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Iron Is Required To Relieve Inhibitory Effects of NifL on Transcriptional Activation by NifA in *Klebsiella pneumoniae*

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In *Klebsiella pneumoniae*, products of the nitrogen fixation *nifLA* operon regulate transcription of the other *nif* operons. NifA activates transcription by σ^{54}-holoenzyme. In vivo, NifL antagonizes the action of NifA under aerobic conditions or in the presence of combined nitrogen. In contrast to a previous report, we show that depletion of iron (Fe) from the growth medium with the chelating agent *o*-phenanthroline (20 μM) mimics aerobiosis or combined nitrogen in giving rise to inhibition of NifA activity even under anaerobic, nitrogen-limiting conditions. Adding back Fe in only twofold molar excess over phenanthroline restores NifA activity, whereas adding other metals fails to do so. By using strains that lack NifL, we showed that NifA activity itself does not require Fe and is not directly affected by phenanthroline. Hence, Fe is required to relieve the inhibition of NifA activity by NifL in vivo. Despite the Fe requirement in vivo, we have found no evidence that NifL contains Fe or an iron-sulfur (Fe-S) cluster. Determination of the molecular mass of an inhibitory form of NifL overproduced under aerobic conditions indicated that it was not posttranslationally modified. When NifL was synthesized in vitro, it inhibited transcriptional activation by NifA even when it was synthesized under anaerobic conditions in the presence of a high Fe concentration or of superoxide dismutase, which is known to protect some Fe-S clusters. Moreover, overproduction of superoxide dismutase in vivo did not relieve NifL inhibition under aerobic conditions, and attempts to relieve NifL inhibition in vitro by reconstituting Fe-S clusters with the NifS enzyme (*Azotobacter vinelandii*) were unsuccessful. Since we obtained no evidence that Fe acts directly on NifL or NifA, we postulate that an additional Fe-containing protein, not yet identified, may be required to relieve NifL inhibition under anaerobic, nitrogen-limiting conditions.

In the free-living diazotroph *Klebsiella pneumoniae*, expression of nitrogen fixation (*nif*) genes is regulated by the products of the *nifLA* operon (3, 8, 14, 32). NifA activates transcription of all *nif* genes (except *nifLA*) by the alternative holoenzyme form of RNA polymerase σ^{54}-holoenzyme. NifA binds to an upstream activation sequence (34) and contacts promoter-bound σ^{54}-holoenzyme by means of a DNA loop (9). It catalyzes the ATP-dependent isomerization of closed complexes between σ^{54}-holoenzyme and a *nif* promoter to transcriptionally productive open complexes (25, 35). NifL, which is a negative regulator, inhibits NifA activity in vivo in response to molecular oxygen and/or combined nitrogen (22, 32).

NifL from *K. pneumoniae* is composed of two domains separated by a hydrophilic interdomain linker (Q-linker [12]). We showed recently that the C-terminal domain is sufficient to inhibit transcriptional activation by NifA in vitro and in vivo (37). Thus, the inhibitory function of NifL appears to lie in its C-terminal domain, which presumably interacts with NifA. The N-terminal domain of NifL contains a region of homology (~30% amino acid identity over 130 residues) to the product of the *bat* gene from *Halobacterium halobium* (19, 47, 50). Since Bat is an oxygen-responsive activator of the synthesis of bacterio-opsin, it has been proposed that the region of homology may be involved in oxygen sensing. The mechanism(s) by which NifL senses oxygen and/or combined nitrogen is not understood.

The N-terminal domain of NifL contains one CysXXCys motif (Cys-184–Ala–Asp–Cys-187). The similarity of this motif to sequences involved in binding metal clusters in proteins such as ferrodoxins and rubredoxins (1, 4, 24) suggested a possible metal-binding role of this region in NifL (21). On the basis of this prediction, Henderson et al. (21) examined the influence of metal ion deficiency on NifL activity. They concluded that iron (Fe) deficiency reduced the inhibitory activity of NifL expressed at high levels in *Escherichia coli.*

To further study the function of a possible metal ion or iron-sulfur (Fe-S) cluster in oxygen-sensing by NifL, we examined the effect of Fe deficiency on NifL inhibition of NifA activity in vivo in a strain of *K. pneumoniae* carrying a chromosomal *nifK-lacZ* fusion and a single chromosomal copy of *nifLA*. Determination of the differential rates of β-galactosidase synthesis in this strain showed that the effect of Fe deficiency was opposite that reported by Henderson et al. (21); namely, Fe is required to relieve the inhibition of NifA activity by NifL under anaerobic, nitrogen-limiting conditions.

We have previously shown that NifL overproduced under aerobic conditions in *E. coli* inhibits NifA activity in a purified transcription system even in the absence of oxygen and ammonium or amino acids (28). We now find that the inhibitory form of NifL is not posttranslationally modified and that NifL synthesized anaerobically in a coupled transcription-translation system is also inhibitory. Attempts to relieve inhibition with Fe or by formation or protection of Fe-S clusters were unsuccessful, and thus we have no evidence that the Fe required to relieve the inhibitory effect of NifL in vivo is present in NifL itself.

