JOURNAL OF BACTERIOLOGY, Feb. 2002, p. 812–820 Vol. 184, No. 3
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.3.812–820.2002

Role of GlnK in NifL-Mediated Regulation of NifA Activity in *Azotobacter vinelandii*

Paul Rudnick, Christopher Kunz, Malkanthi K. Gunatilaka, Eric R. Hines, and Christina Kennedy*

Department of Plant Pathology, The University of Arizona, Tucson, Arizona 85721

Received 21 August 2001/Accepted 6 November 2001

In several diazotrophic species of *Proteobacteria*, P_{II} signal transduction proteins have been implicated in the regulation of nitrogen fixation in response to NH_4^+ by several mechanisms. In *Azotobacter vinelandii*, expression of *nifA*, encoding the *nif*-specific activator, is constitutive, and thus, regulation of NifA activity by the flavoprotein NifL appears to be the primary level of nitrogen control. In vitro and genetic evidence suggests that the nitrogen response involves the P_{II}-like GlnK protein and GlnD (uridylyltransferase/uridylyl-removing enzyme), which reversibly uridylylates GlnK in response to nitrogen limitation. Here, the roles of GlnK and GlnK-UMP in *A. vinelandii* were studied to determine whether the Nif$^-$ phenotype of *glnD* strains was due to an inability to modify GlnK, an effort previously hampered because *glnK* is an essential gene in this organism. A *glnKY51F* mutation, encoding an unuridylylatable form of the protein, was stable only in a strain in which glutamine synthetase activity is not inhibited by NH_4^+, suggesting that GlnK-UMP is required to signal adenylyltransferase/adenylyl-removing enzyme-mediated deadenylylation. *glnKY51F* strains were significantly impaired for diazotrophic growth and expression of a *nifH-lacZ* fusion. NifL interacted with GlnK and GlnKY51F in a yeast two-hybrid system. Together, these data are consistent with those obtained from in vitro experiments (Little et al., EMBO J., 19:6041–6050, 2000) and support a model for regulation of NifA activity in which unmodified GlnK stimulates NifL inhibition and uridylylation of GlnK in response to nitrogen limitation prevents this function. This model is distinct from one proposed for the related bacterium *Klebsiella pneumoniae*, in which unmodified GlnK relieves NifL inhibition instead of stimulating it.

Transcriptional control of nitrogenase synthesis is a common mechanism for the regulation of nitrogen fixation in many diazotrophic bacteria. In the *Proteobacteria*, regulation of nitrogen fixation genes (*nif* genes) occurs by controlling the expression and/or activity of the σ^{54}-dependent activator, NifA. Within the γ subgroup of the *Proteobacteria*, which includes the aerobic diazotroph *Azotobacter vinelandii* and the facultative diazotroph *Klebsiella pneumoniae*, a second protein, NifL, controls NifA activity. The *nifL* gene is cotranscribed with *nifA*, and together, the two encoded proteins comprise an unusual two-component system in which NifL inhibits NifA activity stoichiometrically by the formation of an inactive complex (17, 21); inactivation of *nifL* can lead to overexpression of *nif* genes (6, 32). Inhibition of NifA by NifL occurs in response to increased oxygen tension or fixed-nitrogen excess (13); ADP also increases the inhibitory properties of NifL in vitro, suggesting that NifL may respond to the energy state of the cell (16). Oxygen control of NifL activity involves the oxidation and reduction of a flavin moiety bound to the N-terminal PAS domain of NifL (22, 39). The nitrogen response is thought to involve components of a nitrogen-sensing system homologous to those best characterized in the nondiazotrophic organism *Escherichia coli* and is less well understood in *A. vinelandii*.

Nitrogen sensing in *E. coli* and other bacteria involves the concerted activities of the glutamine sensor, GlnD (uridylyltransferase/uridylyl-removing enzyme), and one or more trimeric signal transduction components generally referred to as P_{II} proteins (2, 36). In *E. coli*, nitrogen limitation, sensed as an internal glutamine deficiency (24), results in the uridylylation of GlnB (P_{II}) by GlnD and also in expression and uridylylation of a second P_{II}-like protein, GlnK (44, 45). Uridylylation of GlnB and GlnK prevents activation of the phosphatase activity of NtrB by unmodified GlnB, leading to the phosphorylation of NtrC and an Ntr response wherein phosphorylated NtrC (NtrC-P) activates a subset of genes required for growth under these conditions (33). Importantly, the expression of *glnK* is controlled by NtrC-P, which has implications for using *E. coli* as a heterologous system to study regulation of NifA activity. Another key target of the Ntr response is the ammonia-assimilatory enzyme glutamine synthetase (GS), encoded by *glnA*. When P_{II} and GlnK are uridylylated, both the expression, activated by NtrC-P, and activity of GS are high (33, 44). The activity of GS is controlled by GlnE, which catalyzes its reversible adenylylation (inactivation) in response to nitrogen excess (1).

In *K. pneumoniae*, the expression of *nifLA* is tightly controlled by NtrC-P, which limits expression to conditions of low fixed-N supply (14). *K. pneumoniae*, like *E. coli*, has both GlnB and GlnK, and expression of *glnK* requires NtrC-P (23). Recently, it was also discovered that GlnK, but not GlnB (P_{II}), modulates NifA activity (20, 23). Moreover, the uridylylation state of GlnK is apparently irrelevant with respect to NifA activity in experiments using a *glnKY51N* allele, which encodes a protein that cannot be uridylylated, or in a *glnD* background of *E. coli* (20) or *K. pneumoniae* (15). These results indicate that in *K. pneumoniae*, effective transcriptional control of *glnK*

* Corresponding author. Mailing address: Department of Plant Pathology, Forbes 204, The University of Arizona, Tucson, AZ 85721. Phone: (520) 621-9835 or (520) 621-3263. Fax: (520) 621-9290. E-mail: ckennedy@u.arizona.edu.

and *nifLA* and modulation of NifL inhibition by GlnK are important factors for the regulation of nitrogenase biosynthesis.

There are several differences between regulation of nitrogenase expression in *A. vinelandii* and in *K. pneumoniae*. For example, in *A. vinelandii*, *nifLA* expression is not activated by NtrC-P, nor does *glnK* expression appear to be N regulated (8, 31); hence, control of NifA activity appears to be the main mechanism regulating nitrogenase expression in this organism. Early mutagenesis experiments in *A. vinelandii* identified GlnD, previously named NfrX, as a key regulator of NifA activity because mutations in the 3′ end of *glnD* resulted in a Nif^- phenotype that could be suppressed to Nif^+ by deletion of *nifL* (12, 38). This result suggested that NifA activity depends on the uridylylation of a P_{II}-like protein. Complicating these results, recent experiments have shown that *glnD* null mutations are conditionally lethal in the wild-type *A. vinelandii* background because homogenous replacement of the wild-type *glnD* allele with a null allele does not occur in the absence of extragenic suppressors which elevate GS activity. These stabilizing suppressor mutations include (i) a site-directed mutation of GS (Y407F) preventing adenylylation and (ii) unlinked suppressor mutations (11). The inability to homogenously replace the wild-type *glnD* allele supports the hypothesis that in cells lacking *glnD*, GS is always inactivated by adenylylation. Since in *A. vinelandii* GS is thought to be the sole ammonia-assimilatory pathway and *glnA* (encoding GS) null mutants cannot be isolated (43), an inability to deadenylylate GS represents a lethal event. One spontaneous *glnD* null suppressor, *gln-71*, is probably in the *glnE* gene, encoding adenylyltransferase/adenylyl-removing enzyme (ATase/AR), because introduction of a wide-host-range plasmid carrying the *E. coli glnE* gene into *gln-71 glnD*$^+$ strains results in reestablishment of normal GS regulation (GS adenylylation) by NH_4^+ (11). Nevertheless, *glnD* null mutants are Nif^-, as were the original *nfrX* isolates, and fail to uridylylate a P_{II}-like protein, indicating that, unlike in *K. pneumoniae*, uridylylation of a P_{II}-like protein may be required for NifA activity as well as GS deadenylylation.

