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NtrC Is Required for Control of *Klebsiella pneumoniae* NifL Activity

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In response to molecular oxygen and/or fixed nitrogen, the product of the *Klebsiella pneumoniae* nitrogen fixation L (*nifL*) gene inhibits NifA-mediated transcriptional activation. Nitrogen regulation of NifL function occurs at two levels: transcription of the *nifLA* operon is regulated by the general Ntr system, and the activity of NifL is controlled by an unknown mechanism. We have studied the regulation of NifL activity in *Escherichia coli* and *Salmonella typhimurium* by monitoring its inhibition of NifA-mediated expression of a *K. pneumoniae* Φ(*nifH′-′lacZ*) fusion. The activity of the NifL protein transcribed from the *tac* promoter is regulated well in response to changes of oxygen and/or nitrogen status, indicating that no *nif*- or *K. pneumoniae*-specific product is required. Unexpectedly, strains carrying *ntrC* (*glnG*) null alleles failed to release NifL inhibition, despite the fact that synthesis of NifL was no longer under Ntr control. Additional evidence indicated that it is indeed the transcriptional activation capacity of NtrC, rather than its repression capacity, that is needed, and hence it is a plausible hypothesis that NtrC activates transcription of a gene(s) whose product(s) in turn functions to relieve NifL inhibition under nitrogen-limiting conditions.

In diazotrophs that are members of the proteobacteria, transcription of the nitrogen fixation *nif* genes is mediated by the *nif*-specific activator protein NifA, a member of the σ^{54}-dependent family of activators (22). Both the expression and the activity of NifA can be regulated in response to the cellular nitrogen status, but the mechanism of this regulation varies according to the organism. In *Klebsiella pneumoniae nifA* is cotranscribed with *nifL*, whose product modulates NifA activity in response to both combined nitrogen and molecular oxygen (28, 45). Thus, inhibition of nitrogen fixation by fixed nitrogen occurs at two levels in *K. pneumoniae*: prevention of *nifLA* transcription, which is known to be controlled by the general Ntr system (19, 22), and inhibition of NifA activity by NifL. The way in which NifL senses the cellular nitrogen status is presently unknown.

A study of purified *Azotobacter vinelandii* NifL protein revealed that it was a flavoprotein with flavin adenine dinucleotide (FAD) as the prosthetic group (27). In vitro the oxidized but not the reduced form of NifL inhibited the ability of NifA to catalyze the formation of open complexes at *nif* promoters. The same appears to be true of *K. pneumoniae* NifL (58). This implies a mechanism by which NifL responds to oxygen supply. We recently demonstrated that iron is required to relieve NifL inhibition of NifA activity in vivo and hypothesized that an unidentified Fe-containing protein might be required (59), e.g., to reduce the FAD cofactor of NifL. Whether limitation of combined nitrogen is also manifested through reduction of the FAD cofactor of NifL is not known.

Although both the *A. vinelandii* and *K. pneumoniae* NifL proteins contain FAD as the prosthetic group, the regulation of NifL activity by fixed nitrogen seems to be controlled differently in the two organisms. Genetic studies in *A. vinelandii* implicated the product of *nfrX*, a homolog of the enteric *glnD* product (P_{II} uridylyltransferase-uridylyl removing enzyme [UT/UR]), in regulation of NifL activity (13, 57), but the link between the NfrX and NifL proteins is unclear. By contrast, studies from Merrick's group (20, 29) led them to conclude that neither UT/UR nor its substrate P_{II}, the product of the *glnB* gene, was involved in regulation of *K. pneumoniae* NifL activity. However, the latter conclusion was based on studies of NifL activity in *glnD* strains that also carried a particular *ntrB* (*glnL*) allele that allowed the constitutive expression of *nifLA*. Because the *ntrB* product in this strain is thought to be insensitive to the P_{II} protein, results of these studies did not address the issue of whether null alleles in *glnD* or *ntrB* individually affected regulation of NifL activity; rather, they showed that neither appeared to be essential for such regulation (but see Discussion).

Constitutive, *ntr*-independent expression of *nifLA* has been used to simplify the study of nitrogen regulation of *K. pneumoniae* NifL activity. In 1989 Henderson et al. (26) developed an *Escherichia coli* system employing two compatible plasmids to study the regulation of NifL activity: plasmid pRT22 carries a *K. pneumoniae* Φ(*nifH′-′lacZ*) translational fusion, and plasmid pNH3 carries the *nifLA* genes under control of the *tac* promoter. Since NifLA synthesis is not dependent on Ntr components in this system, nitrogen regulation of transcription from the *nifH* promoter should depend only on the activity of NifL. Henderson et al. (26) concluded that the activity of the NifL protein was not responsive to environmental signals in their system (but see below).

In this study we constructed another *E. coli* strain that contains the same plasmid carrying the constitutively expressed *nifLA* genes, pNH3, and a single chromosomal copy of a *K. pneumoniae* Φ(*nifH′-′lacZ*) translational fusion instead of plasmid pRT22. Contrary to the conclusion of Henderson et al. (26), we found that the activity of the constitutively expressed NifL protein is regulated well in response to changes of nitrogen status, just as it is in *K. pneumoniae*, and is also regulated in response to oxygen status. Hence we were able to study the regulation of NifL activity in various genetic backgrounds. Surprisingly, we found that in all strains in which transcriptional activation by NtrC is decreased, relief of NifL inhibition under nitrogen-limiting conditions is also decreased. The same is true in *Salmonella typhimurium* strains carrying the Φ(*nifH′-′lacZ*) fusion and the *nifLA* plasmid pNH3. Hence, beyond activating *nifLA* transcription, NtrC is also required to relieve NifL inhibition of NifA activity under nitrogen-limiting conditions.

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Although Henderson et al. (26) ascribed their failure to see relief of NifL inhibition in *E. coli* to the fact that the *nifLA* genes were present in multiple copies, we surmise that it was due to the fact that the strain they used carried a deletion of the *glnA-ntrB-ntrC* region.

MATERIALS AND METHODS

Strains and strain constructions. Bacterial strains and plasmids used in this work are listed in Table 1. Plasmid DNA (including linear DNA) was transformed into *E. coli* cells according to the method of Chung and Miller (12) and into *S. typhimurium* cells by electroporation (41). Transduction by P1*vir* was performed as described previously (60). P22 phage (HT *int-201*)-mediated transduction was performed as described by Ikeda et al. (30), and phage-free transductants were isolated on Evans-Blue Uranine indicator plates (41). Ampicillin, kanamycin, tetracycline, and chloramphenicol were generally used at 100, 25, 10, and 12.5 μg/ml, respectively. Tryptophan (0.1 mM) was added when *E. coli* strains carrying Φ(*nifH′-′lacZ*) were grown in minimal medium.

(i) *E. coli*. *E. coli* RK4353, which grows well under anaerobic conditions, was chosen as the parental strain to study Φ(*nifH′-′lacZ*) transcription. Strain NCM1809 (*glnA1859 zih102*::Tn*10*), which was used as an intermediate in the construction of several of the strains listed below, was obtained by transducing strain NCM1529 to tetracycline resistance with phage grown on strain ET12560 and then screening for glutamine auxotrophy. Strains carrying the *ntrC*(*glnG*)*10*::Tn*5*, *ntrB*(*glnL*)*302*, Δ*ntrBC*, *ntrB1*, and *ntrC3* alleles were derived from strain NCM1809 by P1-mediated transduction to glutamine prototrophy and screening for the ability to use arginine as the sole nitrogen source (Aut$^-$ for all except *ntrB302*).

The *glnD99*::Tn*10* allele was transferred from strain RB9040 into other *E. coli* strains by P1-mediated transduction with selection for tetracycline resistance. Spontaneous Aut$^+$ suppressors of strain NCM1686 carrying *glnD99*::Tn*10* were selected on solid minimal medium M9 (55) containing 0.4% glucose as the carbon source and 2.5 mM arginine as the sole nitrogen source (3). Whereas strains RB9040 and NCM1686 failed to grow after 5 days of incubation at 37°C, suppressor strains NCM1897 and NCM1829 grew as well as parental strain NCM1529 (Aut$^+$); strains NCM1778 and NCM1781 grew faster than strain NCM1529. The suppressor mutation in strain NCM1897 was shown to be linked to *glnD99*::Tn*10* by backcross from NCM1897 to NCM1529 with selection for tetracycline resistance. Lesions from other suppressor strains, which are not linked to *glnD99*::Tn*10*, have not been further studied. Strains NCM1778 and NCM1781, which carry the *ntr-1* and *ntr-2* suppressor mutations, respectively, may also carry additional suppressor mutations, but this is not certain, because we were unable to isolate strains with the same properties a second time.