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MATERIALS AND METHODS

Plasmids and bacterial strains. Plasmid pJES839 was constructed by inserting a tetracycline resistance cassette (11) into the *Hin*dIII site of plasmid pNH3 (21), which carries *nifLA* from *K. pneumoniae* under control of the *tac* promoter.

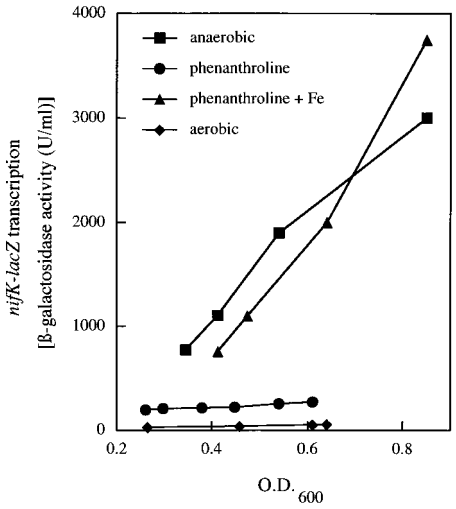

FIG. 1. The iron chelator *o*-phenanthroline prevents NifA-mediated activation of transcription in *K. pneumoniae* carrying a single chromosomal copy of *nifLA*. The activity of β-galactosidase was plotted as a function of OD_{600} for cultures of strain UN4495, which carries a chromosomal *nifK-lacZ* fusion and a single chromosomal copy of *nifLA*. Differential rates of transcription from the *nifHDK* promoter (see text), which reflect NifA activity, were determined from the slopes of these plots. All cultures were grown at 30°C in minimal medium containing glutamine (2 mM) as the sole nitrogen source. Cultures were grown anaerobically in medium containing 20 μM $FeCl_3$ (squares), anaerobically in medium lacking $FeCl_3$ and containing 20 μM *o*-phenanthroline (circles), anaerobically in medium containing 20 μM *o*-phenanthroline and 40 μM $FeCl_3$ (triangles), and aerobically in medium containing 20 μM $FeCl_3$ (diamonds).

Plasmid pJES939, which carries *nifA* from *K. pneumoniae* under the control of the *tac* promoter, was constructed as follows. (i) Plasmid pJES839 was digested with *Eco*RI and *Hin*dIII; (ii) the 5.2-kb vector fragment was isolated and religated to the 2.1-kb *Hin*dIII-*Eco*RI fragment of pJES293 (45), which contains the last 68 bp of *nifL* and a complete *nifA* gene; and (iii) the tetracycline resistance cassette described above was transferred to the resulting plasmid as indicated. Plasmids pJES283 and pJES294 carry *nifL* and *nifA*, respectively (28); each is under control of a strong T7 promoter and translational start site. Plasmid pDT1-22, which carries *sodA* from *E. coli* under control of the *tac* promoter, $lacI^q$, and a gene conferring chloramphenicol resistance, was a gift from Daniele Touati (49).

K. pneumoniae UN4495 [Φ(*nifK-lacZ*)*5935* Δ*lac-4001 his D4226* Gal^r] (30) was provided by Gary Roberts. Plasmids pJES839 and pJES939 were transformed into *K. pneumoniae* UN4495 by electroporation and selection for tetracycline resistance (10 μg/ml).

UN4495 Δ*nifLA* {=UN5476 [Φ(*nifK-lacZ*)*5935* Δ(*nifLA*)*6293*::Km Δ*lac-4001 hisD4226* Gal^r]}, which carries a chromosomal deletion of *nifLA* was constructed from UN4495 in the following steps. First, a 6-kb *nif′FLABQ Eco*RI-*Hin*dIII fragment from pDO503 (38) was cloned into the corresponding sites of the pUC19 vector to produce pJES869. The *Eco*RV fragment containing most of *nifLA* was then replaced by a 2.1-kb *Hin*dIII cassette (made blunt ended with the Klenow fragment of DNA polymerase I) containing an Ω interposon fragment with a kanamycin resistance gene to yield plasmid pJES875. (The 2.1-kb *Hin*dIII cassette was derived from plasmid pHP45Ω-Km [13]. The Ω fragment is flanked in inverted orientation by transcriptional and translational termination signals [41].) Plasmid pJES875 carries a deletion of *nifLA* that encodes only the first 113 amino acids of the N-terminal domain of NifL and the last 6 amino acids of the C terminus of NifA (2). Finally, the deletion in plasmid pJES875 was transferred to the chromosome of *K. pneumoniae* UN4495. To effect transfer, the *Eco*RI-*Hin*dIII fragment containing *nifL′*::Km^r-*′nifABQ* was isolated and electroporated into *K. pneumoniae* UN4495, and recombinants (generated by means of a double crossover) were selected for kanamycin resistance. Unlike the parental strain, three of three kanamycin-resistant colonies did not turn blue on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates under anaerobic, nitrogen-limiting conditions. That *nifLA* had been deleted by homologous recombination was confirmed by Western blot (immunoblot) analysis, which showed that both the NifL and NifA proteins were undetectable in cells grown under anaerobic, nitrogen-limiting conditions.