Only one P_{II}-like protein has been identified in *A. vinelandii*, and it is named GlnK because the gene encoding this protein is linked to the methylammonium transporter gene *amtB*, as occurs in many other bacteria (42). Unfortunately, efforts to demonstrate a role for this protein in *A. vinelandii* have been hampered because *glnK*, but not *amtB*, is an essential gene (31). Therefore GlnK has been studied in vitro and in a heterologous *E. coli* system (28, 37, 40). In *E. coli* cells carrying a *K. pneumoniae nifH-lacZ* reporter and expressing *A. vinelandii nifLA* in *trans*, the NifL-NifA system responds to both oxygen and fixed nitrogen (40). In this system, *E. coli* P_{II} (GlnB) and not GlnK is required for NifL-mediated inhibition in response to fixed nitrogen (37). This result is in contrast to what occurs in *K. pneumoniae*, where NifL inhibition is relieved and not stimulated by GlnK and not GlnB (20, 23). For the *A. vinelandii* heterologous system, NtrC also appears to have some role in limiting NifA activity in response to excess fixed N; how this occurs was not examined (37). In vitro, formation of an inactive *A. vinelandii* NifL-NifA complex, as measured by a decrease in open complex formation at the *nifH* promoter, is stimulated by *E. coli* P_{II} and *A. vinelandii* GlnK in their unuridylylated forms. The corresponding decrease in NifA activity depends on the presence of NifL, demonstrating that NifL, and not NifA, responds to these P_{II}-like proteins. Interestingly, 2-oxoglutarate, at physiological concentrations, was shown to favor dissociation of the NifL-NifA complex, possibly indicating an integrated role for carbon sensing (28).

In this report, the following two questions are addressed: is GlnD-mediated uridylylation of GlnK required for *A. vinelandii* NifA activity in vivo, and does GlnK potentiate NifL inhibition directly? These questions were addressed in order to compare nitrogen regulation of NifA activity in *A. vinelandii* to that in *K. pneumoniae* and also to study the consequence of GlnK uridylylation under natural gene dosage. To this end, the *glnD* suppressor strain MV72 (*gln-71*) was used to construct a *glnKY51F* mutation, encoding a protein that cannot be uridylylated. This mutation was not stable in a wild-type strain, indicating a requirement for GlnK-UMP for deadenylylation of GS. Strains carrying *glnKY51F* failed to derepress nitrogenase expression in response to nitrogen limitation, and GlnK protein-protein interactions with NifL were examined in a yeast two-hybrid system. Together, results from these experiments support a model for regulation of NifA activity involving GlnK where uridylylation prevents unmodified GlnK from stimulating the inhibitory properties of NifL, a model that is clearly distinct from that proposed for the related *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. vinelandii* strains were grown in Burk's medium supplemented with 1% sucrose (BS) at 30°C (35). Liquid cultures were grown with shaking in air (200 rpm). Strains carrying *gln-71* were grown with 5 or 10 mM ammonium acetate (BSN_5 and BSN_{10}); higher concentrations decrease the growth rate, probably due to depletion of glutamate pools, as observed for a *glnE* strain of *Salmonella enterica* serovar Typhimurium (26). Growth of *A. vinelandii* strains for determination of β-galactosidase activity is described below. *A. vinelandii* was made competent by plating on BS lacking Fe and Mo (competence medium [CM]) (6). Cells were resuspended in liquid CM containing 16 mM $MgSO_4$ prior to transformation. *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C with shaking (250 rpm). P1*vir* transduction was performed as described in reference 30. *E. coli* was transformed by standard techniques (29). *Saccharomyces cerevisiae* strains were maintained or grown in or on rich yeast extract-peptone-dextrose medium or minimal SD medium (yeast nitrogen base, dextrose, ammonium sulfate) with shaking for selection of strains carrying plasmids encoding prototrophy markers according to the manufacturer's protocol book for the Matchmaker two-hybrid system (Clontech, Palo Alto, Calif.). Liquid cultures of yeast were grown at 30°C with shaking (200 rpm). Yeast transformations were conducted by the lithium acetate method (Clontech). Media were supplemented with antibiotics where appropriate: for *A. vinelandii*, ampicillin (25 μg/ml), chloramphenicol (50 μg/ml), kanamycin (1 μg/ml), streptomycin (1 μg/ml), and tetracycline (5 μg/ml); for *E. coli*, ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), kanamycin (50 μg/ml), spectinomycin (30 μg/ml), streptomycin (50 μg/ml), and tetracycline (10 μg/ml).

Construction of a plasmid carrying *glnKY51F*. Forward primer glnB F H3 (5′-TCCATAACCAC<u>AAGCTT</u>GATAGGGG-3′), which introduces a *Hin*dIII site (underlined) for cloning, and reverse mutagenic primer glnB Y-F (5′-GCA GAAA<u>GTCGAC</u>TAC<u>GAA**TTC**</u>CG-3′), which introduces an *Eco*RI site at tyrosine 51 (TAC → TTC) and contains a recognition site for *Hin*cII (both underlined), were used to amplify the 5′ half of *glnK* from pPR101 with cloned *Pfu* polymerase (Stratagene, La Jolla, Calif.). pPR101 was then digested with *Hin*dIII, which cuts in the vector, and *Hin*cII and ligated to a similarly digested *glnKY51F* PCR product to give pPR102. The presence of the *glnKY51F* mutation and the absence of other mutations was verified by sequencing of the pPR102 (LMSE sequencing facility, University of Arizona). Standard recombinant techniques were performed essentially as described by Maniatis et al. (29). To construct pPR118 (Fig. 1), a 1.4-kb *Eco*RI/*Ava*I fragment containing the Tet^r gene from pBR322 was blunt-ended with the addition of deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I and cloned into

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or characteristics	Reference or source
Strains		
A. vinelandii		
UW136	Rif^r derived from strain UW (ATCC 13705)	7
UW136H[a]	*nifH1-lacZ*/KSS	This work; 46
MV71	*gln-71 glnD*::Ω	11
MV72	*gln-71* (spontaneous *glnD1*::Ω suppressor)	11
MV378	*nifL1*::KIXX *nifH1-lacZ*/KSS	6
MV577	*gln-71 glnKY51F amtB3*::Tet^r	This work
MV577H	*gln-71 glnKY51F amtB3*::Tet^r *nifH1-lacZ*/KSS	This work
MV578	*gln-71 amtB3*::Tet^r	This work
MV578H	*gln-71 amtB3*::Tet^r *nifH1-lacZ*/KSS	This work
MV579	*gln-71 glnKY51F amtB3*::Tet^r *nifL1*::KIXX	This work
MV579H	*gln-71 glnKY51F amtB3*::Tet^r *nifL1*::KIXX *nifH1-lacZ*/KSS	This work
MV580	*gln-71 amtB3*::Tet^r *nifL1*::KIXX	This work
MV580H	*gln-71 amtB3*::Tet^r *nifL1*::KIXX *nifH1-lacZ*/KSS	
MV581	*gln-71 nifL1*::KIXX	This work
MV581H	*gln-71 nifL1*::KIXX *nifH1-lacZ*/KSS	
E. coli		
DH5α	*supE44 hsdR17 recA1 thi-1* Δ*lacU169* (ϕ*80lacZ*Δ*M15*) *endA1 gyrA96 relA1*	18
MC4100	[Δ(*lac*)U169 *araD139 rpsL thi*]	10
CK1005	MC4100 with (Δ*glnB*)::Cam^r	
CK1007	MC4100 with (Δ*glnB*)::Cam^r *glnK*::Spc^r	This work
CK1008	MC4100 with (Δ*glnB*)::Cam^r *glnK*::Spc^r *glnD99*::Tn*10*	This work
NCM1971	*glnK*::Spc^r	20
NCM1686	*glnD99*::Tn*10*	19
NCM1736	(Δ*glnB*)::Cam^r	19
S. cerevisiae		
SFY526	UAS-*GAL1-lacZ* reporter (His$^-$ Trp$^-$ Leu$^-$)	Clontech
HF7c	UAS$_{G\ 17\text{-}mer(X3)}$ *CYC1-lacZ* UAS-*GAL1-HIS3*	Clontech
Plasmids		
pAB29	pTZ19 derivative carrying carrying *nifL1*::KIXX	6
pBR322	Tet^r vectors source of Tet^r gene	9
pGAD424[b]	GAL4 activation domain fusion two-hybrid vector	Clontech
pGBT9[b]	GAL4 binding domain fusion two-hybrid vector	Clontech
pJAW2	Suicide plasmid carrying *nifH1-lacZ*/KSS	46
pPR101	pTZ19 carrying *A. vinelandii glnK amtB*	31
pPR102	pTZ19 carrying *A. vinelandii glnKY51F amtB*	This work
pPR113	Plasmid encoding GlnK^{His6} in pTrcHisB	This work
pPR115	Plasmid encoding GlnKY51F^{His6} in pTrcHisB	This work
pPR118	pPR102 with a 1.4-kb *Eco*RI/*Ava*I Tet^r gene cloned into *amtB*	This work
pTrcHis B	His tag cloning vector	Invitrogen
pTD1	Activation domain control fusion for two-hybrid system	Clontech
pVA3	Binding domain control fusion for two-hybrid system	Clontech

[a] Strain UW136H is a remake of strain MV101 (46).
[b] For descriptions of two-hybrid fusion constructs, see Materials and Methods.

the single *Nsp*V site of the *amtB* gene in pPR102. *glnKY51F* was verified in this clone by sequencing prior to transformation of *A. vinelandii*. A clone carrying the tetracycline resistance gene in the forward orientation, pPR118, was isolated to be used for mutagenesis of the *A. vinelandii* chromosome (Fig. 1).

