⁺ background (Fig. 2A, lane 3 versus lane 1) but not in a *glnD* mutant background (Fig. 2A, lanes 5 and 7). The GlnD protein, which has both uridylyltransferase and uridylyl-removing activities, uridylylates GlnK under N-limiting conditions but less so in the presence of ammonium (N excess conditions). Surprisingly, however, the constitutive expression of *glnK* resulted in relief of NifL inhibition in the presence of ammonium, as well as under N-limiting conditions (Table 3, β -galactosidase activities under N excess conditions). These results implied that uridylylation of GlnK might not be required for relief of NifL inhibition.

We used two additional approaches to investigate the role of uridylylation of the GlnK protein with respect to *nif* regulation. First, we examined the effects of a *glnD::Tn10* allele that is known to reduce uridylyltransferase activity greatly (8, 9). Second, we examined effects of changing the uridylylated tyrosine in GlnK to another residue. Although we had shown previously that *glnD* mutations prevent relief of NifL inhibition (29) (Table 3, NCM1687), it was not clear whether their effects extend beyond control of the activity of NtrC. To assess whether GlnD is directly required to uridylylate GlnK, we transferred plasmid pJES1104 (*Ptet-glnK*⁺) into the *glnD::Tn10* strain. Immunological (Western blot) analysis indicated that GlnK was in its unmodified form in this background (Fig. 2A, lanes 5 and 7). As shown in Table 3, NifL inhibition was relieved and *nif* expression was increased to levels characteristic of a wild-type background under both N-limiting and N excess conditions (Table 3, NCM2093 and NCM1528). The fact that constitutive expression of *glnK*⁺ could suppress the effects of a *glnD* mutation on *nif* expression provided further evidence that uridylylation of GlnK is not required for relief of NifL inhibition.

A second, independent test of the need for uridylylation of GlnK to relieve NifL inhibition investigated the effects of a mutant form of GlnK in which the presumed site of uridylylation, Y51 (1, 11, 13, 23, 36, 50), was altered. Western blot