Determination of the mass of NifL by electrospray mass spectrometry. NifL was purified from a 200-ml culture of *E. coli* NCM632 (27) carrying plasmid pJES283. The culture was grown aerobically at 30°C in maximal induction medium (36). After disruption of cells and centrifugation at 20,000 × *g*, NifL was purified from the pellet as follows. The pellet was suspended in 30 ml of breakage buffer [20 mM potassium *N*-(2-hydroxyethyl)piperazine-*N′*-3-propanesulfonic acid (pH 8.0), 125 mM potassium glutamate, 5% (vol/vol) glycerol, 1.5 mM dithiothreitol (DTT)] (25) containing EDTA (1 mM), lysozyme (6.3 mg), and methionine (100 μM). After incubation at room temperature for 30 min, the suspension was subjected to centrifugation at 20,000 × *g* for 30 min. The resulting pellet was again suspended in 30 ml of breakage buffer containing EDTA, lysozyme, and methionine and again incubated at room temperature for 30 min. After centrifugation at 20,000 × *g* for 30 min, the pellet was suspended in 30 ml of water containing methionine (100 μM) and washed three times with water containing methionine to remove salts. After the last centrifugation, the pellet was extracted with a 1:1 mixture of acetonitrile and water, both containing 0.2% trifluoroacetic acid, and stirred overnight at room temperature. The extracted

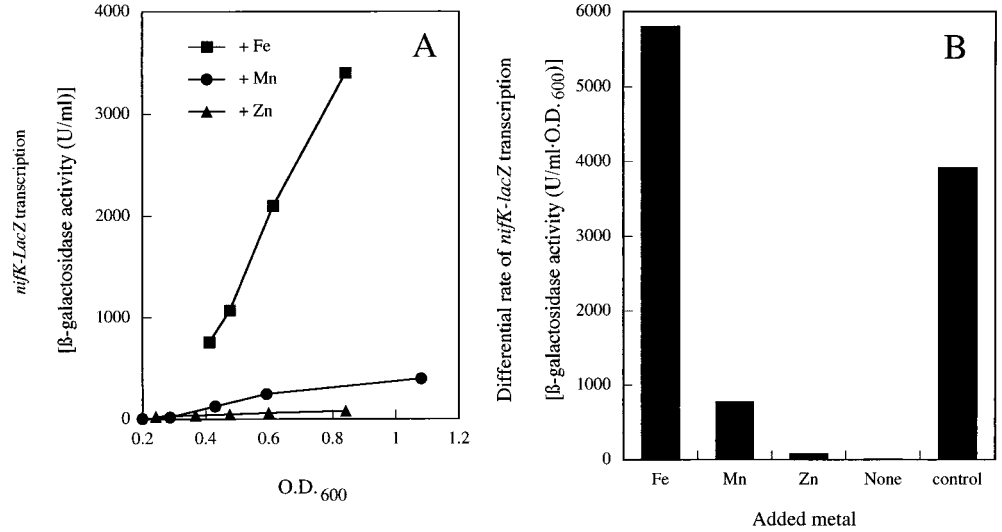

FIG. 2. Inhibitory effects of *o*-phenanthroline on NifA-mediated activation of transcription are reversed by iron. The activity of β-galactosidase was plotted as a function of OD_{600} for *K. pneumoniae* UN4495, which carries a chromosomal *nifK-lacZ* fusion and a single chromosomal copy of *nifLA* (A). Differential rates of transcription from the *nifHDK* promoter (B) were determined from the slopes of these plots. All cultures were grown anaerobically at 30°C in minimal medium with glutamine (2 mM) as the sole nitrogen source. Cultures contained no phenanthroline (control; not shown in panel A), 20 μM *o*-phenanthroline and no added Fe (none; not shown in panel A), 20 μM *o*-phenanthroline and 40 μM $FeCl_3$ (Fe), 20 μM *o*-phenanthroline and 40 μM $MnSO_4$ (Mn), and 20 μM *o*-phenanthroline and 40 μM $ZnCl_2$ (Zn).