Construction of plasmids expressing GlnK^{His6} and GlnKY51F^{His6}. pPR113 was constructed by PCR amplification of *glnK* from pPR101 with the primers GLNK FB (5′-GAGATGGATCCGATGAAGCTAGT-3′) and GLNK RK (5′-AATCCACGGTACCCTCCTG-3′), which introduce a *Bam*HI and a *Kpn*I site (underlined), respectively, for directional cloning into the His tag vector pTrcHisB (Invitrogen, Carlsbad, Calif.). The PCR product was first cloned into the T-A cloning vector pGEM-T EASY as an intermediate (Promega, Madison, Wis.). The *Bam*HI/*Kpn*I fragment was then cloned into similarly digested pTrcHisB to give pPR113. pPR115 was constructed in essentially the same manner, except that *glnKY51F* was amplified from plasmid pPR102 and cloned directly into pTrcHisB following digestion. pPR113 and pPR115 have N-terminal translational fusions that contains six consecutive histidine residues, encoded by pTrcHisB.

Construction of yeast two-hybrid plasmids. Plasmid vectors used in the yeast two-hybrid assays were pGAD424 (activation domain fusion) and pGBT9 (binding domain fusion) from the Matchmaker two-hybrid system. For cloning of *nifL*, *glnK*, or *glnKY51F*, PCR products were amplified from plasmid or chromosomal template DNA and ligated to pGEM-T EASY, again, as an intermediate step. For each of these genes, the entire reading frame was cloned by the introduction of restriction site compatible with sites in the two-hybrid vectors to generate in-frame fusions. *nifL* was amplified with the primers NifL.For.ApoI (5′-TCGCCGAATTTCTTGGATCGACGAGG-3′) and NifL.Rev.BamHI (5′-GGTTGGATCCATGGGCATTCAT-3′) from chromosomal DNA as the template; *glnK* and *glnKY51F* were amplified from pPR101 and pPR102, respectively. Amplification of *glnK* or *glnKY51F* was performed with the primers GlnK.For.EcoRI (5′-TTACACGGAATTCTGTTTCATGAA-3′) and GlnK.R.BamHI (5′-CGGGGGGATCCTGGGCT-3′). The cloned products were fully or partially digested with *Eco*RI and cloned into either pGBT9 or pGAD424. All plasmids were verified by sequencing (LMSE sequencing facility, The University of Arizona).

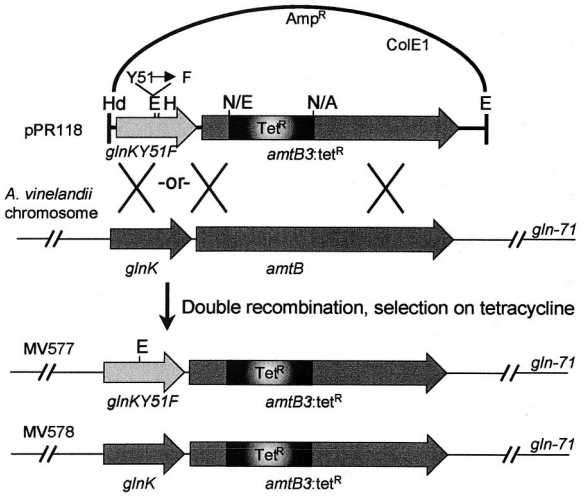

FIG. 1. Map of the *glnK amtB* region and construction of *glnKY51F*. To construct the *glnKY51F* mutation, the 5′ half of *glnK* was amplified with a mutagenic primer that contained the TAC → TTC base change encoding residue 51. The mutation of Y51 prevents uridylylation of the encoded protein and introduces a new *Eco*RI site. For selection of *A. vinelandii* transformants carrying the point mutation, a tetracycline resistance gene was introduced into the 5′ end of the downstream *amtB* gene carried on the plasmid. *A. vinelandii* mutants were selected on the basis of Tet^r and screened for sensitivity to vector-borne Amp^r. Of these, *glnK* was amplified and digested with *Eco*RI to identify mutants carrying the *glnKY51F* allele. The genetic structure of the stable *gln-71* transformants MV577 and MV578 is shown. A, *Ava*I; E, *Eco*RI; H, *Hin*cII; Hd, *Hin*dIII; N, *Nsp*V; P *Pst*I.

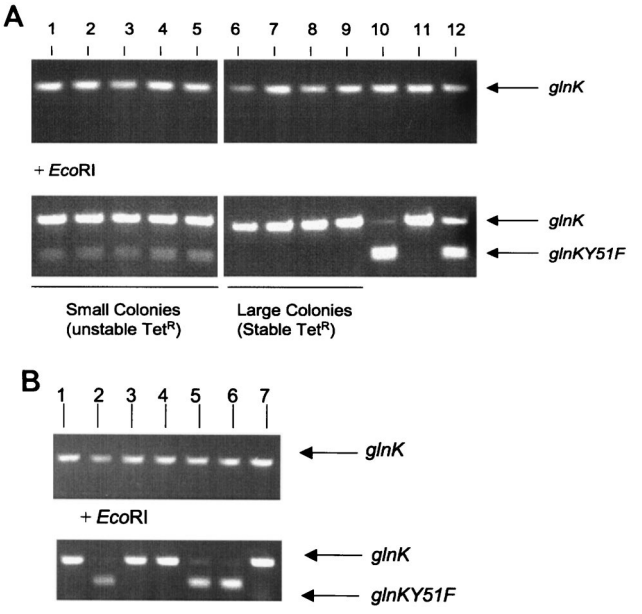

FIG. 2. PCR analysis of *glnKY51F amtB3*::Tet transformants. (A) Large- and small-colony Tet^r Amp^s transformants of wild-type strain UW136. (Top) amplified PCR products; (bottom) products after *Eco*RI digestion. *Eco*RI cleaves *glnKY51F* in half, while *glnK* is *Eco*RI resistant. Lanes: 1 to 5, small-colony transformants; 6 to 9, large-colony isolates; 10, *glnKY51F* PCR product amplified from plasmid pPR118; 11, *glnK* amplified from plasmid pPR101. 12, Amp^r Tet^r isolate resulting from integration of plasmid pPR118 carrying both alleles in tandem. (B) Transformation of *glnD1*::Ω suppressor strain MV72. Lanes: 1 to 5, Tet^r Amp^s isolates; 6, *glnKY51F* amplified from plasmid pPR118; 7, wild-type *glnK* from pPR101.

Recombination of *glnKY51F* onto the *A. vinelandii* chromosome. The suicide plasmid pPR118 was used to transform *A. vinelandii* strains UW136 and MV72 followed by selection on tetracycline. To screen for allelic replacement mutants, Tet^r transformants were patched onto BSN plates supplemented with ampicillin. Tet^r Amp^s clones were purified and analyzed for the presence of the *glnKY51F* mutation by PCR amplification of the ~350-bp *glnK* gene using primers K101U (5′-ACTTGAATCGGGATCGTTT-3′) and K101D (5′-GCCTTTGCGCAGCG TCAT-3′) from single colonies as templates followed by *Eco*RI digestion. *nifL1*::KIXX strains were constructed by transformation with pAB29 (6) followed by selection on kanamycin and screening for sensitivity to ampicillin.