The (Δ*glnB*)::Cam^r mutation in plasmid pJES998 (see below) was introduced onto the *E. coli* chromosome as follows. After linearization with *Aat*II, pJES998 was transformed into strain NCM1469 [see below for Φ(*nifH′-′lacZ*) construction]. A Southern blot with the 1.3-kb *glnB* fragment of pDK601 (see below) as probe confirmed that strain NCM1742, one of the Cam^r Amp^s transformants, carried the (Δ*glnB*)::Cam^r mutation in its chromosome (data not shown). Then, the mutation was transferred to strain NCM1529 by P1-mediated transduction with selection for chloramphenicol resistance to yield strain NCM1736. Strain NCM1736 was Gln$^+$ and Aut$^+$, as was strain NCM1529, but strains carrying both the (Δ*glnB*)::Cam^r and *glnD99*::Tn*10* mutations were Gln$^+$ Aut$^-$; i.e., the *glnB* mutation was able to suppress the glutamine bradytrophy but not the Aut$^-$ phenotype caused by the *glnD* mutation (9).

(ii) *S. typhimurium*. Strains that were used to monitor NifL inhibition are congenic with parental strain SK2979 (30). The *ntrC74* (NtrC^{repressor}) allele in strain SK1799, which results in glutamine auxotrophy, has been described previously (50, 68). The *ntrC74* allele was transferred to strain SK2979 by P22-mediated transduction with selection for a linked Tn*10* insertion and screening for Gln$^-$ and Aut$^-$ phenotypes. Strain SK1690, which carries the *ntrB509* (NtrB$^-$) and *ntrC599*(NtrC^{constitutive}) alleles, grows faster than usual on arginine as the sole nitrogen source (23). The *ntrB509* and *ntrC599* alleles were transferred into strain SK2980 [Δ(*glnA-ntrB-ntrC*)*60*] by P22-mediated transduction with selection for glutamine prototrophy and screening for arginine fast growth. The *glnD96* allele in strain SK3071 was transduced into strain SK3276 (*ntrB509 ntrC599*) by P22-mediated transduction with selection for a linked Tn*10* insertion and subsequent genetic analysis (backcrossing the *glnD* allele into wild-type strain SK2979 and screening for glutamine bradytrophy and the Aut$^-$ phenotype). Strain SK3301, which carries the *ntrB509 ntrC599* and *glnD96* alleles is Gln$^+$ and Aut$^+$, but its colony size is smaller than that of strain SK3276 on both enriched medium lacking glutamine (nutrient broth [NB]) and minimal medium with arginine as the sole nitrogen source.

Construction of a single copy Φ(*nifH′-′lacZ*) fusion. (i) Construction in *E. coli*. Strain NCM1469, which carries a Φ(*nifH′-′lacZ*) fusion in single copy at the *trp* locus, was constructed according to the method of Elliott (21, 30), as described below. A 2.2-kb *Eco*RI-*Sac*I fragment from pJES384 (56), which carries the *nifH* promoter region of *K. pneumoniae* lacking both the integration host factor- and NifA-binding sites for the divergent *nifJ* operon and carries the first codon of *nifH* joined to the eighth codon of *lacZ* (64), was ligated to the 10-kb *Eco*RI-*Sac*I fragment of pRS551 (61), which carries the remainder of *lacZ*, to yield plasmid pJES863. The gene order around the *nifH* insert is Kan^r-Φ(*nifH′-′lacZ*)*YA*. After linearization with *Xho*I, pJES863 was transformed into the *E. coli recD* strain TE2680 (21), which carries a nested set of insertions in the *trp* operon: *trpDC700*::*putPA1303* (derived from *S. typhimurium*)::[Kan^s-Cam^r-*lacZYA*]. Selection was for kanamycin resistance, which was achieved by homologous recombination between the Kan and *lac* regions of plasmid and chromosome, yielding kanamycin-resistant and chloramphenicol-sensitive transformants; one of those carrying the Φ(*nifH′-′lacZ*) fusion was denoted NCM1469. The Φ(*nifH′-′lacZ*) fusion was transferred from strain NCM1469 to other *E. coli* strains by P1-mediated transduction with selection for kanamycin resistance (Table 1).

(ii) Construction in *S. typhimurium*. Phage sensitivities of *S. typhimurium galE* strains were exploited to transfer the Φ(*nifH′-′lacZ*) fusion from *E. coli* into *S. typhimurium*. As summarized by Maloy (42), when no exogenous galactose is added, *galE* mutant strains make a truncated lipopolysaccharide (LPS) and are sensitive to phage P1 but resistant to P22 (P1^s P22^r). By contrast, when exogenous galactose is added, *galE* mutants make the complete LPS and are P1^r P22^s. To receive the Φ(*nifH′-′lacZ*) fusion carried by P1 phage, *S. typhimurium* SL4213 (*galE* [14]) was grown in LB medium containing 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.2% glucose (60) but lacking galactose. Transductants were selected for kanamycin resistance. One of the transductants, designated SK3164, was then grown with 1% glucose and 0.2% galactose to allow completion of the LPS and adsorption of phage P22 and was used as the donor to transduce the Φ(*nifH′-′lacZ*) fusion into other strains of *S. typhimurium* (Table 1).

Construction of plasmids. Plasmid pJES998 (Δ*glnB*::Cam^r) was derived from plasmid pDK601, which carries the *E. coli glnB* region (66). To construct the *glnB* mutation, a 1.3-kb *Age*I-*Eco*NI fragment of pDK601 internal to *glnB* (*Eco*NI blunt ended by Klenow fragment of DNA polymerase I) was replaced with the chloramphenicol resistance gene from pJES1002 (a pT7-3 vector carrying the Cam^r gene at its *Sal*I site [25]).

Plasmid pJES851, which carries *nifA* from *K. pneumoniae* under control of the *tac* promoter, was constructed by ligating a 2.1-kb *Eco*RI-*Hin*dIII fragment carrying the *nifA* gene from pJES293 (59) to the 5.2-kb vector fragment from pNH3 digested with the same enzymes. This plasmid was described by Schmitz et al. (59) but was not named.

Plasmid pJES935, which encodes an internal, in-frame NifL deletion [Δ(Ile114-Asp135)] and intact NifA, was derived from pNH3 in the following several steps. To construct the *nifL* deletion, plasmid pJES282 (36), which carries the *nifLA* genes, was first digested with *Pst*I, and the largest fragment was religated to yield pJES868. Then, the two remaining *Eco*RV fragments in pJES868 were deleted to yield pJES874, which carries an in-frame deletion in *nifL*. (Loss of the *Cla*I site indicated that the small upstream *Eco*RV fragment had been deleted, and a decrease in size of the *Sma*I-*Csp*I fragment indicated that the larger downstream fragment had also been deleted; hence, the deletion results in loss of the peptide Ile114-Asp135 in the N terminus of NifL.) To place the deleted *nifL* allele under control of the *tac* promoter, the *Sma*I-*Csp*I fragment from pJES832 (47), which carries *nifL* under control of the *tac* promoter derived from pNH3, was replaced with the corresponding fragment from pJES874, which carries the *nifL* deletion, to yield plasmid pJES883. Finally, to reconstruct *nifA*, the *Nco*I-*Sca*I fragment of pJES883 was replaced by the corresponding fragment of pJES871 to yield pJES935. (Plasmid pJES871, which carries *nifA*, was derived from pJES282 by inserting a tetracycline resistance cassette [15] into the *Hin*dIII site.) Since the *Nco*I site lies downstream of the *Eco*RV sites in *nifL* and the *Sca*I site in pJES883 lies in the *bla* gene of the vector, the replacement yields a plasmid (pJES935) that carries an in-frame deletion of *nifL* and intact *nifA* under control of the *tac* promoter and that confers resistance to tetracycline but not β-lactam antibiotics. By contrast to the case for pJES851, the amount of NifA synthesized from pJES935 should not be greater than that from pNH3, because translational coupling between *nifL* and *nifA* has been maintained (24). Although the deleted NifL protein produced from pJES935 retains slight ability to inhibit NifA activity (47), a very large excess of the deleted NifL over NifA is required to see inhibitory effects.