TABLE 1. The iron chelator *o*-phenanthroline inhibits NifA-mediated activation of transcription in a *K. pneumoniae* strain that overproduces NifL and NifA from a plasmid[a]

Growth conditions	β-Galactosidase activity (U/ml/OD_{600})	Doubling time (h)
0.0 μM IPTG		
Aerobic	23	2.0
Anaerobic	200	4.2
Anaerobic + phenanthroline	30	5.0
Anaerobic + phenanthroline + $FeCl_3$	200	4.2
0.1 μM IPTG		
Aerobic	21	2.0
Anaerobic	232	4.8
Anaerobic + phenanthroline	ND	4.8
Anaerobic + phenanthroline + $FeCl_3$	ND	4.8
1.0 μM IPTG		
Aerobic	27	2.0
Anaerobic	680	5.0
Anaerobic + phenanthroline	64	5.0
Anaerobic + phenanthroline + $FeCl_3$	820	5.0
10.0 μM IPTG		
Aerobic	20	2.0
Anaerobic	2,134	4.8
Anaerobic + phenanthroline	250	7.5
Anaerobic + phenanthroline + $FeCl_3$	1,725	4.8

[a] Differential rates of transcription from the *nifHKD* promoter were determined in strain UN4495/pJES839. This strain carries a chromosomal *nifK-lacZ* fusion and both a chromosomal copy of *nifLA* and a plasmid encoding NifL and NifA under the control of the *tac* promoter (pJES839). Cells were grown aerobically or anaerobically, as indicated, in minimal medium with 2 mM glutamine as the nitrogen source in the presence of different concentrations of IPTG. When *o*-phenanthroline was added, it was at 20 μM, and iron was omitted from the medium. When both *o*-phenanthroline and iron were added, they were at 20 and 40 μM, respectively. ND, not determined.

NifL was desalted by reversed-phase high-performance liquid chromatography using a gradient of acetonitrile in water, with both acetonitrile and water containing 0.2% trifluoroacetic acid. Its mass was determined by electrospray ionization mass spectrometry on a Hewlett-Packard 5989A quadrupole mass spectrometer equipped with an electrospray ion source.

Cell extracts containing NifA. Crude supernatants containing NifA were prepared from *E. coli* NCM700 (NCM632/pJES294). Even though NifA was insoluble when overproduced by using 500 μM isopropylthiogalactopyranoside (IPTG), the small amount that remained in the crude supernatant was active (28).

Coupled transcription-translation assays. Coupled transcription-translation assays were performed as previously described (27, 28, 43). S30 extract was prepared from an aerobically grown culture of *Salmonella typhimurium* SK419. NifL was synthesized from the driver plasmid pJES283 (5 μg) under anaerobic or aerobic conditions in a 50-μl reaction volume at 30°C. Synthesis under anaerobic conditions was performed in closed 8-ml serum bottles (Wheaton) under a nitrogen atmosphere in the presence of DTT (5 mM), in the presence of DTT (5 mM) and various concentrations of $FeSO_4$ (2 to 200 μM), or in the presence of DTT (5 mM) and manganese-containing superoxide dismutase (Mn-SOD) from *E. coli* (10 U; Sigma). Serum bottles containing all of the required small molecules (S30 mix) and the driver plasmid were degassed and gassed with nitrogen gas 40 times, and then the DTT was added with a gastight syringe. S30 extract was degassed and gassed in a separate serum bottle and supplemented with 2 mM DTT. After a period of 10 min to allow for reduction, 15 μl of anaerobic S30 extract was added to the other components. Synthesis under aerobic conditions was also performed in closed serum bottles with DTT present at 1 mM. Synthesis of NifL was started by adding T7 RNA polymerase (40 U/50 μl of reaction mixture) and shifting from 4°C to 30°C. After 10, 20, and 30 min, 10-μl samples were taken with gastight syringes and assayed for the inhibition of NifA activity in separate reaction mixtures. Plasmid p318 (5 μg/50 μl of reaction mixture) carrying a fusion of *nifH* to *lacZ* (39, 43) was used as a reporter to monitor inhibition of NifA activity by NifL. The assay tubes also contained σ^{54} (100 nM). When NifL was synthesized anaerobically, the assay tubes were closed and then degassed and gassed with nitrogen 40 times, and DTT was added with a gastight syringe to a final concentration of 5 mM. After the addition of NifL to the assay tubes, NifA was provided from a crude supernatant of *E. coli* NCM700 (NCM632/pJES294). For anaerobic assays, the crude supernatant containing NifA was degassed and gassed with nitrogen gas and supplemented with 2 mM DTT.

Attempts to reconstitute Fe-S clusters with NifS protein from *Azotobacter vinelandii*. NifL was overproduced in *E. coli* NCM1097 (NCM632/pJES283) and purified under aerobic conditions as described previously (28). Purified NifL (1.4 nmol) was incubated under anaerobic conditions with NifS protein (0.32 nmol) from *A. vinelandii*, which catalyzes the reconstitution of Fe-S clusters. Both proteins were incubated at 30°C in 12.5 mM Tris-HCl buffer (pH 7.4) in the presence of 1 mM L-cysteine (Sigma), 2.5 mM $MgCl_2$, 2.5 mM ATP, 2 mM $FeSO_4$, and 3 mM DTT in a total volume of 100 μl. As a control, NifL (1.4 nmol) was incubated anaerobically in the absence of NifS, DTT, and L-cysteine. After 1 h, samples were taken with gastight syringes and assayed for inhibition of NifA activity in the coupled system under anaerobic conditions. NifS protein from *A. vinelandii* was kindly provided by Dennis R. Dean (Virginia Polytechnic Institute and State University, Blacksburg).