Construction of *nifH1-lacZ*/KSS reporter strains. *A. vinelandii* strains UW136, MV72, MV577, MV578, and MV579 were transformed with pJAW2 (47) to generate *nifH1-lacZ*/KSS transcriptional fusions on the chromosome. pJAW2 is a suicide vector carrying the *nifH1-lacZ*/KSS fusion, which carries a *nifH-lacZ* fusion followed by the kanamycin and streptomycin marker genes from Tn*5*. Transformants were selected on streptomycin and screened for sensitivity to chloramphenicol to identify allelic exchange mutations. The fusions in Sm^r Cam^s isolates were verified by PCR amplification of a *nifH-lacZ* product from the chromosome (not shown).

Construction of *E. coli* strains. To construct an *E. coli* strain devoid of both *glnB* and *glnK* in strain MC4100, Δ*glnB*::Cam^r was moved from strain NCM1736 (19) by P1 transduction to generate CK1005. CK1005 was then transduced with *glnK*::Spc^r with a lysate grown on strain NCM1971 (20) to generate strain CK1007. A triple *glnB glnK glnD* mutant strain was constructed by transducing *glnD99*::Tn*10* from NCM1686 (19) followed by selection on tetracycline and screening for glutamine bradytrophy.

Growth curve and β-galactosidase assays. Cells used for growth curves were first grown overnight in 10-ml starter cultures in BSN_5. The next morning, the cultures were diluted to 10 Klett units (optical density at 600 nm [OD_{600}] ≈ 0.1) in either BS or BSN_{10} and grown for 27 h. Samples were read periodically on a Klett-Summerson colorimeter through a no. 54 green filter measuring the OD_{526} in sidearm flasks, and values were plotted on a semilog graph. For analysis of *nifH1-lacZ* fusions in *A. vinelandii*, 10-ml overnight cultures were grown in BS supplemented with 5 mM urea (BSU_5) and the appropriate antibiotics. The following day, the cultures were diluted to approximately 20 Klett units in fresh BSN_{10} for repression or BSU_2 for derepression. Five samples were taken throughout a 12-h period, at which time the OD_{600} was recorded for each sample. Samples were immediately frozen and stored at −20°C. β-Galactosidase activities were determined for each sample in units per milliliter according to the following calculation, which has been modified for an enzyme-linked immunosorbent assay microtiter plate reader: $1{,}000[OD_{414} - (1.75 \times OD_{540})]/(\Delta t \times v)$, where ΔT is the time of the reaction and v is the volume of extract. Each time point was assayed in duplicate, and the experiment was repeated for each strain at least three times (34). The activities for each time point were then plotted against OD_{600}. This analysis indicated that the differential rate of β-galactosidase synthesis was constant throughout the growth curve and therefore the slope of each line was approximated to give rates, which are reported in Table 2. β-Galactosidase assays of yeast strains were performed by the protocols supplied by the manufacturer (Clontech). Briefly, single colonies of strains carrying the appropriate plasmids were inoculated into SD selective media and grown to saturation at 30°C. Cultures were then diluted into 8 ml of yeast extract-peptone-dextrose and grown to an OD_{600} of 0.5 to 0.8, and 1.5 ml of culture was pelleted, washed, concentrated, and assayed for determination of β-galactosidase activity.

Preparation of cell extracts, in vitro uridylylation assays, and Western blotting. For preparation of cell extracts, CK1007 or CK1008 carrying plasmid pPR113 or pPR115 was grown in 25 ml of LB in a 125-ml flask to an OD_{600} of ~0.5, at which time the cultures were induced with 500 μM IPTG (isopropyl-β-D-galactopyranoside) for 2 to 3 h. Strain CK1008 grew markedly slower than CK1007; plasmid pPR115 inhibited growth of CK1007, CK1008, and MC4100, with greater inhibition observed after induction. The cells were harvested, washed once in an equal volume of uridylylation buffer (50 mM Tris-Cl, 100 mM KCl, 10 mM $MgCl_2$ [pH 7.5]), and resuspended in 0.5 or 1.0 ml of the same buffer (4). The cell suspensions were lysed with three 5-s bursts on a Branson sonicator on medium-high power followed by three cycles of rapid freezing in liquid N_2 and rapid thawing at 37°C. Cell debris was then pelleted by centrifugation at 12,000 × *g* for 15 min at 4°C, and the soluble proteins were recovered in the supernatant. All extracts were quantitated for protein content by a modified Bradford method (Bio-Rad, Hercules, Calif.). Uridylylation of GlnK^{His6} was carried out in vitro basically as described in reference 25. Briefly, 30 μg of soluble protein containing

$GlnK^{His6}$ or $GlnKY51F^{His6}$ was incubated in uridylylation buffer plus 0.2 mM ATP. [α^{32}-P]UTP (1 μl; 800Ci/mmol) and 1 mM 2-oxoglutarate (pH 7.5) were added to initiate labeling of GlnK. The reaction mixture was incubated for 30 min. at 30°C. Duplicate samples were incubated with unlabeled UTP for Western analysis and were processed in parallel. All samples were mixed in 1× sodium dodecyl sulfate sample buffer and boiled for 5 min, and half of the reaction mixture was separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (5). The gel containing labeled proteins was dried overnight between cellulose sheets and exposed to Kodak Biomax X-ray film for approximately 30 min. The nonradioactive gel was blotted to a nitrocellulose membrane with a semidry transblotter (Bio-Rad) following equilibration in Bjerrum and Schafer-Nielson transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol [pH 9.2]) (Bio-Rad electroblotter manual). Polyclonal *E. coli* P_{II} rabbit antiserum was applied as the primary antibody. Alkaline phosphatase conjugate was then used to detect the proteins, by using an AP substrate pack for colorimetric development (Bio-Rad).

RESULTS

Uridylylation of GlnK is essential for growth of wild-type strain UW136. One hypothesis for the lethality of *glnD* null mutations in *A. vinelandii* is that uridylylation of GlnK is required for deadenylylation of GS. Inactivation of GS in *A. vinelandii* by adenylylation would be lethal, as are *glnA* null mutations in this organism, attributed to the lack of an alternate ammonia-assimilatory mechanism and an inability to transport glutamine (43). This is apparently the case, since suppressors of *glnD* mutations (i.e., *gln-71*) resemble *glnE* strains of other organisms, and *E. coli glnE* complements this suppressor strain for restoration of normal regulation of GS activity by NH_4^+ (11). Therefore, attempts were made to construct *glnK* null mutations in a *gln-71* strain (data not shown). These attempts failed, which indicates that GlnK is essential for at least two functions in *A. vinelandii*: deadenylylation of GS and another, unknown function. Nevertheless, if the second essential function of GlnK does not require the uridylylated form but only the unmodified form, then it might be possible to construct *glnK* mutations altered only at the site of uridylylation. This protein would perform the functions of the unmodified form but lack the activities of GlnK-UMP, including those that may include regulation of NifA activity. To test this hypothesis, a *glnKY51F* mutation was constructed by elongation of a mutagenic primer (see Materials and Methods). The point mutation at Y51 of *glnK* created a new *Eco*RI site, which was used to screen for the presence of the mutation. To select for *A. vinelandii* strains carrying this mutation, a Tet^r cassette was cloned into the 5′ end of the adjacent *amtB* gene to give pPR118 (Fig. 1). Previous studies in this organism, *K. pneumoniae*, and heterologous *E. coli* systems have determined that *amtB* mutations have little or no effect on the regulation of nitrogenase expression (23, 31, 37). Therefore, we expected these two mutations together to reflect the effects of the *glnKY51F* allele. However, since the complete function of AmtB has not been defined, it cannot be ruled out that loss of *amtB* may cause differences in *nif* expression in response to ammonium in the growth medium (41). Therefore, $glnK^+$ strains carrying *amtB3*::Tet^r were examined in parallel.