We confirmed by sequencing that the two tandem NtrC-binding sites required for activation of *nifLA* transcription from the natural (σ^{54}-dependent) promoter are absent in pNH3 (and, by implication, in pJES935) (26), although the promoter itself is present downstream of the *tac* promoter. Hence, expression of *nifLA* from pNH3 and pJES935 should not be influenced by nitrogen status. The natural translational start of the *nifL* gene in pNH3 is 168 nucleotides downstream of the *tac* promoter (unpublished data).

Growth conditions and β-galactosidase assay. Cells were grown in modified K medium (39) (see below for modifications) supplemented with tryptophan (0.1 mM), Na_2CO_3 (1 mM), glucose (0.4%) as the carbon source, and the nitrogen sources specified. Modified K medium contained (per liter) Na_2HPO_4 (18.75 g), KH_2PO_4 (2.2 g), NaCl (2 g), $Na_2MoO_4 \cdot 2H_2O$ (0.25 mg), $MgSO_4 \cdot 7H_2O$ (0.2 g), and $FeCl_3 \cdot 6H_2O$ (4.9 mg) and had a final pH of 7.6. NifA-mediated activation of transcription from the *nifH* promoter was determined 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* transcription. Precultures were grown aerobically overnight with 2 mM glutamine and 4 mM NH_4Cl as the nitrogen sources. To relieve NifL inhibition, cells were grown in closed bottles with N_2 as gas phase at 30°C with glutamine (2 mM) as the nitrogen source. To achieve NifL inhibition, cells were grown under the same conditions with additional NH_4Cl (4 mM) as the nitrogen source or were grown aerobically. All cultures were shaken at 250 rpm. Expression of *nifA* or *nifLA* was

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or characteristic(s)[a]	Construction, reference, source, and/or description[b]	Strain congenic with pNH3 (NifL NifA)
E. coli strains			
RK4353	*araD139* Δ(*argF-lacU*)*169 flhD5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1* (wild type)	63	
TE2680	F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 recD1903*::Tn*10d*-Tet *trpDC700*::*putPA1303*::[Kan^s-Cam^r-*lac*]	21	
YMC12	*endA1 thi-1 hsdR17 supE44* Δ*lacU169* *hutC*$_{Klebs}$ *glnG*(*ntrC*)*10*::Tn*5*	R. Bender, 4	
YMC15	*endA1 thi-1 hsdR17 supE44* Δ*lacU169* *hutC*$_{Klebs}$ *glnL*(*ntrB*)*302*[c]	R. Bender, 11	
RB9040	Mu lysogen; *endA1 thi-1 hsdR17 supE44* Δ*lacU169* *hutC*$_{Klebs}$ *glnD99*::Tn*10*	R. Bender, 9	
ET12560	*glnV*$_{44}$(AS) λ$^-$ *endA1 thi-1 hsdR17 glnA1859 zih-102*::Tn*10*	*E. coli* Genetic Stock Center, 40	
ET6017	[*araD139*] Δ(*argF-lac*)*205 flhD5301 fruA25 relA1 rpsL150*(*strR*) *rha-10 deoC1* (*glnG*?-*glnL-glnA*)*229* [(*ntrC*?-*ntrB-glnA*)*229*]	*E. coli* Genetic Stock Center	
SN24	*endA1 thi-1 hsdR17 supE44* Δ*lacU169* *hutC*$_{Klebs}$ *lacI*^{q} Δ(*glnLG*) [Δ(*ntrBC*)]	L. Reitzer	
NCM130	*ntrB1*(*glnL208*) *galT23*	44	
NCM131	*ntrC3*(*glnG210*) *galT23*	43	
NCM1469	*recD1903*::Tn*10d*-Tet *trpDC700*::*putPA1303*::[Kan^r-Φ(*nifH'-'lacZ*)]	See Materials and Methods	
NCM1529	*trpDC700*::*putPA1303*::[Kan^r-Φ(*nifH'-'lacZ*)]	RK4353, NCM1469[d]	NCM1528[e]
NCM1686	*glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1529, RB9040[f]	NCM1687[g]
NCM1736	(Δ*glnB*)::Cam^r [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1529, NCM1742[h]	NCM1737
NCM1739	(Δ*glnB*)::Cam^r *glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1686, NCM1742[h]	NCM1740
NCM1742	*recD1903*::Tn*10d*-Tet (Δ*glnB*)::Cam^r [Kan^r-Φ(*nifH'-'lacZ*)]	See Materials and Methods	
NCM1778	*glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)] *ntr-1*	NCM1686[i]	NCM1779
NCM1781	*glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)] *ntr-2*	NCM1686[i]	NCM1782
NCM1798	Δ(*argF-lac*)*205* Δ(*ntrC*?-*ntrB*)*229* [Kan^r-Φ(*nifH'-'lacZ*)]	ET6017, NCM1686[d]	NCM1799
NCM1801	Δ(*argF-lac*)*205*	ET6017, NCM1686[j]	
NCM1802	Δ(*argF-lac*)*205* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1801, NCM1686[d]	NCM1803
NCM1809	*glnA1859 zih-102*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1529, ET12560[f,k]	
NCM1829	*glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)] *ntr*-3/pNH3	NCM1687[i]	
NCM1846	Δ*ntrBC* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1809, SN24[j,l]	NCM1847
NCM1850	*ntrC10*::Tn*5* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1809, YMC12[j,m]	NCM1851[n]
NCM1856	*ntrB302* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1809, YMC15[j,o]	NCM1857
NCM1860	*ntrC3* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1809, NCM131[j,l]	NCM1866
NCM1863	*ntrB1* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1809, NCM130[j,l]	NCM1872
NCM1897	*glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)] *ntr-4*	NCM1686[i]	NCM1898
NCM1900	*glnD99*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)] *ntr-4*	NCM1529, NCM1897[f]	NCM1901
NCM1932	*glnD99*::Tn*10 ntrC10*::Tn*5* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1850, NCM1686[f]	NCM1934
NCM1933	*glnD99*::Tn*10 ntrB302* [Kan^r-Φ(*nifH'-'lacZ*)]	NCM1856, NCM1686[f]	NCM1935
S. typhimurium strains			
SL4213	*galE496 metA22 metE55 rpsL120 xyl-404* (Fels2)$^-$ *H1-b nml*$^-$ *H2-enx* (*ilv*?) *hsdL6 hsdSA29*	14	
SK1159	*ntrB509 hisF645*	23	
SK1690	*ntrB509 ntrC599*(Con) *hisF645*	23	
SK1799	*ntrC74*(Rep) *zig220*::Tn*10 hisF645*	68	
SK2979	Wild type	30	
SK2980	Δ(*glnA-ntrB-ntrC*)*60*	30	
SK3019	*ntrB137*::Tn*10*	30	
SK3020	*ntrC352*::Tn*10*	30	
SK3071	*glnD96 zag208*::Tn*10*	30	
SK3164	*galE496 putPA1303*::[Kan^r-Φ(*nifH'-'lacZ*)]	SL4213, NCM1469[d]	
SK3166	*putPA1303*::[Kan^r-Φ(*nifH'-'lacZ*)]	SK2979, SK3164[d]	SK3236
SK3247	*ntrB137*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	SK3019, SK3164[d]	SK3248
SK3250	*ntrC352*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	SK3020, SK3164[d]	SK3251
SK3253	*glnD96 zag208*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	SK3071, SK3164[d]	SK3254
SK3276	*ntrB509 ntrC599*(Con)	SK2980, SK1690[j,p]	
SK3277	*ntrC74*(Rep) *zig220*::Tn*10*	SK2979, SK1799[f,k]	
SK3278	*ntrB509 ntrC599*(Con) [Kan^r-Φ(*nifH'-'lacZ*)]	SK3276, SK3164[d]	SK3279
SK3281	*ntrC74*(Rep) *zig220*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	SK3277, SK3164[d]	SK3282
SK3301	*ntrB509 ntrC599*(Con) *glnD96 zag208*::Tn*10*	SK3276, SK3071[f,q]	
SK3302	*ntrB509 ntrC599*(Con) *glnD96 zag208*::Tn*10* [Kan^r-Φ(*nifH'-'lacZ*)]	SK3301, SK3164[d]	SK3303
SK3317	*ntrB509*	SK2980, SK1159[j]	
SK3318	*ntrB509* [Kan^r-Φ(*nifH'-'lacZ*)]	SK3317, SK3164[d]	SK3319
Plasmids			
pRS551	*bla*-Kan^r-T1$_4$-*lacZYA*	61	
pJES384	Φ(*nifH'-'lacZ*) in pMC1403	56	
pJES863	*bla*-Kan^r-T1$_4$-Φ(*nifH'-'lacZ*)	See Materials and Methods	
pNH3	*K. pneumoniae nifLA* controlled by *tac* promoter	26	
pJES851	*K. pneumoniae nifA* controlled by *tac* promoter	59	
pJES935	Δ*nifL nifA* derived from pNH3	See Materials and Methods, 47	
pDK601	1.5-kb *Eco*RI-*Sal*I fragment of *E. coli glnB* region in pUC18	D. Kahn, 66	
pJES1002	Cam^r gene in pT7-3 vector	25	
pJES998	(Δ*glnB*)::Cam^r	See Materials and Methods	