In vivo assays. NifA-mediated activation of transcription from the *nifHDK* promoter was assayed in *K. pneumoniae* UN4495, in this strain carrying a plasmid encoding NifL and NifA (pJES839), or in UN4495 Δ*nifLA* carrying a plasmid encoding NifA alone (pJES939). Expression of *nifLA* or *nifA* was induced from the *tac* promoter with different concentrations of IPTG. Cells were grown with N_2 as the gas phase at 30°C in minimal medium (K medium [30]) supplemented with 0.4% sucrose as the carbon source, 2 mM glutamine as the nitrogen source, 0.004% histidine, 10 mM Na_2CO_3, 0.3 mM Na_2S (to help maintain anaerobic conditions), and 0.002% resazurin (to monitor anaerobiosis). When the cells were grown in the presence of *o*-phenanthroline (20 μM), Fe was not added to the medium. The stock solution of *o*-phenanthroline (phenanthroline; 20 mM) was freshly prepared in 100% ethanol. Precultures were grown overnight in closed bottles with N_2 as the gas phase in the same medium lacking sulfide and resazurin and supplemented with 4 mM ammonium acetate. Samples of the growing cultures were taken every 2 h to determine the optical density at 600 nm (OD_{600}) and the β-galactosidase activity [U/ml = $(1{,}000 \times OD_{420} - 1.75 \times OD_{550})/(t \times v)$] (33). Differential rates of β-galactosidase synthesis (units per milliliter per OD_{600}) were calculated by plotting the β-galactosidase activity (units/milliliter) as a function of the OD of the culture and determining the slope. Aerobic cultures were grown in the same medium lacking sulfide and resazurin. Cultures (25 ml) were incubated in 250-ml flasks with rapid shaking (250 rpm).

For the shift from anaerobic to aerobic growth conditions, 25-ml cultures of *K. pneumoniae* UN 4495/pJES839 were grown under anaerobic conditions with 4 mM glutamine as the nitrogen source in the presence of 30 μM sulfide. Expression of *nifLA* was induced with 10 or 100 μM IPTG. At an OD_{600} of 0.6, the cultures were shifted to aerobiosis by transferring them to 500-ml flasks and shaking them rapidly (250 rpm).

K. pneumoniae UN4495/pDT1-22 (pDT1-22 [49]) was grown under aerobic conditions in minimal medium containing 0.4% sucrose as the carbon source, 2 mM glutamine as the nitrogen source, and 0.004% histidine. Superoxide dismutase was induced to different levels with IPTG. NifA-mediated activation of transcription was assayed by determining the differential rate of synthesis of β-galactosidase (see above). Superoxide dismutase activity was monitored by measuring its inhibition of xanthine oxidase activity in an optical test assay as described by McCord and Fridovich (31): by competing for the superoxide radical generated upon xanthine oxidation, superoxide dismutase inhibits the reduction of ferricytochrome. Between 50 and 100 μl of crude extract was used, and the extract was diluted if necessary. One unit of superoxide dismutase is defined as the quantity which decreases the apparent xanthine oxidase activity by 50%. Crude extracts were prepared as follows. Samples of the culture were harvested at different times during growth, and the cells were suspended in potassium phosphate buffer (100 mM; pH 7.0). They were disrupted by five cycles of freezing and thawing (dry ice-42°C) (10). Protein concentrations were determined by the method of Bradford (7) with the Bio-Rad protein assay (6), using bovine serum albumin (BSA) as standard.

Phosphorylation and adenylylation assays. The central domain of NifA was purified as described by Berger et al. (5). NifL (5 μM monomer) and NifA central domain (1.2 μM dimer) were incubated separately and in combination in 50 mM Tris-acetate buffer (pH 8.0) containing 400 μM ATP, 40 mM potassium chloride, 5.4 mM magnesium chloride, 0.1 mM EDTA, 3% glycerol, 1 mM DTT, 0.1 mM acetylated BSA, and 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol) or [α-^{32}P]ATP (3,000 Ci/mmol). (These are the conditions used for single-cycle transcription assays [44].) After 5, 10, and 60 min at 30°C, samples (5 μl) were taken, diluted 1:2 with double-strength sodium dodecyl sulfate (SDS) loading buffer, and subjected to electrophoresis on a 10% polyacrylamide gel in the presence of SDS. The amount of phosphate incorporation into NifA and NifL was monitored qualitatively by exposure of the gel to X-ray film. As a control, NtrC (1 μM dimer) was incubated with NtrB (100 nM) under the conditions described above, and phosphate incorporation was monitored.