To place the *glnKY51F* mutation on the chromosome, suicide plasmid pPR118 was used to transform the wild-type *A. vinelandii* strain UW136. Transformation gave rise to small and large Tet^r Amp^s (allelic exchange mutants) colony types, suggesting that the *glnKY51F* mutation, perhaps present in the smaller of the two colony types, might be unstable or have a dominant negative effect on colony growth, since *amtB* mutations do not impair growth on the selective medium (31). To determine if the *glnKY51F* mutation had recombined into the chromosome along with *amtB3*::Tet^r, *glnK* was PCR amplified from several transformants. Following amplification, the PCR products were digested with *Eco*RI, which cuts the *glnKY51F* allele in half, while $glnK^+$ is *Eco*RI resistant. This analysis revealed that cells in the larger of the two colony types, as hypothesized, were $glnK^+$ (Fig. 2A, lanes 6 to 9). The smaller colonies contained both alleles, demonstrating an inability of *glnKY51F* to homogeneously replace *glnK* in these cells (lanes 1 to 5) and consistent with other presumed lethal mutations in *A. vinelandii* reported by this and other laboratories (11, 31, 43, 49). The controls indicate that the enzyme digestion was specific for *glnKY51F* and was nearly complete (lanes 10 and 11). Lane 12 contains *glnK* amplification products from a Tet^r Amp^r transformant carrying and integrated copy of pPR118. When these products are digested, the concentration of the smaller *glnKY51F* band appears to be higher than in the unstable small-colony transformants. This result indicates that the number of copies of the *glnKY51F*-containing chromosome in the unstable colonies may be lower than that obtained by integrating a second copy of *glnK* on the chromosome by single recombination.

TABLE 2. Expression of *nifH1-lacZ* in *glnKY51F* strains of *A. vinelandii*

Strain[b]	Relevant genotype	β-Galactosidase activity (U/ml/OD_{600} unit)[a]	
		$+N^c$	$-N^d$
UW136H	Wild type	200	16,100
MV72H	*gln-71*	240	15,130
MV71H	*gln-71 glnD1*::Ω	160	160
MV577H	*gln-71 amtB3*::Tet^r *glnKY51F*	260	860
MV578H	*gln-71 amtB3*::Tet^r	191	12,010
MV579H	*gln-71 amtB3*::Tet^r *glnKY51F nifL1*::KIXX	4,880	14,610
MV580H	*gln-71 amtB3*::Tet^r *nifL1*::KIXX	NT[e]	5,870
MV581H	*gln-71 nifL1*::KIXX	5,260	9,570

[a] See Materials and Methods for determination of β-galactosidase activities.
[b] All strains carry a *nifH1-lacZ*/KSS transcriptional fusion (46).
[c] BS medium supplemented with 10 mM ammonium acetate.
[d] BS containing 2 mM urea as a nonrepressive nitrogen source.
[e] NT, not tested.

In cases of unstable alleles, the drug resistance carried on the drug cassette is lost when the selective pressure for the mutated copy is removed. Therefore, UW136 *glnKY51F* transformants were tested for stability of the tetracycline resistance marker by plating on BSN_{10} in the absence of tetracycline followed by reculturing on tetracycline-containing medium. This experiment revealed that the antibiotic resistance in strains carrying *glnKY51F amtB3*::Tet^r was unstable in the wild-type UW136 background; cells from the small colonies did not grow when recultured on tetracycline, in contrast to the large $glnK^+$ transformants. These analyses indicate that the *glnKY51F* mutation is lethal in the wild-type background.

Strains carrying the *gln-71* suppressor mutation stabilize *glnKY51F*. Recent work on the *glnD* gene of *A. vinelandii* revealed that null mutations, where the 5′ or central region is removed or replaced by gene cassettes (Ω or KIXX), are lethal

and cannot be stabilized in the wild-type background. One of these alleles (*glnD1*::Ω) was stabilized by a suppressor mutation, *gln-71*, identified after spontaneous appearance of a large, stably antibiotic-resistant (Spc^r) colony, in which GS activity is not regulated by ammonium. The *gln-71* mutation is apparently in the *glnE* gene, encoding ATase/AR, because GS activity becomes regulated in MV72 (a *glnD*$^+$ derivative of MV71 carrying only *gln-71*) (i.e., is inactivated by ammonium) after introduction of a plasmid carrying the *E. coli glnE* gene (11). To determine whether the *glnKY51F* allele could be stabilized by the *gln-71* suppressor, MV72 was transformed with pPR118. Transformants were selected on BSN_5 with tetracycline, and all were of a uniform large colony size. About 60% of the Amp^s Tet^r transformants carried only the *glnKY51F* allele; the others were *glnK*$^+$, as determined by PCR amplification followed by *Eco*RI digestion of the PCR products (Fig. 2B, lanes 1 to 5). In contrast to the wild-type *A. vinelandii* pPR118 transformants, none of the MV72 transformants carried both the mutated and wild-type *glnK* alleles. In addition, Tet^r in the *glnKY51F amtB3*::Tet^r transformants was stable and not lost after subculturing on antibiotic-free medium (data not shown). One *gln-71 glnKY51F amtB3*::Tet^r transformant was named MV577, and one corresponding *glnK*$^+$ transformant was named MV578 (Fig. 1). These results support the hypothesis that GlnK-UMP is required for control of GS activity, almost certainly by signaling deadenylylation of GS by ATase/AR. Importantly, they also indicate that MV72 could probably be used as a host strain to study the role of GlnK in the regulation of NifA activity in *A. vinelandii*.

***glnKY51F* encodes a protein which cannot be uridylylated.** To test whether the GlnKY51F protein could be modified by uridylylation, *glnKY51F* and wild-type *glnK* were expressed in *E. coli* from plasmids pPR113 and pPR115 (see Materials and Methods). These two plasmids express the complete GlnK or GlnKY51F proteins fused to a six-residue N-terminal His tag. Since *E. coli* expresses GlnB and GlnK which can be uridylylated, a strain was constructed that lacked both *glnB* and *glnK* (CK1007) or *glnB glnK* and *glnD* (CK1008) (see Materials and Methods) (Table 1). For the uridylylation experiments, strains CK1007 and CK1008 carrying either pPR113 or pPR115 were grown to mid-log phase in LB or LB plus glutamine (100 μg/ml), and expression of GlnK or GlnK^{His6} was induced from the plasmid with 0.5 mM IPTG. In extracts, GlnK^{His6} could be uridylylated in a GlnD-dependent fashion while GlnKY51F^{His6} could not (Fig. 3, bottom). Western blotting of the extracts used in the uridylylation experiments indicated that both plasmids expressed stable proteins which cross-reacted with *E. coli* P_{II} antisera (Fig. 3, top) (gift from W. vanHeeswijk). In the upper panel of Fig. 3 it is also evident that GlnKY51F is not detected at the same level as GlnK. This may be because the level of expression from pPR115 is lower or that the Y51F mutation disrupts a region of important antigenicity.

Uridylylated GlnK is required for growth in N-free media and for NifA activity. MV577 (*glnKY51F amtB3*::Tet^r *gln-71*) formed only very small colonies on N-free medium, indicating that the *glnKY51F* mutation affected nitrogen fixation and/or assimilation (data not shown). Growth rates were determined for strains MV577, MV578, and MV579 and control strains in order to quantitate the effects of the *glnKY51F* mutation on growth in the presence and absence of fixed nitrogen (Fig. 4).

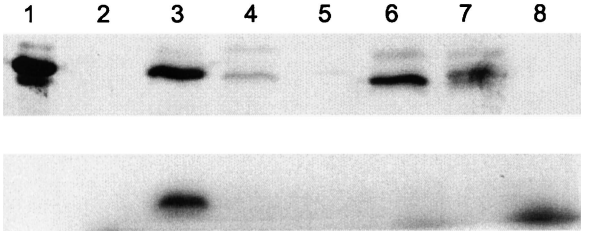

FIG. 3. Western analysis and uridylylation of *A. vinelandii* GlnK and GlnKY51F in *E. coli*. (Top) Western blot of *E. coli* extracts incubated with 2-oxoglutarate and UTP for 30 min. (Bottom) Autoradiograph of the same extracts labeled with [α^{32}-P]UTP. Lanes: 1, purified GlnK^{His6} as a size standard; 2, CK1007 (*glnB glnK*); 3, CK1007(pPR113); 4, CK1007(pPR115); 5, CK1008 (*glnB glnK glnD*); 6, CK1008(pPR113); 7, CK1008(pPR115); 8, no protein.