Continued on following page

induced from the *tac* promoter with 10 μM isopropyl-β-D-thiogalactopyranoside (IPTG) unless specified otherwise. Samples of the growing cultures were taken every 2 to 3 h to determine the optical density at 600 nm (OD_{600}) and the β-galactosidase activity (in units per milliliter), calculated as $1{,}000(OD_{420} - 1.75 \times OD_{550})/(\Delta t \times v)$, where Δt is time of the reaction in minutes and v is the volume of culture in milliliters used in the assay (46). The differential rates of β-galactosidase synthesis reported here were obtained by determining the slopes of plots of β-galactosidase activity versus the optical density of the culture (in units per milliliter per OD_{600}).

Glutamine synthetase assay. Cells were grown in minimal medium M9 (42) containing glutamine (3 mM) (derepressing conditions) or glutamine (3 mM) and NH_4Cl (10 mM) (repressing conditions), or they were grown in nutrient broth medium plus glutamine (1 mM) (very repressing conditions). Cultures were inoculated to an OD_{600} of 0.08 and grown at 37°C to an OD_{600} of 0.5. Cells were harvested and disrupted as described previously, and glutamine synthetase activity in cell extracts was determined as described previously (35). Precultures were grown overnight under repressing conditions.

Western blotting. Cells were grown anaerobically in modified, supplemented K medium with glutamine as the nitrogen source and with or without IPTG induction of *nifLA* expression. At an OD_{600} of 0.8 to 1.0, cells were harvested from 1 ml of culture and concentrated 20-fold into sodium dodecyl sulfate gel-loading buffer (54). Samples were subjected to electrophoreses in sodium dodecyl sulfate–10% polyacrylamide gels and transferred to nitrocellulose membranes as described previously (54). Membranes were exposed to polyclonal rabbit antisera directed against the NifL or NifA protein of *K. pneumoniae*, and bands were detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to alkaline phosphatase (Bio-Rad). Purified NifL and NifA and prestained protein markers (Broad Range; New England Biolabs) were used as standards.

RESULTS

The activity of constitutively expressed NifL protein responds to environmental signals. Potential roles of Ntr and Gln proteins in regulating NifL activity are not easily studied when *nifLA* is under the control of its own promoter, because synthesis of the NifL and NifA proteins requires phosphorylated NtrC (19). To simplify the study of nitrogen regulation of NifL activity, we used plasmids pNH3 and pJES851, which carry, respectively, the *K. pneumoniae nifLA* or *nifA* genes under the control of the *tac* promoter (26, 59), and plasmid pJES935, which is derived from pNH3 and contains an in-frame deletion in the amino (N)-terminal domain of NifL (see Materials and Methods). To avoid effects of multiple copies of *nifH* (8), we examined NifL activity in *E. coli* strains carrying a single chromosomal copy of a *K. pneumoniae* Φ(*nifH′-′lacZ*) translational fusion. Transcription from the *nifH* promoter was assessed by measuring the differential rate of β-galactosidase synthesis.

As expected based on previous reports (56, 64), there was no β-galactosidase synthesis in the absence of NifA (strain NCM1529) (Table 2), indicating that the NifA homolog NtrC cannot activate transcription from the *K. pneumoniae nifH* promoter. In the presence of NifA alone, the differential rate of synthesis of β-galactosidase was high (approximately 5,000 U/ml/OD_{600}) and remained at similar high levels regardless of the growth conditions (strain NCM1527) (Fig. 1A and Table 2). Thus, as observed previously (7), the NifA protein itself does not sense environmental signals. Similarly, *nifH* expression was high under all growth conditions in a strain carrying NifA and the NifL deletion protein (strain NCM1538) (Fig. 1B and Table 2).

We monitored NifL activity by monitoring the inhibition of Φ(*nifH′-′lacZ*) transcription activated by NifA. In the presence of NifL and NifA, transcription of *nifH* occurred only when cells were grown anaerobically in minimal medium with 2 mM glutamine (K medium plus glutamine) as the sole nitrogen source (strain NCM1528) (Fig. 1C and Table 2). (These are so-called derepressing conditions.) The differential rate of synthesis of β-galactosidase was more than 2,000 U/ml/OD_{600}, whereas when the cells were grown aerobically in the same medium or when 4 mM NH_4Cl was added to the medium under anaerobic conditions, transcription from the *nifH* promoter decreased several hundredfold (Fig. 1C and Table 2). (The latter two conditions are called repressing conditions.) These results indicated that inhibitory effects of ammonium and/or oxygen on *nifH* expression depended on the presence of NifL and thus provided evidence that they were due to inhibitory effects of NifL on NifA activity. Contrary to a previous report (26), the activity of the NifL protein expressed from the *tac* promoter is regulated well in response to environmental signals in *E. coli*, as it is in *K. pneumoniae* (38, 59), even at the same high level of induction as that used previously (data not shown).

In addition, in our *E. coli* system depletion of iron (Fe) from the growth medium with the chelating agent *o*-phenanthroline (20 μM) resulted in failure to relieve NifL inhibition even under derepressing conditions, whereas supplementation of phenanthroline-containing medium with a small excess of iron reversed this effect (data not shown). These are the same as our recent findings in *K. pneumoniae* (59). These results demonstrate that no *K. pneumoniae* gene product(s)—*nif* specific or otherwise—is required to regulate NifL activity in response to cellular nitrogen or oxygen availability, and thus, regulation of NifL activity can be studied in *E. coli.*

NtrC is required for release of NifL inhibition under derepressing conditions. Since the regulation of NifL activity in *E. coli* appears to be the same as that in *K. pneumoniae* and the synthesis of the NifL and NifA proteins in our *E. coli* strains is independent of the Ntr system, we were able to study the effect of *ntrC* mutations on the regulation of NifL activity. An *ntrC* null allele (*ntrC10*::Tn*5*) was introduced by P1 transduction into a wild-type *E. coli* strain carrying the Φ(*nifH′-′lacZ*) fusion (see Materials and Methods), and as expected, the resulting strain (NCM1850) could not utilize arginine as the sole nitrogen source (Aut$^-$ phenotype). (Although the pathway for arginine catabolism has not been defined, growth on arginine as

[a] *glnG* and *glnL* in *E. coli* are referred to as *ntrC* and *ntrB*, respectively, in other organisms, and we employ the latter terminology in the text, figures, and Tables 2 to 7.
[b] For each transductional cross, we list the recipient and the donor.
[c] Called *glnL2302* by Ninfa et al. (49) and Atkinson and Ninfa (3).
[d] Selected for kanamycin resistance.
[e] Strain NCM1527 is NCM1529 carrying pJES851 (NifA), and NCM1538 is NCM1529 carrying pJES935 (ΔNifL NifA).
[f] Selected for tetracycline resistance.
[g] Strain NCM1688 is NCM1686 carrying pJES851.
[h] Selected for chloramphenicol resistance.
[i] Spontaneous suppressor selected on arginine as nitrogen source.
[j] Selected for glutamine prototrophy.
[k] Screened for glutamine auxotrophy.
[l] Screened for Aut$^-$.
[m] Screened for Aut$^-$; confirmed by phleomycin resistance.
[n] Strain NCM1852 is NCM1850 carrying pJES851.
[o] Screened for Aut^{++} (faster growth than the wild type on K medium with arginine as the nitrogen source).
[p] Screened for Aut^{++}.
[q] The presence of *glnD96* in strain SK3301 was confirmed by genetic analysis.