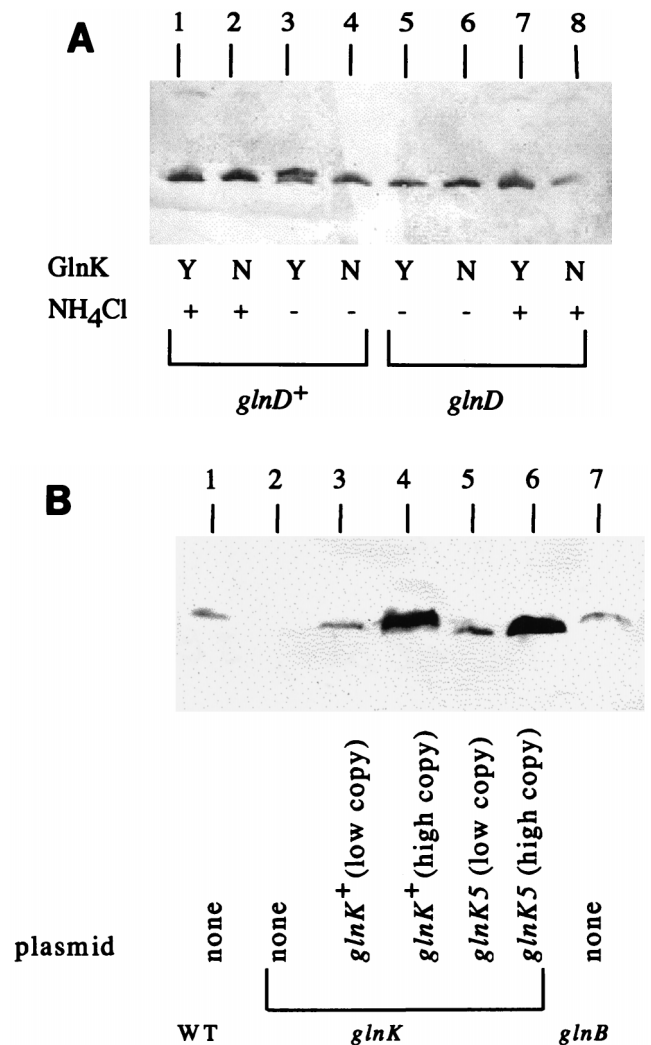


FIG. 2. Uridylation (A) and different levels of accumulation (B) of GlnK (Y) and GlnK^{Y51N} (N). (A) Cultures were grown in K medium under anaerobic conditions with (+) or without (–) NH₄Cl (see Materials and Methods). Proteins were separated by SDS-PAGE in the Tris-Tricine system (see Materials and Methods) and detected with anti-GlnB serum. Lanes: 1 to 4, *glnD*⁺ background (NCM1851); 5 to 8, *glnD* mutant background (NCM1687). The strains used for lanes 1, 3, 5, and 7 contained pJES1104 (*Ptet-glnK* in vector pACYC184); the strains used for lanes 2, 4, 6, and 8 contained plasmid pJES1106 (*Ptet-glnK5*; GlnK^{Y51N} in vector pACYC184). When cell extracts were treated with snake venom phosphodiesterase to remove uridylyl groups prior to electrophoresis, only the lower band was detected (data not shown). Treatment with alkaline phosphatase had no effect. (B) Cultures were grown in K medium under derepressing conditions (see Materials and Methods). Proteins were separated by SDS-PAGE in the 2× Tris-glycine system (see Materials and Methods) and detected with anti-GlnK serum. Lanes: 1, strain NCM1528 (wild type); 2, strain NCM1974 (*glnK* mutant); 3, strain NCM2069 (*glnK*/pJES1161 [*PglnK-glnK*⁺ in mini-F vector]); 4, strain NCM2087 (*glnK*/pJES1104 [*Ptet-glnK*⁺ in vector pACYC184]); 5, strain NCM2081 (*glnK*/pJES1162 [*PglnK-glnK5*; GlnK^{Y51N} in mini-F vector]); 6, strain NCM2088 (*glnK*/pJES1106 [*Ptet-glnK5*; GlnK^{Y51N} in vector pACYC184]). In this panel, bands were not resolved well enough for assessment of uridylylation.

analysis (Fig. 2A, lanes 2, 4, 6, and 8) and in vitro assays (3, 42) confirmed that GlnK^{Y51N} was not uridylylated. In agreement with results obtained under N excess conditions and in a *glnD* mutant background, GlnK^{Y51N} allowed relief of NifL inhibition not only in a *glnK* mutant background but also in *ntrC* and *glnD* mutant backgrounds (Table 3, NCM2089, NCM2090, NCM2091, and NCM2094). As was the case for

TABLE 4. Effects of low levels of GlnK and GlnB on NifL activity

Strain ^a	Relevant genotype	Expression of <i>nifH'</i> - <i>lacZ</i> ^b (U/ml/OD ₆₀₀)	
		-N	+N
NCM1528	Wild type	5,780	<10
NCM1974	<i>glnK</i>	<10	<10
NCM2070	<i>glnK</i> /pJES1163	<10	<10
NCM2069	<i>glnK</i> /P <i>glnK</i> - <i>glnK</i> ⁺ (mini-F vector)	5,200	<10
NCM2081	<i>glnK</i> /P <i>glnK</i> - <i>glnK5</i> (GlnK ^{Y51N}) (mini-F vector)	3,770	<10
NCM2080	<i>glnD</i> /P <i>glnK</i> - <i>glnK</i> ⁺ (mini-F vector)	60	ND ^c
NCM2082	<i>glnD</i> /P <i>glnK</i> - <i>glnK5</i> (GlnK ^{Y51N}) (mini-F vector)	60	ND
NCM2048	<i>glnK</i> /P <i>glnB</i> - <i>glnB</i> ⁺ (in pACYC184)	30	<10
NCM2068	<i>glnK</i> /P <i>glnB</i> - <i>glnB</i> ⁺ (mini-F vector)	<10	<10

^a All strains carry a single chromosomal copy of a *nifH'*-*lacZ* fusion at the *trp* locus and plasmid pNH3 (*P_{trac}-nifLA*).