In these experiments, the doubling time of MV577 was greater than 11 h in N-free media. MV578, the corresponding *glnK*$^+$ strain, doubled in about 2.5 h (Fig. 4A). An increase in the growth rate of MV577 was observed with the addition of 10 mM ammonium acetate to the cultures (Fig. 4B), although there was no increase in the rate of MV578, which may indicate effects of GlnKY51F on other cellular functions in N-rich me-

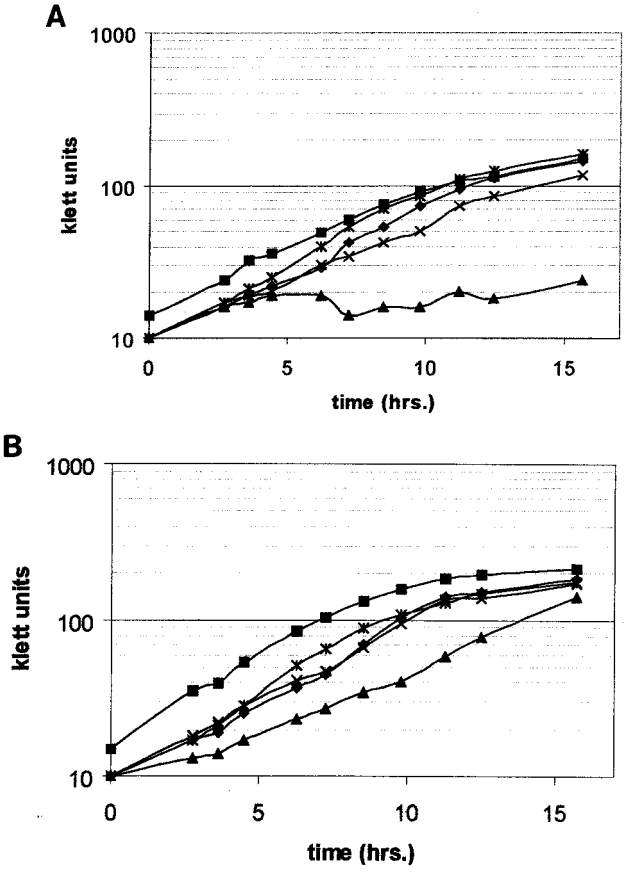

FIG. 4. Growth of *glnKY51F* strains in BS. (A) N-free growth; (B) growth with the addition of 10 mM ammonium acetate. ■, UW136 (wild-type strain); ◆, MV72 (*gln-71*); ▲, MV577 (*gln-71 glnKY51F amtB3*::Tet^r); ×, MV578 (*gln-71 amtB3*::Tet^r); (✳), MV579 (*gln-71 glnKY51F amtB3*::Tet^r *nifL1*:: KIXX).

dium. Nonetheless, the *glnKY51F* mutation severely impaired growth under diazotrophic conditions. Also in these experiments, a *nifL* mutation was epistatic to the *glnKY51F* mutation (MV579). This result is consistent with the hypothesis that the unmodified GlnK positively influences NifL inhibition and GlnK does not effect nitrogenase activity per se, as measured by diazotrophic growth.

To examine the effect of the *glnKY51F* mutation on NifA activity, *nifH1-lacZ* reporter strains were constructed (Table 1) and the differential rates of β-galactosidase synthesis were determined (see Materials and Methods). These data indicate that the *gln-71* suppressor mutation, used to stabilize the *glnKY51F* mutation, had little effect on *nifH* expression under derepressed conditions (MV72H), as did the combined *gln-71* and *amtB3*::Tet^r mutations in strain MV578H. Therefore, any effects in the isogenic *glnKY51F* strains are a consequence of the *glnK* mutation and not other mutations in these strains. For comparison, a *nifH1-lacZ* fusion in MV71 (*glnD1*::Ω *gln-71*) was constructed and assayed. Compared to MV578H (*glnK*$^+$), strain MV577H (*glnKY51F*) expressed about 5% of the amount of β-galactosidase under derepressing conditions but still more than the *glnD* strain (compare MV577H and MV71H), indicating that uridylylated GlnK is required for full activation of the *nifH* promoter in vivo (Table 2).

To determine if the effect of GlnK on NifA activity was mediated through NifL, *nifL* in MV577 was disrupted with a KIXX cassette encoding kanamycin resistance to give strain MV579 and the corresponding *nifH1-lacZ* reporter strain, MV579H. Both growth on N-free medium (Fig. 4A) and *nifH1-lacZ* expression (Table 2) were restored to wild-type levels under derepressing conditions by mutation of *nifL*, and as observed with other *nifL* strains, expression of *nifH1-lacZ* was not fully repressed in the presence of NH_4^+ (Table 2) (6). As controls, *nifL1*::KIXX was introduced into strains MV72H and MV578H for comparison (MV580H and MV581H). Taken together, these data indicate that unmodified GlnK negatively regulates NifA activity by a mechanism involving NifL.

GlnK and GlnKY51F interact with NifL in a yeast two-hybrid assay. To assess if GlnK interacts directly with NifL, translational fusions to the GAL4 DNA binding and activation domains were made using *nifL*, *glnK*, and *glnKY51F*. The resulting plasmids and pairs of plasmids were used to transform yeast strain SFY526, carrying a *lacZ* reporter downstream of the GAL1 upstream activation sequence (Matchmaker system). In this system, activation of the reporter is dependent on the fusion proteins coming into proximity long enough to activate transcription, a function that is dependent on interaction of the fused target proteins. The results indicate that none of the plasmids carrying fusions activated expression when paired with either vector alone; in these strains, β-galactosidase activity was less than 2 U (Table 3). As a positive control, p53 and simian virus 40 large T antigen (supplied with the kit) together activated transcription sufficiently to give ~100 β-galactosidase units. In test assays, GlnK and NifL interacted in both vector combinations (16 to 49 β-galactosidase units). GlnKY51F interacted with NifL, but only when NifL was fused to the GAL4 DNA-binding domain (Table 3). These results indicate that NifL and unuridylylated GlnK may physically interact in vivo, demonstrating a mechanism for activating the inhibitory properties of NifL in response to nitrogen sufficiency.

TABLE 3. GlnK interactions in a yeast two-hybrid assay

Strain[a]	Fusion[b]		β-Galactosidase activity (U/ml/OD_{600} unit)[c]
	BD	AD	
S122[d,e]	p53	SV40 large T-antigen	110
S112[e]	NifL	GlnK	18
S113[e]	GlnK	NifL	49
S114[e]	NifL	GlnKY51F	16
S124	GlnKY51F	NifL	<2
S103	Vector only	Vector only	<2
S123	GlnK	Vector only	<2
S125	GlnKY51F	Vector only	<2
S132	Vector only	NifL	<2
S134	NifL	Vector only	<2
S135	Vector only	GlnK	<2
S136	Vector only	GlnKY51F	<2

[a] All strains are derivatives of yeast strain SFY526 carrying two plasmids.
[b] BD, fusion to GAL4 DNA-binding domain; AD, fusion to GAL4 transcriptional activation domain.
[c] See Materials and Methods for determination of β-galactosidase activities.
[d] Strain S122 carries plasmid pVA3 and pTD1 from the Clontech kit as positive controls.
[e] This strain also gives sufficient expression of a GAL4-*his* reporter in host strain HF7c to allow growth on His$^-$ DO (Drop Out) medium (Clontech, Palo Alto, Calif.).

DISCUSSION

GlnK-UMP is essential for activation of GS in *A. vinelandii*. In this work, we proposed that *gln-71*, a likely allele of *glnE*, would stabilize *glnKY51F*, as it did *glnD1*::Ω, because either *glnD* or a *glnKY51F* mutation would create a state in which GS would be irreversibly inactivated by adenylylation, if GlnK positively influences the adenylylation of GS (11). The allelic instability of these mutations mutation, due to the effects on GS, might be expected because *A. vinelandii* does not transport glutamine and lacks a glutamate dehydrogenase activity as an alternate ammonia-assimilatory pathway (43). The *gln-71* mutation did stabilize the *glnKY51F* allele, suggesting a dependence on the uridylylated form of GlnK for deadenylylation of GS. However, the effects of the *glnKY51F* are probably not restricted to those of GS, because a significant decrease in growth rate of the *glnKY51F* strain (MV577) over the *glnK*$^+$ strain (MV578) was noted when fixed N was present in the culture medium (Fig. 4B). This result illustrates the complexity of the mutation and suggests the possible occurrence of other GlnK targets in *A. vinelandii* and in other organisms.