TABLE 2. Response of *K. pneumoniae* NifL protein to oxygen and ammonium in a wild-type strain of *E. coli*

Strain[a]	Plasmid	β-Galactosidase activity[b] ($U/ml/OD_{600}$)			
		Aerobic[c]		Anaerobic[c]	
		−N	+N	−N	+N
NCM1529	None	3	3	3	3
NCM1528	pNH3 (NifL NifA)	48	3	2,500	4
NCM1527	pJES851 (NifA)	5,118	5,332	5,342	5,586
NCM1538	pJES935 (ΔNifL NifA)	2,907	2,954	3,469	3,884

[a] All strains carry a single chromosomal copy of a Φ(*nifH'*-*'lacZ*) fusion at the *trp* locus.
[b] The slope of a plot of β-galactosidase activity versus the OD of the culture.
[c] Cultures were grown in K medium under aerobic or anaerobic conditions with 2 mM glutamine (−N) or 2 mM glutamine plus 4 mM NH_4Cl (+N) (see Materials and Methods).

the sole nitrogen source has been used as being diagnostic of the amount and activity of NtrC [33, 34, 40, 51].) Surprisingly, in the *ntrC* strain carrying the *nifLA* plasmid (NCM1851), the differential rate of β-galactosidase synthesis from the *nifH* promoter was only about 8 $U/ml/OD_{600}$ under derepressing conditions, which is more than 200-fold lower than that in the wild-type strain (Table 3). In the *ntrC* strain carrying only *nifA* (NCM1852) the differential rate of β-galactosidase synthesis remained high under all growth conditions (Table 3), similar to that in the wild-type strain (NCM1527) (Table 2). These results indicated that the poor expression from the *nifH* promoter in the *ntrC* mutant strain was apparently due to loss of the ability to release NifL inhibition under derepressing conditions. In addition, results in Table 3 indicate that strains carrying other *ntrC* null alleles—an *ntrBC* deletion mutation (NCM1847) or the *ntrC3* point mutation (NCM1866)—gave the same results as the strain carrying the *ntrC*::Tn*5* allele.

To determine whether the effect of NtrC on the regulation of NifL activity depended on the specific strain background, we tested NifL activity in another *E. coli* background containing a *glnA ntrBC* deletion mutation and a congenic wild-type strain (see Materials and Methods for strain construction). Expression from the *nifH* promoter in the *glnA ntrBC* deletion strain (strain NCM1799) (Table 3) was low. Therefore, the effect of NtrC on regulation of NifL activity is not dependent on strain background. Beyond controlling expression of the *nifLA* operon in response to the cellular nitrogen status, NtrC also appears to play a role in modulating NifL activity.

To confirm that the failure of *ntrC* mutant strains to express the Φ(*nifH'*-*'lacZ*) fusion under derepressing conditions was, in fact, due to NifL inhibition of NifA activity rather than to the absence of NifA protein or the presence of excess NifL protein, we compared the amounts of the Nif regulatory proteins in *ntrC* and congenic wild-type strains by Western blotting (see Materials and Methods). Under derepressing conditions and at our usual level of induction with 10 μM IPTG, the *ntrC* strain NCM1851 had about one-fourth as much NifL and NifA proteins as the wild-type strain NCM1528 (Fig. 2A, compare lanes 3 and 7 to lanes 1 and 5). When IPTG was omitted, both the wild-type strain and the *ntrC* strain had about one-fourth as much NifL and NifA proteins as when IPTG was present (Fig. 2A, lanes 2 and 6 for wild type and lanes 4 and 8 for *ntrC*). Thus, when IPTG was absent for the wild-type strain (lanes 2 and 6) but present for the *ntrC* strain (lanes 3 and 7), the two had approximately equal amounts of NifL and NifA. Since the differential rates of expression of β-galactosidase in the two strains under these conditions were 1,077 and 13 $U/ml/OD_{600}$, respectively (Fig. 2B), the lack of activity in the *ntrC* strain appears to be due to inhibitory effects of NifL on NifA activity.

NifL activity in strains with other *ntr* and *gln* mutations. Since we knew that NtrC is one component of the general Ntr system, we could predict that other mutations in the Ntr system which alter the amount of phosphorylated NtrC should also alter the ability to release NifL inhibition under derepressing conditions. To test this hypothesis, we examined NifL activity in a set of congenic strains carrying such mutations (see Materials and Methods) under anaerobic, nitrogen-limiting conditions. The results are summarized in Tables 4 to 6 and analyzed below.

(i) *ntrB* null allele. A strain carrying the *ntrB1* mutation was shown to lack the NtrB protein but not the NtrC protein (44), and hence the *ntrB1* mutation appears to be a nonpolar null allele. In a strain carrying the *ntrB1* allele, NifL remained inhibitory even under derepressing conditions (strain NCM1872) (Table 4), as was the case for strains carrying *ntrC* null alleles; expression of β-galactosidase from the *nifH* promoter was only 6 $U/ml/OD_{600}$. Because the *ntrB1* strain grows poorly on arginine as the sole nitrogen source, we infer that the phosphorylation level of NtrC in this strain may not be high enough to release NifL inhibition.

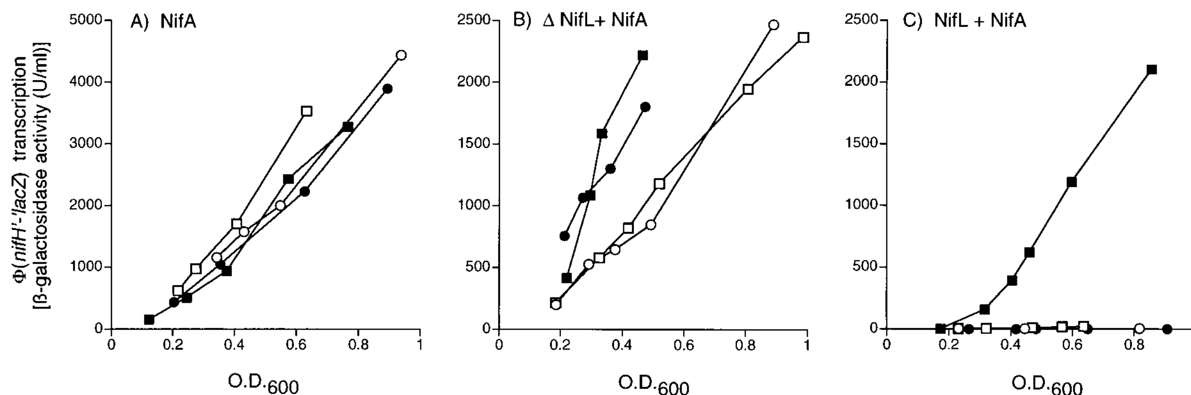

FIG. 1. Expression of a Φ(*nifH'*-*'lacZ*) fusion in wild-type *E. coli* strains. The activity of β-galactosidase was plotted as a function of OD_{600} for cultures grown in K medium under anaerobic (solid symbols) or aerobic (open symbols) conditions with 2 mM glutamine as the nitrogen source (squares) or with 2 mM glutamine plus 4 mM NH_4Cl (circles) (see Materials and Methods). All strains carried a single chromosomal copy of a Φ(*nifH'*-*'lacZ*) fusion at the *trp* locus. (A) Strain NCM1527 (NifA); (B) strain NCM1538 (ΔNifL NifA); (C) strain NCM1528 (NifL NifA).