TABLE 2. *o*-Phenanthroline does not inhibit NifA-mediated activation of transcription in a *K. pneumoniae* strain that overproduces NifA but lacks NifL[a]

Growth conditions	β-Galactosidase activity (U/ml/OD_{600})	Doubling time (h)
0.0 μM IPTG		
Aerobic	3,455	1.6
Anaerobic	3,850	4.6
Anaerobic + phenanthroline	4,250	6.6
Anaerobic + phenanthroline + $FeCl_3$	4,400	4.6
0.1 μM IPTG		
Aerobic	3,640	1.6
Anaerobic	3,850	4.6
Anaerobic + phenanthroline	2,950	9.6
Anaerobic + phenanthroline + $FeCl_3$	3,640	4.6
0.5 μM IPTG		
Aerobic	4,000	1.6
Anaerobic	3,500	4.8
Anaerobic + phenanthroline	2,700	12.0
Anaerobic + phenanthroline + $FeCl_3$	3,850	4.8

[a] Differential rates of transcription from the *nifHKD* promoter were determined in strain UN4495 Δ*nif*LA/pJES 939. This strain carries a chromosomal *nifK-lacZ* fusion, a deletion of chromosomal *nifLA*, and a plasmid encoding NifA under the control of the *tac* promoter (pJES939). Cells were grown in minimal medium with 2 mM glutamine as the nitrogen source in the presence of different concentrations of IPTG. Other aspects of growth were as described for Table 1.

RESULTS

Fe depletion results in loss of NifA activity under anaerobic, nitrogen-limiting growth conditions. We determined the effect of Fe depletion on NifA activity in *K. pneumoniae* UN4495, which carries a chromosomal *nifK-lacZ* fusion and a single chromosomal copy of *nifLA*. To achieve induction of *nif* transcription, cultures were grown anaerobically in minimal medium with 2 mM glutamine as the sole (limiting) nitrogen source. (Contrary to previous reports [30, 32], glutamine at concentrations of up to 15 mM allows full induction of *nif* expression in *K. pneumoniae* [46]. We can only surmise that when used at 15 mM in previous studies, the glutamine was partially hydrolyzed and some ammonia was present.) Under these conditions, the differential rate of β-galactosidase synthesis from the *nifHDK* promoter was 4,000 U/ml/OD_{600} and the doubling time was 4 h. By contrast, when cells were grown aerobically, the differential rate of synthesis of β-galactosidase was only 25 U/ml/OD_{600}. When cells were grown anaerobically in the presence of the Fe chelator phenanthroline at 20 μM and Fe was omitted from the medium, the differential rate of β-galactosidase synthesis from the *nifHDK* promoter was only 30 U/ml/OD_{600}, very similar to that seen under aerobic conditions. At 20 μM, phenanthroline does not inhibit β-galactosidase activity and has only a small effect on the anaerobic or aerobic growth rate (48); doubling times for strain UN4495 were 5.5 and 4 h in the presence and absence of phenanthroline, respectively, under anaerobic conditions and 2.4 and 1.3 h under aerobic conditions. To demonstrate that the effect of phenanthroline was, in fact, due to Fe depletion, we added different metal ions (to 40 μM) to the phenanthroline-containing medium (Fig. 1 and 2). Fe restored NifA activity completely (differential rate of expression from the *nifHDK* promoter 122% of that in the absence of phenanthroline), whereas manganese restored activity only partially (to 19%) and zinc failed to restore it (differential rate ~2% of that in the absence of phenanthroline). Thus, Fe is specifically required for NifA activity under anaerobic, nitrogen-limiting conditions. This requirement apparently does not entail function of an essential protein, since phenanthroline caused inhibition of NifA activity at low concentrations (20 μM) that had little effect on growth. At a higher concentration (100 μM), phenanthroline completely inhibited anaerobic growth of *K. pneumoniae* UN4495, and adding back Fe to 140 μM restored growth (doubling time of 4 h [not shown]).

When cells were grown aerobically in the presence of phenanthroline (20 μM) and Fe was omitted from the medium, the differential rate of β-galactosidase synthesis from the *nifHDK* promoter (20 U/ml/OD_{600}) remained as low as that seen under aerobic conditions in the presence of Fe. Thus, Fe is not required to achieve inhibition of NifA activity by NifL.