^b See footnote *b* to Table 3.

^c ND, not determined.

GlnK itself (NCM2086, NCM2087, NCM2088, and NCM2093), the altered protein allowed relief of NifL inhibition under both N excess and N-limiting conditions.

To determine whether the efficacy of GlnK^{Y51N} in relieving NifL inhibition depend on its being overproduced from a multicopy vector (pACYC184; copy number, about 10 to 15 per cell [43]), we further studied the effects of this GlnK protein when it was expressed from its native promoter carried on a very low-copy-number vector (pZC320 [49], a mini-F plasmid; 0.2 to 1.4 copies per chromosome [27]). Plasmids pJES1161 and pJES1162, which encode GlnK and GlnK^{Y51N}, respectively, are compatible with pNH3 (pBR322 based; 15 to 20 copies per cell [43]), which encodes NifL and NifA, and direct analysis showed that the amounts of the mini-F derivatives (pJES1161 and pJES1162) were much lower than that of pNH3 when the two coexisted in one strain (NCM2069 or NCM2081) (data not shown). Moreover, Western blot analysis showed that the accumulation of GlnK or GlnK^{Y51N} expressed from the mini-F vector was similar to that obtained when GlnK was expressed from the chromosome in the wild-type background (NCM1528) and was less than that obtained when GlnK was expressed from the pACYC184 vector (Fig. 2B). As shown in Table 4, *glnK* (encoding wild-type GlnK) and *glnK5* (encoding GlnK^{Y51N}) carried on the mini-F vector could complement a *glnK* null allele for relief of NifL inhibition under N-limiting conditions (NCM2069 and NCM2081, respectively), indicating that large amounts of the GlnK protein are not required for this purpose. As expected, neither pJES1161, which encodes GlnK, nor pJES1162, which encodes GlnK^{Y51N}, allowed relief of NifL inhibition under N excess conditions or in a *glnD* or *ntrC* background (Table 4, strains NCM2080 and NCM2082, respectively; data not shown) because *glnK* was transcribed from its native promoter. Taken together, the results in this section indicate that uridylylation of GlnK is not required for relief of NifL inhibition.

The functions of GlnK have diverged from those of GlnB. Although the amino acid sequence of GlnK is 67% identical to that of GlnB (52), it is known that *glnB* null alleles have no effect on NifL inhibition of NifA activity in either *K. pneumoniae* (33, 34) or *E. coli* (29). In addition, a *glnB*⁺ allele did not suppress effects of a *glnK* null mutation on NifL inhibition, whether the *glnB* gene was carried by a multicopy vector or a very low-copy vector (Table 4, NCM2048 and NCM2068, respectively). Moreover, GlnB was not able to suppress effects of *ntrC* or *glnD* mutations on *nif* gene regulation (data not

shown). These findings indicate that the functions of GlnK have diverged from those of GlnB.

DISCUSSION

GlnK is required to relieve inhibition of NifA activity by the *K. pneumoniae* NifL protein. A major question concerning regulation of *nif* transcription is how signals of O₂ and fixed-N availability are transmitted to the transcriptional machinery. Although it has long been known that N status regulates transcriptional activation by NifA through the NifL protein in the γ -proteobacteria (32, 40), it was not clear whether NifL senses signals of N availability directly or additional components are required. Our recent finding that transcriptional activation by the general nitrogen regulator NtrC is essential for relief of NifL inhibition of NifA activity under derepressing conditions led us to propose the existence of other components in the signal transduction pathway (29). In this study, we have demonstrated that, indeed, a previously unrecognized protein, GlnK, acts as the missing link between the nitrogen sensor NtrC and the coupled redox/nitrogen sensor NifL (or a complex of NifL and NifA) (Tables 2, 3, and 4). Whereas NifL is a negative regulatory factor, GlnK acts positively to counteract inhibitory effects of NifL specifically under derepressing (N-limiting) conditions. Epistasis tests performed with constitutively expressed GlnK indicated that GlnK is the only link between NtrC and NifL.