TABLE 3. Effects of *ntrC* null alleles on NifL activity in *E. coli*

Strain[a]	Relevant genotype	Relevant phenotype	Plasmid	β-Galactosidase activity[b] (U/ml/OD_{600})	
				−N[c]	+N[c]
NCM1528	Wild type	Gln^+ Aut^+	pNH3 (NifL NifA)	2,123	3
NCM1851	*ntrC10*::Tn*5*	Gln^+ Aut^-	pNH3 (NifL NifA)	8	6
NCM1852	*ntrC10*::Tn*5*	Gln^+ Aut^-	pJES851 (NifA)	4,096	4,421
NCM1847	Δ*ntrBC*	Gln^+ Aut^-	pNH3 (NifL NifA)	3	4
NCM1866	*ntrC3*	Gln^+ Aut^-	pNH3 (NifL NifA)	3	3
NCM1803	Wild type	Gln^+ Aut^+	pNH3 (NifL NifA)	3,050	4
NCM1799	Δ(*ntrC?-ntrB-glnA*)*229*	Gln^- Aut^-	pNH3 (NifL NifA)	51	4

[a] All strains carry a single chromosomal copy of a Φ(*nifH'-'lacZ*) fusion at the *trp* locus. Strains NCM1851, NCM1852, NCM1847, and NCM1866 are congenic with wild-type strain NCM1528, whereas strain NCM1799 is congenic with wild-type strain NCM1803.
[b] The slope of a plot of β-galactosidase activity versus the OD of the culture.
[c] Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine (−N) or 2 mM glutamine plus 4 mM NH_4Cl (+N) (see Materials and Methods).

(ii) *glnD* "null" allele. The *glnD99*::Tn*10* mutation is one of the original, defining mutations for *glnD* in *E. coli* (6, 9). This mutation reduces uridylylation of P_{II} by 90% (62) and, as a consequence, lowers the amounts of phosphorylated NtrC. Strains carrying this allele are glutamine bradytrophs on enriched medium and fail to grow on arginine as a nitrogen source (Aut^- phenotype). A strain carrying the *glnD99*::Tn*10* allele and the *nifLA* plasmid (NCM1687) showed greatly reduced transcription from the *nifH* promoter relative to the congenic $glnD^+$ strain (NCM1528): the differential rate of expression of β-galactosidase in strain NCM1687 was only 173 U/ml/OD_{600} under derepressing conditions (Table 4). By contrast, when the NifL protein was absent, NifA activity itself was not decreased in the *glnD* mutant strain (NCM1688). The results indicated that, contrary to the conclusion reached by Edwards and Merrick (20), the product of *glnD* (UT/UR), though apparently not essential for relief of NifL inhibition, does play a role in the regulation of NifL activity (at least indirectly). Strains carrying both the *ntrC*::Tn*5* and *glnD99*::Tn*10* alleles decreased the differential rate of β-galactosidase synthesis to the level characteristic of the *ntrC*::Tn*5* mutation (Table 4). These results indicated that an *ntrC* null allele was epistatic to the *glnD* null allele and, hence, left open the possibility that effects of UT/UR on NifL activity might be accounted for entirely by indirect effects on the amount of phosphorylated NtrC (but see Discussion).

(iii) *glnD* suppressors. Previous reports showed that phenotypes caused by *glnD* mutations could be suppressed by other mutations in *ntr* and *gln* genes (3, 5, 9). We further investigated the regulation of NifL activity in strains with different suppressors of the *glnD99*::Tn*10* allele.

(a) *ntrB302 glnD99*::Tn*10* strain. The *ntrB302* allele encodes an altered NtrB protein whose activity is insensitive to P_{II} (49). Strains carrying both the *ntrB302* and *glnD99*::Tn*10* alleles (NCM1933 and NCM1935) were Gln^+ and Aut^+, although their colony sizes on both NB and M9-Arg plates were smaller than that of either a congenic wild-type strain or a strain carrying the *ntrB302* mutation alone (NCM1857). Under derepressing conditions, β-galactosidase activity in the *ntrB302 glnD* double mutant strain carrying the *nifLA* plasmid (NCM1935) was back up to a level similar to that in a wild-type strain or in strains carrying the *ntrB302* mutation alone (NCM1857) (Table 4). This indicated that the *ntrB302* mutation suppressed the effect of the *glnD* mutation and allowed release of NifL inhibition under derepressing conditions. It is interesting that NifL inhibition was also partially relieved under repressing conditions in the double mutant strain (Table 4).

(b) (Δ*glnB*)::Cam^r *glnD99*::Tn*10* strain. As found by Bueno et al. (9), a *glnB* deletion mutation is also able to suppress the glutamine requirement of strains carrying the *glnD99*::Tn*10* mutation on NB medium. A strain carrying a *glnB* deletion-insertion mutation [(Δ*glnB*)::Cam^r], was constructed (see Ma-

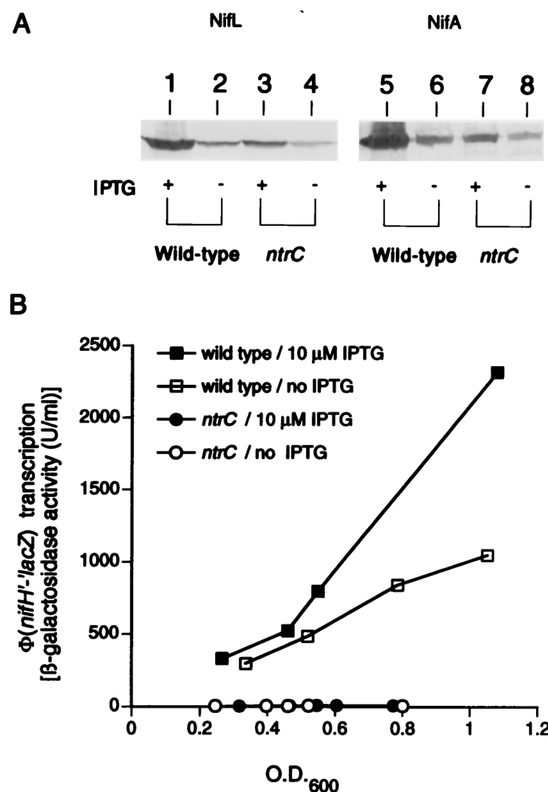

FIG. 2. Amounts of NifL and NifA in wild-type and *ntrC* strains of *E. coli* (A) and expression of a Φ(*nifH'-'lacZ*) fusion in these strains (B). Cultures were grown in K medium under derepressing conditions (see Materials and Methods). All strains carried a single chromosomal copy of a Φ(*nifH'-'lacZ*) fusion at the *trp* locus and plasmid pNH3 encoding NifL and NifA under the control of the *tac* promoter. Expression of NifL and NifA was induced with 10 µM IPTG (plus symbols [A]) or was not induced (minus symbols [A]) in wild-type strain NCM1528 (squares [B]) or a strain carrying an *ntrC* null allele, NCM1851 (circles [B]). (A) Amounts of NifL and NifA were determined by Western blotting as described in Materials and Methods. Only the relevant bands are shown. (B) The activity of β-galactosidase was plotted as a function of OD_{600} of the cultures.

TABLE 4. Effects of *ntrB* and *glnD* mutations on NifL activity in *E. coli*

Strain[a]	Relevant genotype	Relevant phenotype	Plasmids	β-Galactosidase activity[b] ($U/ml/OD_{600}$)	
				−N[c]	+N[c]
NCM1528	Wild type	Gln^+ Aut^+	pNH3 (NifL NifA)	2,123	3
NCM1872	*ntrB1*	Gln^+ Aut^-	pNH3 (NifL NifA)	6	6
NCM1687	*glnD99*::Tn*10*	Gln^{-d} Aut^-	pNH3 (NifL NifA)	173	13
NCM1688	*glnD99*::Tn*10*	Gln^- Aut^-	pJES851 (NifA)	6,257	8,524
NCM1934	*glnD99*::Tn*10 ntrC10*::Tn*5*	Gln^- Aut^-	pNH3 (NifL NifA)	6	16
NCM1857	*ntrB302*	Gln^+ Aut^+	pNH3 (NifL NifA)	1,264[e]	55
NCM1933	*glnD99*::Tn*10 ntrB302*	Gln^+ Aut^{+f}	None	3	3
NCM1935	*glnD99*::Tn*10 ntrB302*	Gln^+ Aut^{+f}	pNH3 (NifL NifA)	1,745	246

[a] All strains carry a single chromosomal copy of a Φ(*nifH'*-*'lacZ*) fusion at the *trp* locus.
[b] The slope of a plot of β-galactosidase activity versus the OD of the culture.
[c] Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine (−N) or 2 mM glutamine plus 4 mM NH_4Cl (+N) (see Materials and Methods).
[d] Glutamine bradytroph on enriched medium.
[e] Expression was delayed until the end of exponential phase.
[f] Colony size smaller than that of the wild type.

terials and Methods), and NifL activity was examined in a strain carrying this mutation alone or in combination with the *glnD99*::Tn*10* allele (Table 5). NifL activity was regulated well in the *glnB* single mutant strain (NCM1737), which indicated that the P_{II} protein was not required to regulate NifL activity in response to nitrogen status. The *glnB* mutation only partially suppressed the effect of *glnD* on NifL inhibition: expression of β-galactosidase from the *nifH* promoter was approximately 315 $U/ml/OD_{600}$ (strain NCM1740), which was much less than that of the wild-type strain under derepressing conditions, although greater than that of the *glnD* strain. Because the *glnB glnD* double mutant strain remained Aut^-, as has been observed previously (9), the amount of phosphorylated NtrC may not be high enough to relieve NifL inhibition completely (but see Discussion).