Loss of NifA activity in the absence of iron is due to inhibition by NifL. To determine whether Fe was required directly for NifA activity or was required to relieve inhibition by NifL under anaerobic nitrogen-limiting conditions, we tested the effect of phenanthroline on strains of *K. pneumoniae* that had or lacked NifL. These strains carried the same chromosomal *nifK-lacZ* fusion described above. Control and experimental strains carried *nifLA* or just *nifA*, respectively, under control of the *tac* promoter on a multicopy plasmid (pJES839 or pJES939, respectively). (We used these plasmids rather than disrupting chromosomal *nifL* because NifA expression is known to be translationally coupled to that of NifL [17, 42].) We first studied the control strain UN4495/pJES839 to determine the level of IPTG required for maximal levels of induction of the *nifK-lacZ* fusion under anaerobic, nitrogen-limiting conditions and to show that the effect of phenanthroline seen with a single chromosomal copy of *nifLA* was also seen with the plasmid-bearing strain. Maximal levels of induction were achieved with 10 μM IPTG (Table 1). Lower concentrations (0, 0.1, and 1.0 μM) gave partial induction, and 100 μM IPTG gave the same level of induction as 10 μM (see below). (For reasons that we discuss later, induction was never as high as with a single chromosomal copy of *nifLA* [Fig. 1], even though the chromosomal copy remained intact.) As was the case with a single chromosomal copy of *nifLA*, growth under aerobic conditions inhibited expression of the *nifK-lacZ* fusion almost completely. Phenanthroline at 20 μM decreased expression under anaerobic conditions by 10-fold, and adding back Fe to 40 μM restored expression. We noted that the effect of phenanthroline on NifA activity was not as great as that of

TABLE 3. Overproduction of Mn-SOD in *K. pneumoniae* does not relieve NifL inhibition of NifA activity under aerobic growth conditions[a]

Strain	[IPTG] (μM)	β-Galactosidase activity (U/ml/OD_{600})	Superoxide dismutase activity (U/mg of crude protein)
UN4495	0	24	360
UN4495/pDT1-22	0	15	633
UN4495/pDTI-22	20	24	726
UN4495/pDTI-22	100	34	850
UN4495/pDTI-22	500	27	1150
UN4495/pDTI-22	2,000	29	1,600

[a] Differential rates of transcription from the *nifHDK* promoter were determined in strain UN4495 and in strain UN4495/pDT1-22. Strain UN4495 carries a chromosomal *nifK-lacZ* fusion and a single chromosomal copy of *nifLA*. Plasmid pDT1-22 encodes the Mn-SOD from *E. coli* under the control of the *tac* promoter. Cells were grown aerobically in minimal medium containing 2 mM glutamine as the sole nitrogen source at different concentrations of IPTG. The activity of superoxide dismutase was determined in crude cell extracts as described in Materials and Methods.

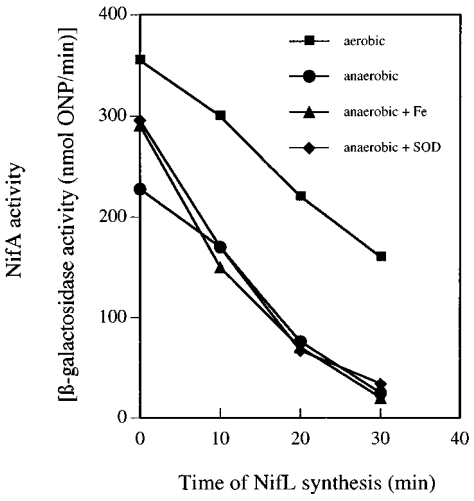

FIG. 3. Inhibition of NifA activity by NifL synthesized in a coupled transcription-translation system. NifL was synthesized in vitro at 30°C from the driver plasmid pJES283 under different conditions: aerobic, in closed tubes containing 5 mM DTT under a nitrogen atmosphere (anaerobic), in closed tubes containing 5 mM DTT and 200 μM $FeSO_4$ under a nitrogen atmosphere (anaerobic + Fe), and in closed tubes containing 5 mM DTT and Mn-SOD (10 U/50 μl of reaction mixture) under a nitrogen atmosphere (anaerobic + SOD). Synthesis of NifL was started by adding T7 RNA polymerase (40 U/50 μl of reaction mixture), and samples (10 μl) were taken at the times indicated and assayed for inhibition of NifA activity at the *nifH* promoter in separate reaction mixtures. Western blots indicated that the amounts of NifL synthesized aerobically and anaerobically were the same. (The sample at time zero was taken before addition of T7 polymerase, and hence no NifL was present.) In addition to S30 extract and the required low-molecular-weight components, assay tubes contained the *nifH-lacZ* reporter plasmid p318 (5 μg), σ^{54} (100 nM), and NifA, which was added after NifL. NifA (24 μg of protein per 50 μl of reaction mixture) was provided as a crude cell supernatant (~6 mg/ml) of the overproducing strain NCM700 (NCM632/pJES294). When NifL was synthesized anaerobically (circles, triangles, and diamonds), assay tubes contained 2 mM DTT, were closed, and contained a nitrogen atmosphere. ONP, *o*-nitrophenol.

aerobiosis, particularly at the higher levels of *nifLA* expression achieved at high IPTG concentrations (see below).