To determine whether the only reason for the lethality of *glnK* mutations was this requirement, the null allele *glnK*::KIXX (31) was used to transform MV72 (*gln-71*). Kan^r Amp^s transformants were unstable (high-frequency loss of Kan^r after growth without kanamycin), indicating that GlnK in its unmodified form is required for an as-yet-unidentified function in *A. vinelandii*. The reason why *glnK* null mutants cannot be isolated remains unknown, although *A. vinelandii* is well suited for a study that may reveal potentially new regulatory targets for this class of proteins, possibly involved in carbon or energy metabolism or DNA processing, as may occur in *Rhodobacter capsulatus*, where new P_{II} targets, including dinitrogenase reductase ADP-ribosyl transferase (DRAT), have

been identified by using a yeast two-hybrid system (Werner Klipp, personal communication). In these experiments, as in other two-hybrid screens, caution should be used before any conclusions can be made concerning the validity of these new targets, and each will have to be verified in vivo or in vitro. On a positive note, however, an interaction with DRAT in this organism might be predicted from genetic evidence in the related organism *Rhodospirillum rubrum* (48).

GlnKY51F cannot relieve the inhibitory properties of NifL. An advantage of these in vivo experiments over other approaches is that in assays for NifA activity, gene expression was limited by both the chromosomal location and the preservation of native promoters for the regulatory proteins. These results should therefore reflect natural changes in nitrogenase expression with respect to growth phase and fixed nitrogen content without the ambiguity that might arise from the use of heterologous or in vitro systems or highly expressed promoters. The most significant finding of this work is that, in contrast to what occurs in *K. pneumoniae*, uridylylation of *A. vinelandii* GlnK is apparently required for relief of NifL inhibition of NifA activity. However, this requirement is not absolute because significant *nifH-lacZ* expression was detected with strain MV577H (*glnKY51F*) under N-limited conditions. The residual activity might indicate that GlnKY51F may not form as tight a complex with NifL as does unmodified wild-type GlnK, such that NifL inhibition can be partially relieved by a reduction in fixed nitrogen, perhaps by the binding of 2-oxoglutarate (28) or other mechanisms affecting GlnK activity.

As has been proposed for *A. vinelandii* and other organisms which probably have only one P_{II}-like protein, the composition of *A. vinelandii* GlnK may be an amalgam of residues such that a single protein can function with all regulatory targets (2). In light of this, it is also not surprising that while GlnK of *A. vinelandii* and GlnK of *E. coli* are more similar (83% identity) than are *A. vinelandii* GlnK and *E. coli* GlnB (75% identity), the T-loop region of *A. vinelandii* GlnK containing Y51 is more characteristic of GlnB proteins (3). This may be why *E. coli* GlnB and *A. vinelandii* GlnK apparently interact with NifL while *E. coli* GlnK does not (28). If GlnK is an essential protein in *A. vinelandii*, its rapid uridylylation in response to nitrogen flux mediates protein activities and would rely less on changes in the level of GlnK by transcriptional control. This is consistent with the observation that neither *glnA* (encoding GS) nor *nifL* gene expression is regulated by NH_4^+ in *A. vinelandii* (8, 43). In contrast, *K. pneumoniae* harbors two P_{II}-like proteins, probably because they provide a selective advantage over a single protein. In this organism, P_{II} (GlnB), whose expression is not regulated by nitrogen status, may be specific for targets involved in more subtle environmental tuning. GlnK, on the other hand, may be specific to nitrogen fixation and/or severe N starvation responses. In this scenario, it would seem sufficient to express *glnK* in the absence of combined nitrogen to activate NifA, which has been suggested for *K. pneumoniae* (20, 23) and for *E. coli* (44).

Unmodified GlnK interacts with NifL in a yeast two-hybrid assay. In this work it was proposed that *A. vinelandii* GlnK might exert its effects directly on the NifL. While a GlnK NifL-NifA interaction has been observed in vitro (28), it was important to study the interaction of the proteins in vivo and to determine which protein GlnK might target. In the yeast two-hybrid system, both GlnK and GlnKY51F interacted significantly with *A. vinelandii* NifL; that NifL and NifA interact in yeast was shown previously (27). The possibility that GlnK interacts with NifA as well could not be ruled out. This, however, seems unlikely based on in vitro data (28). Nevertheless, the demonstration of interaction between GlnK and NifL is further evidence that these two proteins interact in such a way that GlnK stabilizes the NifL-NifA nonactivating complex or promotes an inhibitory conformation of NifL and uridylylation prevents either function.

Since no *glnK* null mutants of this organism are available, it is difficult to predict what may be the result of loss of GlnK with respect to NifA activity and to other cellular functions in *A. vinelandii*. However, experiments with *E. coli* indicate that NifL is less inhibitory to NifA activity in the absence of both GlnB and GlnK than in their presence (37). This suggests that in *A. vinelandii*, GlnK may act solely as a negative regulator of NifA activity by stimulating the inhibitory properties of NifL and that in its absence, *nif* gene expression would occur constitutively, regardless of N status. It will be important to learn if interaction of GlnK with NifL is dependent on the uridylylation state of GlnK and which residues are important for such an interaction. This question can be addressed either in vitro or by using a two-hybrid approach. It is also of interest to discover new regulatory targets for *A. vinelandii* GlnK. Finding these targets may explain the apparent essentiality of this protein and have implications for all other organisms whose genomes contain one or more homologs of this interesting group of signal transduction proteins.

ACKNOWLEDGMENTS

We thank Luhong He and Sydney Kustu for *E. coli* strains and Wally van Heeswijk for the generous gift of P_{II} antibody. We also thank Stephen Billington and B. Helen Jost for a critical reading of the manuscript and Stefani Gilbert for technical assistance and critical comments.

This work was supported by a grant from USDA-NRI (95-37305-2067) to C.K.

REFERENCES

1. **Adler, S. P., D. Purich, and E. R. Stadtman.** 1975. Cascade control of *Escherichia coli* glutamine synthetase: properties of the P_{II} regulatory protein and the uridylyltransferase-uridylyl removing enzyme. J. Biol. Chem. **250:**6264–6272.
2. **Arcondeguy, T., R. Jack, and M. Merrick.** 2001. P(II) signal transduction proteins, pivotal players in microbial nitrogen control. Microbiol. Mol. Biol. Rev. **65:**80–105.
3. **Arcondeguy, T., D. Lawson, and M. Merrick.** 2000. Two residues in the T-loop of *Klebsiella pneumoniae* GlnK determine NifL-dependent nitrogen control of *nif* gene expression. J. Biol. Chem. **275:**38452–38456.
4. **Atkinson, M. R., E. S. Kamberov, R. L. Weiss, and A. J. Ninfa.** 1994. Reversible uridylylation of the *Escherichia coli* PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC). J. Biol. Chem. **269:**28288–28293.
5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl, P. Wang-Iverson, and S. G. Bonitz.** 1989. Short protocols in molecular biology. John Wiley & Sons, New York, N.Y.
6. **Bali, A., G. Blanco, S. Hill, and C. Kennedy.** 1992. Excretion of ammonium by a *nifL* mutant of *Azotobacter vinelandii* fixing nitrogen. Appl. Environ. Microbiol. **58:**1711–1718.
7. **Bishop, P. E., and W. J. Brill.** 1977. Genetic analysis of *Azotobacter vinelandii* mutant strains unable to fix nitrogen. J. Bacteriol. **130:**954–956.
8. **Blanco, G., M. Drummond, P. Woodley, and C. Kennedy.** 1993. Sequence and molecular analysis of the *nifL* gene of *Azotobacter vinelandii*. Mol. Microbiol. **9:**869–879.
9. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.

10. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. **104:**541–555.
11. **Colnaghi, R., P. Rudnick, L. He, A. Green, D. Yan, E. Larson, and C. Kennedy.** 2001. Lethality of *glnD* null mutations in *Azotobacter vinelandii* is suppressible by prevention of glutamine synthetase adenylylation. Microbiology **147:**1267–1276.
12. **Contreras, A., M. Drummond, A. Bali, G. Blanco, E. Garcia, G. Bush, C. Kennedy, and M. Merrick.** 1991. The product of the nitrogen fixation regulatory gene *nfrX* of *Azotobacter vinelandii* is functionally and structurally homologous to the uridylyltransferase encoded by *glnD* in enteric bacteria. J. Bacteriol. **173:**7741–7749.
13. **Dixon, R.** 1998. The oxygen-responsive NIFL-NIFA complex: a novel two-component regulatory system controlling nitrogenase synthesis in gamma-proteobacteria. Arch. Microbiol. **169:**371–380.
14. **Drummond, M., J. Clements, M. Merrick, and R. Dixon.** 1983. Positive control and autogenous regulation of the *nifLA* promoter in *Klebsiella pneumoniae*. Nature **301:**302–307.
15. **Edwards, R., and M. Merrick.** 1995. The role of uridylyltransferase in the control of *Klebsiella pneumoniae nif* gene regulation. Mol. Gen. Genet. **247:**189–198.
16. **Eydmann, T., E. Soderback, T. Jones, S. Hill, S. Austin, and R. Dixon.** 1995. Transcriptional activation of the nitrogenase promoter in vitro: adenosine nucleotides are required for inhibition of NIFA activity by NIFL. J. Bacteriol. **177:**1186–1195.
17. **Govantes, F., J. A. Molina-Lopez, and E. Santero.** 1996. Mechanism of coordinated synthesis of the antagonistic regulatory proteins NifL and NifA of *Klebsiella pneumoniae*. J. Bacteriol. **178:**6817–6823.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **He, L., E. Soupene, and S. Kustu.** 1997. NtrC is required for control of *Klebsiella pneumoniae* NifL activity. J. Bacteriol. **179:**7446–7455.
20. **He, L., E. Soupene, A. Ninfa, and S. Kustu.** 1998. Physiological role for the GlnK protein of enteric bacteria: relief of NifL inhibition under nitrogen-limiting conditions. J. Bacteriol. **180:**6661–6667.
21. **Henderson, N., S. A. Austin, and R. A. Dixon.** 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.
22. **Hill, S., S. Austin, T. Eydmann, T. Jones, and R. Dixon.** 1996. *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. Proc. Natl. Acad. Sci. USA **93:**2143–2148.
23. **Jack, R., M. De Zamaroczy, and M. Merrick.** 1999. The signal transduction protein GlnK is required for NifL-dependent nitrogen control of *nif* gene expression in *Klebsiella pneumoniae*. J. Bacteriol. **181:**1156–1162.
24. **Jiang, P., J. A. Peliska, and A. J. Ninfa.** 1998. Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. Biochemistry **37:**12782–12794.
25. **Kamberov, E. S., M. R. Atkinson, and A. J. Ninfa.** 1995. The *Escherichia coli* PII signal transduction protein is activated upon binding 2-oxoglutarate and ATP. J. Biol. Chem. **270:**17797–17807.
26. **Kustu, S., J. Hirschman, D. Burton, J. Jelesko, and J. C. Meeks.** 1984. Covalent modification of bacterial glutamine synthetase: physiological significance. Mol. Gen. Genet. **197:**309–317.
27. **Lei, S., L. Pulakat, and N. Gavini.** 1999. Genetic analysis of *nif* regulatory genes by utilizing the yeast two-hybrid system detected formation of a NifL-NifA complex that is implicated in regulated expression of *nif* genes. J. Bacteriol. **181:**6535–6539.
28. **Little, R., F. Reyes-Ramirez, Y. Zhang, W. C. van Heeswijk, and R. Dixon.** 2000. Signal transduction to the *Azotobacter vinelandii* NIFL-NIFA regulatory system is influenced directly by interaction with 2-oxoglutarate and the PII regulatory protein. EMBO J. **19:**6041–6050.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Manoil, C., B. Bassler, and J. Slauch.** 1999. Advanced bacterial genetics laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Meletzus, D., P. Rudnick, N. Doetsch, A. Green, and C. Kennedy.** 1998. Characterization of the *glnK-amtB* operon of *Azotobacter vinelandii*. J. Bacteriol. **180:**3260–3264.
32. **Merrick, M., S. Hill, H. Hennecke, R. Hahn, R. Dixon, and C. Kennedy.** 1982. Repressor properties of the *nifL* gene product in *Klebsiella pneumoniae*. Mol. Gen. Genet. **185:**75–81.
33. **Merrick, M. J., and R. A. Edwards.** 1995. Nitrogen control in bacteria. Microbiol. Rev. **59:**604–622.
34. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Newton, W. E., P. W. Wilson, and R. H. Burris.** 1953. Direct demonstration of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. J. Biol. Chem. **204:**445–451.
36. **Ninfa, A. J., and M. R. Atkinson.** 2000. PII signal transduction proteins. Trends Microbiol. **8:**172–179.
37. **Reyes-Ramirez, F., R. Little, and R. Dixon.** 2001. Role of *Escherichia coli* nitrogen regulatory genes in the nitrogen response of the *Azotobacter vinelandii* NifL-NifA complex. J. Bacteriol. **183:**3076–3082.
38. **Santero, E., A. Toukdarian, R. Humphrey, and C. Kennedy.** 1988. Identification and characterization of two nitrogen fixation regulatory regions, *nifA* and *nfrX*, in *Azotobacter vinelandii* and *Azotobacter chroococcum*. Mol. Microbiol. **2:**303–314.
39. **Schmitz, R. A.** 1997. NifL of *Klebsiella pneumoniae* carries an N-terminally bound FAD cofactor, which is not directly required for the inhibitory function of NifL. FEMS Microbiol. Lett. **157:**313–318.
40. **Soderback, E., F. Reyes-Ramirez, T. Eydmann, S. Austin, S. Hill, and R. Dixon.** 1998. The redox- and fixed nitrogen-responsive regulatory protein NifL from *Azotobacter vinelandii* comprises discrete flavin and nucleotide-binding domains. Mol. Microbiol. **28:**179–192.
41. **Soupene, E., L. He, D. Yan, and S. Kustu.** 1998. Ammonia acquisition in enteric bacteria: physiological role of the ammonium/methylammonium transport B (AmtB) protein. Proc. Natl. Acad. Sci. USA **95:**7030–7034.
42. **Thomas, G., G. Coutts, and M. Merrick.** 2000. The *glnKamtB* operon. A conserved gene pair in prokaryotes. Trends Genet. **16:**11–14.
43. **Toukdarian, A., G. Saunders, G. Selman-Sosa, E. Santero, P. Woodley, and C. Kennedy.** 1990. Molecular analysis of the *Azotobacter vinelandii glnA* gene encoding glutamine synthetase. J. Bacteriol. **172:**6529–6539.
44. **van Heeswijk, W. C., S. Hoving, D. Molenaar, B. Stegeman, D. Kahn, and H. V. Westerhoff.** 1996. An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. Mol. Microbiol. **21:**133–146.
45. **van Heeswijk, W. C., B. Stegeman, S. Hoving, D. Molenaar, D. Kahn, and H. V. Westerhoff.** 1995. An additional PII in *Escherichia coli*: a new regulatory protein in the glutamine synthetase cascade. FEMS Microbiol. Lett. **132:**153–157.
46. **Walmsley, J., and C. Kennedy.** 1991. Temperature-dependent regulation by molybdenum and vanadium of expression of the structural genes encoding three nitrogenases in *Azotobacter vinelandii*. Appl. Environ. Microbiol. **57:**622–624.
47. **Walmsley, J., A. Toukdarian, and C. Kennedy.** 1994. The role of regulatory genes *nifA*, *vnfA*, *anfA*, *nfrX*, *ntrC*, and *rpoN* in expression of genes encoding the three nitrogenases of *Azotobacter vinelandii*. Arch. Microbiol. **162:**422–429.
48. **Zhang, Y., E. L. Pohlmann, C. M. Halbleib, P. W. Ludden, and G. P. Roberts.** 2001. Effect of P_{II} and its homolog GlnK on reversible ADP-ribosylation of dinitrogenase reductase by heterologous expression of the *Rhodospirillum rubrum* dinitrogenase reductase ADP-ribosyl transferase-dinitrogenase reductase-activating glycohydrolase regulatory system in *Klebsiella pneumoniae*. J. Bacteriol. **183:**1610–1620.
49. **Zheng, L., and D. R. Dean.** 1994. Catalytic formation of a nitrogenase iron-sulfur cluster. J. Biol. Chem. **269:**18723–18726.