(c) Spontaneous suppressors of *glnD99*::Tn*10*. From results in Tables 4 and 5 we can see that there is a correlation between arginine utilization and regulation of NifL activity: mutant strains that are Aut^- (i.e., *ntrB1* or *glnD* or *glnB glnD* double mutant strains) cannot relieve NifL inhibition even under derepressing conditions, whereas a number of strains that were Aut^+ (i.e., wild-type, *glnB*, and *ntrB302 glnD* strains) could relieve NifL inhibition. So we selected Aut^+ suppressors from a strain carrying the *glnD99*::Tn*10* allele (see Materials and Methods) and tested NifL activity in these suppressors. Some of the suppressor mutations resulted in restoration of *nifH* expression in the *glnD99*::Tn*10* background in the presence of NifL and NifA (Table 6), which indicated that NifL inhibition could be relieved in these strains under derepressing conditions. Those that were tested also resulted in elevated glutamine synthetase activity, i.e., increased expression of the *glnA* gene, which is under the control of phosphorylated NtrC. (One suppressor mutation, *ntr-4*, was linked to the Tn*10* element [see Materials and Methods], whereas the locations of the others have not been determined.) Interestingly, two of the strains carrying suppressor mutations (NCM1779 and NCM1782) also relieved NifL inhibition in the presence of ammonium (Table 6) but not in the presence of oxygen (not shown). Although these strains may carry more than one mutation (see Materials and Methods), their properties indicated that UT/UR controls NifL activity in response to combined nitrogen but not molecular oxygen.

The transcriptional activation capacity of NtrC is needed for relief of NifL inhibition. By virtue of its DNA-binding properties, NtrC can act as a negative regulator to repress transcription by σ^{70} holoenzyme, as well as an activator of σ^{54} holoenzyme. To confirm that transcriptional activation by NtrC was needed to relieve NifL inhibition under derepressing conditions, we studied the effect of two special mutant forms of NtrC-$NtrC^{repressor}$ forms, which specifically fail in activating transcription by σ^{54}-holoenzyme but retain the ability to repress transcription by σ^{70}-holoenzyme (50), and $NtrC^{constitutive}$ forms, which can activate transcription from the *glnA* promoter in vitro without being phosphorylated (23). Because we have studied the properties of such mutant forms of NtrC in *S. typhimurium*, we examined their effects on the regulation of NifL activity in *S. typhimurium* strains carrying a single copy of the *K. pneumoniae* Φ(*nifH'*-*'lacZ*) fusion on the chromosome and

TABLE 5. Effect of a *glnB* mutation on NifL activity in *E. coli*

Strain[a]	Relevant genotype	Relevant phenotype	Plasmid	β-Galactosidase activity[b] ($U/ml/OD_{600}$)	
				−N[c]	+N[c]
NCM1528	Wild type	Gln^+ Aut^+	pNH3 (NifL NifA)	3,266	1
NCM1737	(Δ*glnB*)::Cam^r	Gln^+ Aut^+	pNH3 (NifL NifA)	3,504	6
NCM1740	*glnD99*::Tn*10* (Δ*glnB*)::Cam^r	Gln^+ Aut^-	pNH3 (NifA NifA)	315	12
NCM1687	*glnD99*::Tn*10*	Gln^{-d} Aut^-	pNH3 (NifL NifA)	70	11

[a] All strains carry a single chromosomal copy of a Φ(*nifH'*-*'lacZ*) fusion at the *trp* locus.
[b] The slope of a plot of β-galactosidase activity versus the OD of the culture.
[c] Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine (−N) or 2 mM glutamine plus 4 mM NH_4Cl (+N) (see Materials and Methods).
[d] Glutamine bradytroph on enriched medium.

TABLE 6. Expression of a Φ(*nifH'*-*'lacZ*) fusion and *glnA* in *E. coli* strains carrying spontaneous suppressors of the *glnD99*::Tn*10* allele

Strain[a]	Relevant genotype	Relevant phenotype[b]	β-Galactosidase activity[c] (U/ml/OD_{600})		Glutamine synthetase activity[d] (μmol/min/mg of protein)		
			−N[e]	+N[e]	−N[f]	+N[f]	NBgln[g]
NCM1528	Wild type	Gln^+ Aut^+	2,600	2	1.2	0.13	0.1
NCM1687	*glnD99*::Tn*10*	Gln^{-h} Aut^-	140	3	0.4	0.06	0.08
NCM1779	*glnD99*::Tn*10 ntr-1*	Gln^+ Aut^{++}	2,900	1,840	1.3	ND[i]	1.4
NCM1782	*glnD99*::Tn*10 ntr-2*	Gln^+ Aut^{++}	1,900	2,000	1.3	ND	0.9
NCM1829	*glnD99*::Tn*10 ntr-3*	Gln^+ Aut^+	1,730	60	1.2	0.45	0.2
NCM1898	*glnD99*::Tn*10 ntr-4*	Gln^+ Aut^+	2,300	2	ND	ND	ND
NCM1901	*glnD99*::Tn*10 ntr-4* in E1529	Gln^+ Aut^+	2,300	3	ND	ND	ND

[a] All strains carry a single chromosomal copy of a Φ(*nifH'*-*'lacZ*) fusion at the *trp* locus and plasmid pNH3 (NifL NifA).
[b] See Materials and Methods.
[c] The slope of a plot of β-galactosidase activity versus the OD of the culture.
[d] Specific activity determined in cell extracts (see Materials and Methods).
[e] Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine (−N) or 2 mM glutamine plus 4 mM NH_4Cl (+N) (see Materials and Methods).
[f] Cultures were grown in M9 medium under aerobic conditions with 3 mM glutamine (−N) or 3 mM glutamine plus 10 mM NH_4Cl (+N).
[g] NB plus 1 mM glutamine.
[h] Glutamine bradytroph on enriched medium (NB).
[i] ND, not determined.

the *nifLA* plasmid (see Materials and Methods for construction of strains). The results (Table 7) are summarized as follows.

First, NifL activity is regulated well in response to nitrogen and molecular oxygen in our wild-type strain of *S. typhimurium* (SK3236) (see Materials and Methods): expression of β-galactosidase from the *nifH* promoter was approximately 800 U/ml/OD_{600} under derepressing conditions and decreased to only 9 U/ml/OD_{600} under repressing conditions (Table 7). As was true in *E. coli*, mutations in *S. typhimurium* that eliminated NtrC, such as the *ntrB137*::Tn*10* or *ntrC352*::Tn*10* alleles (43), or mutations that reduced the amount of phosphorylated NtrC, such as the *glnD96* (5) and *ntrB509* (23) mutations, mimicked repressing conditions in causing a great decrease of β-galactosidase expression under derepressing conditions (strains SK3248, 3251, 3254, and 3319, respectively) (Table 7). These results provided further evidence that the general Ntr system is indeed involved in controlling NifL activity in response to nitrogen status.

Second, strains carrying $NtrC^{repressor}$ forms could not relieve NifL inhibition under derepressing conditions. The *ntrC74* allele encodes a form of NtrC with a single amino acid substitution, $NtrC^{A216V}$ (50). When it is phosphorylated, the $NtrC^{A216V}$ protein has as much ATPase activity as phosphorylated wild-type NtrC and it binds well to enhancers. However, it fails to activate transcription by σ^{54}-holoenzyme. Expression of β-galactosidase in a strain carrying the *ntrC74* mutation and the *nifLA* plasmid (SK3282) was 60 U/ml/OD_{600} under derepressing conditions, which is 10-fold lower than that in a wild-type strain (Table 7). Thus NifL inhibition was not relieved even by a form of NtrC that binds normally to DNA, is phosphorylated normally, and has a normal capacity to hydrolyze ATP. To relieve the inhibition of NifL under derepressing conditions requires that NtrC be capable of transcriptional activation.