Despite the fact that GlnK is normally uridylylated under N-limiting conditions, we have several lines of evidence that uridylylation is not required for relief of NifL inhibition, even when GlnK is expressed at normal levels (Tables 3 and 4; Fig. 2A and B). First, constitutively expressed (overexpressed) GlnK can relieve NifL inhibition, even in the presence of ammonium, a circumstance under which GlnK is not highly uridylylated normally. Second, the product of the *glnD* gene, which catalyzes uridylylation of GlnK, is not required under these conditions. Finally, when expressed at normal levels from the native *glnK* promoter, GlnK^{Y51N}, in which the uridylylated tyrosine has been changed to a residue that cannot be modified, allows relief of NifL inhibition.

The surprising finding that uridylylation of GlnK is not required for relief of NifL inhibition leads to two interesting questions. First, why is GlnK interposed between NtrC and NifL when transcription of both GlnK and NifL is under NtrC control? Second, how is NifL inhibition restored when ammonium (combined N) is added back to the medium? With respect to the first question, one can speculate that more extreme conditions of N limitation—which should result in accumulation of more phosphorylated (active) NtrC—are required for transcription of the *glnK* operon than for that of the *nifLA* operon. (It is known that larger amounts of phosphorylated NtrC are required for transcription of *nifLA* than for that of *glnA* [6, 54].) This postulate can be tested in vivo in ammonium-limited chemostat cultures. With respect to the second question, it is known that NifA-dependent transcription of the *nifHDK* operon of *K. pneumoniae* ceases less than 20 min after ammonium is added back to the medium and that cessation is mediated by NifL (10, 14). Because pre-existing GlnK would be diluted less than twofold during this time period, it is not apparent how inhibition would be restored. It is possible that GlnK is proteolyzed or covalently modified by a mechanism other than uridylylation upon replenishment of ammonium.

Role of P_{II}-like proteins in regulation of *nif* gene expression in other organisms. The GlnK and GlnB proteins of enteric bacteria are both “P_{II}-like” protein allosteric effectors that regulate nitrogen metabolism (1, 5, 52). Because their func-

tions have diverged (Table 4)—e.g., only GlnK can relieve NifL inhibition—they are most usefully referred to as paralogues rather than homologues. Interestingly, *A. vinelandii*, another member of the γ -proteobacteria, has only one P_{II}-like protein, which has been designated GlnK (38). In *A. vinelandii*, GlnK is essential—strains carrying disruptions of the *glnK* gene cannot segregate mutant chromosomes (38). Moreover, lesions in *nfrX*, which is homologous to the *glnD* gene of enteric bacteria, cause a Nif⁻ phenotype. If the (single) GlnK protein of *A. vinelandii* is required for relief of NifL inhibition, it is possible that it must be uridylylated to perform this function. Uridylylation of a single P_{II}-like protein may serve as an alternative to the presence of a second such protein.

Finally, there is evidence for pairs of P_{II}-like paralogues in diazotrophs among the α - and β -proteobacteria (7, 17). In *Azospirillum brasilense* (α) and *Herbaspirillum seropedicae* (β), at least one of the two P_{II}-like proteins is required for a Nif⁺ phenotype (7, 16, 17, 37). Whether uridylylation of the pertinent P_{II}-like protein is involved in *nif* regulation in these cases is not known. Finally, in α - and β -proteobacteria, there is no NifL protein (24). Rather, in *A. brasilense*, the P_{II}-like protein appears to interact directly with NifA (4). Like the differences between NifA proteins with regard to the presence of cysteine-rich inserts and, presumably, metal clusters involved in O₂ sensing, differences in the details of N sensing by the NifA protein or the NifA and NifL proteins of different organisms provide evidence for the regulatory volatility thought to be responsible for much phenotypic diversity (19, 20, 22, 28).

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ADDENDUM

When expressed from the *glnK* promoter, the GlnK^{Y51F} protein, which carries a conservative amino acid substitution of phenylalanine for the tyrosine that is normally uridylylated, relieves NifL inhibition under derepressing conditions. Like GlnK^{Y51N}, GlnK^{Y51F} is not uridylylated, and hence, the result confirms the conclusion that uridylylation of GlnK is not required for relief of NifL inhibition.

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