Third, strains carrying $NtrC^{constitutive}$ forms could relieve NifL inhibition even in the absence of NtrB or GlnD (UT/UR). The *ntrC599* allele encodes $NtrC^{S160F}$, which can activate transcription in vitro without being phosphorylated (32, 52). A strain carrying *ntrC599* and the *ntrB509* allele, a null allele in *ntrB* that is semipolar on *ntrC* (23), could relieve NifL inhibition under derepressing conditions (Table 7): in a strain carrying the *nifLA* plasmid (SK3278), expression of β-galactosi-

TABLE 7. Effects of *ntr* and *gln* mutations on NifL activity in *S. typhimurium*

Strain[a]	Relevant genotype	Relevant phenotype	Plasmid	β-Galactosidase activity[b] (U/ml/OD_{600})	
				−N[c]	+N[c]
SK3166	Wild type	Gln^+ Aut^+	None	7	2
SK3236	Wild type	Gln^+ Aut^+	pNH3 (NifL NifA)	800	9
SK3248	*ntrB137*::Tn*10*	Gln^+ Aut^-	pNH3 (NifL NifA)	13	8
SK3251	*ntrC352*::Tn*10*	Gln^+ Aut^-	pNH3 (NifL NifA)	26	8
SK3254	*glnD96*	Gln^{-d} Aut^-	pNH3 (NifL NifA)	24	20
SK3282	*ntrC74* ($NtrC^{A216V}$)	Gln^- Aut^-	pNH3 (NifL NifA)	60	22
SK3319	*ntrB509*	Gln^+ Aut^-	pNH3 (NifL NifA)	28	10
SK3278	*ntrB509 ntrC599* ($NtrC^{S160F}$)	Gln^+ Aut^{++}	None	2	4
SK3279	*ntrB509 ntrC599*	Gln^+ Aut^{++}	pNH3 (NifL NifA)	830[e]	15
SK3303	*ntrB509 ntrC599 glnD96*	Gln^+ Aut^+	pNH3 (NifL NifA)	690[e]	20

[a] All strains carry a single chromosomal copy of a Φ(*nifH'*-*'lacZ*) fusion at the *put* locus.
[b] The slope of a plot of β-galactosidase activity versus the OD of the culture.
[c] Cultures were grown in K medium under anaerobic conditions with 2 mM glutamine (−N) or 2 mM glutamine plus 4 mM NH_4Cl (+N) (see Materials and Methods).
[d] Glutamine bradytroph on enriched medium.
[e] Expression was delayed until the end of exponential growth.

dase from the *nifH* promoter was 800 U/ml/OD_{600}, which is similar to that from a wild-type strain. Thus, NtrB is not required for relief of NifL inhibition under derepressing conditions if NtrC is transcriptionally active in its absence. Furthermore, the combination of the *ntrB509* and *ntrC599* alleles suppressed the effects of a *glnD* mutation on regulation of NifL activity. When the *glnD96* allele was moved into the strain carrying *ntrB509* and *ntrC599*, NifL inhibition was relieved under derepressing conditions, suggesting that UT catalytic activity might not be needed directly for relief of NifL inhibition when NtrC is transcriptionally active (but see Discussion).

DISCUSSION

The existence of the *nifL* gene was first proposed by Kennedy 20 years ago on the basis of transductional mapping of a point mutation, *nifL*2265 (31). Subsequently, NifL was demonstrated to be a negative regulator of *nif*-specific gene expression in response to ammonium or oxygen (28, 45). Although NifL is now known to be an FAD-containing protein (27, 58), the details of how molecular oxygen and, in particular, combined nitrogen are sensed remain a mystery. To further elucidate this mechanism we examined the activity of constitutively expressed NifL protein in *E. coli* and *S. typhimurium* strains carrying a single chromosomal copy of a *K. pneumoniae* Φ(*nifH′*-*′lacZ*) translational fusion. We confirmed that expression of the *nifLA* genes from the *tac* promoter is not dependent on NtrC (Fig. 2A) (26) and that, in the absence of NifL, NifA itself does not respond to changes in growth conditions (Fig. 1 and Table 2) (7). Decreased expression of *nifH* in a wild-type strain carrying both NifA and NifL in the presence of combined nitrogen and/or molecular oxygen is due to the inhibitory effect of NifL on NifA. In contrast to the conclusion of Henderson et al. (26), we demonstrated that wild-type strains of *E. coli* and *S. typhimurium* can relieve NifL inhibition when both combined nitrogen and molecular oxygen are limiting (Fig. 1 and 2 and Tables 2 and 7). Further, depletion of iron from the medium resulted in failure to relieve NifL inhibition even under these otherwise derepressing conditions, as was true in *K. pneumoniae* (59). Thus, the activity of overexpressed NifL is regulated efficiently in both *E. coli* and *S. typhimurium*, and hence, this regulation does not require a *nif*- or *K. pneumoniae*-specific product(s). The latter is commensurate with previous findings that the *nif* genes of *K. pneumoniae* are expressed in other enteric bacteria carrying only the *nif* cluster from *Klebsiella* (10, 18, 53).

Surprisingly, in both *E. coli* and *S. typhimurium* we found that the ability to relieve NifL inhibition under derepressing conditions requires the transcriptional activator NtrC. Thus, both the synthesis and the activity of NifL are controlled by NtrC in response to nitrogen availability. Moreover, failure of an $NtrC^{repressor}$ form of NtrC to relieve NifL inhibition indicated that it is, in fact, the transcriptional activation capacity of NtrC rather than its repressor activity that is needed. Our working hypothesis is that NtrC is needed to activate transcription of one or more genes whose products are required for relief of NifL inhibition under nitrogen-limiting conditions.

Effects of *ntr* and *gln* mutations indicated that there was a correlation between failure to relieve NifL inhibition and failure to grow on arginine as the sole nitrogen source. Although the pathway for arginine catabolism has not been defined, growth on arginine as the sole nitrogen source is known to require large amounts of phosphorylated NtrC (33, 34, 40, 51). The requirement for NtrB to relieve NifL inhibition is probably accounted for on the basis of its role in phosphorylating NtrC: the negative alleles of *ntrB* we employed are known to be null alleles (44), and their effects can be bypassed by $NtrC^{constitutive}$ proteins, which are known to be active in vivo in the absence of NtrB (23, 32). By contrast, it is not clear whether the requirement for GlnD (UT activity) to relieve NifL inhibition can be accounted for solely by its roles in synthesis of phosphorylated NtrC: although effects of negative alleles in *glnD* can be bypassed by alteration of function alleles in *ntrB* or *ntrC*, the *glnD* alleles we employed are not known to be null alleles, and in fact, the Tn*10* insertion allele that we employed in *E. coli* is known to have residual UT activity (62). (The insertion lies in the 3′ end of the gene, whereas it is the 5′ end that encodes UT [48].) In addition to being required for synthesis of large amounts of phosphorylated NtrC, GlnD may also be required for covalent modification (uridylylation) of a product more directly involved in relief of NifL inhibition (e.g., the *glnK* product, which is under NtrC control; see below). By virtue of its roles in controlling the amount of phosphorylated NtrC, GlnD is clearly involved in relief of NifL inhibition, and the possibility remains that it is essential.

In agreement with results of Holtel and Merrick (29), our results showed that the P_{II} protein, a substrate of UT/UR, is not required for regulation of NifL activity in response to nitrogen status. Recently, however, the existence of two different P_{II} proteins (paralogs), encoded by two different structural genes, has been documented in several organisms, including *E. coli* (1, 65, 67), *K. pneumoniae*, and *Azospirillum brasilense* (17). In *E. coli*, the *glnK* gene is known to be under NtrC control and the GlnK protein was detected only under nitrogen-limiting growth conditions (65). Hence, GlnK is a logical candidate for an NtrC-controlled protein required to relieve NifL inhibition. Interestingly, in *A. brasilense*, one of the P_{II} proteins, which is synthesized at a high level under nitrogen-limiting conditions, is involved in regulation of NifA activity (2, 16, 37). Studies of a *glnK* gene disruption in *E. coli* and its effect on NifL activity are ongoing in our laboratory.

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