By contrast to its effects on NifA activity in the presence of NifL, phenanthroline had no inhibitory effect in the absence of NifL (experimental strain UN4495Δ*nifLA*/pJES939 [Table 2]). In this case, the chromosomal copy of *nifLA* was disrupted and NifA alone was provided from a plasmid. Maximum expression of the *nifK-lacZ* fusion, which was the same as that achieved with a single chromosomal copy of *nifLA* (Fig. 1), was achieved without addition of IPTG and was not affected by addition of IPTG to 0.1 or 0.5 μM. Neither aerobiosis nor addition of phenanthroline under anaerobic conditions affected NifA activity, indicating that the phenanthroline effect, like that of molecular oxygen, was mediated by NifL and hence that Fe is required to relieve NifL inhibition under anaerobic, nitrogen-limiting conditions.

Overproduction of superoxide dismutase in vivo does not relieve NifL inhibition of NifA activity aerobically. Since Fe appeared to be required to relieve NifL inhibition of NifA activity under anaerobic, nitrogen-limiting conditions in vivo, we performed a number of experiments in vivo and in vitro to determine whether NifL itself carried Fe or an Fe-S cluster. To test whether NifL (or any unidentified protein that participated in sensing oxygen or combined nitrogen in conjunction with NifL) carried an Fe-S cluster that was labile to superoxide (15, 18, 23), we overproduced Mn-SOD from *E. coli* in *K. pneumoniae* UN4495/pDT1-22 under aerobic nitrogen-limiting conditions to determine whether decreasing the concentration of superoxide would relieve the inhibitory effect of NifL on NifA activity.

The superoxide dismutase activity of strain UN4495 itself was 360 U/mg of cell protein under aerobic, nitrogen-limiting conditions (Table 3). Upon induction of Mn-SOD in strain UN4495/pDT1-22 at different levels of IPTG, superoxide dismutase activity increased to levels as high as 1,600 U/mg of cell protein. However, the differential rate of expression of β-galactosidase from the *nifHDK* promoter remained at the very low level characteristic of aerobic growth. Hence, this experiment did not provide any evidence that NifL or an unidentified protein required to relieve its inhibitory effect under anaerobic conditions contains a superoxide-labile Fe-S cluster.

NifL synthesized in vitro under anaerobic conditions and in the presence of Fe or Mn-SOD still inhibits NifA activity. Previous studies indicated that NifL synthesized aerobically in a coupled transcription-translation system inhibited NifA activity. Given the requirement for Fe to relieve NifL inhibition in vivo, we determined whether synthesis of NifL anaerobically and in the presence of Fe or Mn-SOD would yield a noninhibitory or less inhibitory form. After allowing different times for synthesis of NifL under various conditions, the NifL was tested for its ability to inhibit NifA-mediated expression of a *nifH-lacZ* fusion in a separate reaction. Like NifL synthesized aerobically, NifL synthesized anaerobically in the presence of 5 mM DTT inhibited NifA activity (Fig. 3). The same was true if NifL was synthesized anaerobically in the presence of DTT and various concentrations of Fe (2 to 200 μM $FeSO_4$ [Fig. 3 and data not shown]). Thus, we could not demonstrate an effect of anaerobiosis or anaerobiosis plus Fe in relieving NifL inhibition in vitro. If the plasmid encoding NifL was omitted from the initial reactions, there was no inhibition of NifA activity, indicating that inhibition was indeed due to NifL (not shown).

To attempt to prevent superoxide-mediated destruction of an Fe-S cluster that might be required to relieve NifL inhibition or direct effects of superoxide on NifL, we synthesized NifL anaerobically in the presence of DTT and Mn-SOD (10 U/50-μl assay volume). However, NifL synthesized under these conditions retained its inhibitory function (Fig. 3). Control experiments indicated that Mn-SOD itself had no effect on NifA activity (not shown).

Previous studies indicated that NifL synthesized aerobically in vivo, like the protein synthesized aerobically in vitro, inhibited NifA activity (28). Incubating NifL (1.4 nmol/50 μl) with the NifS enzyme from *A. vinelandii* (0.37 nmol/50 μl) in the presence of ferrous iron, cysteine, and ATP did not relieve inhibition (see Materials and Methods; data not shown). Under these conditions, NifS is known to catalyze the formation of Fe-S clusters in both components of nitrogenase and in other proteins (26, 52–54). We could not synthesize NifL under conditions appropriate for NifS activity because the concentrations of ferrous iron required were inhibitory to the function of the coupled system (46).

Determination of the molecular mass of NifL. To determine whether the inhibitory form of NifL synthesized aerobically in vivo contained a covalently bound cofactor or was posttranslationally modified (e.g., phosphorylated or nucleotidylylated), we determined the molecular mass of NifL by electrospray ionization mass spectrometry. NifL was overproduced under aerobic growth conditions in *E. coli* NCM632/pJES283 and was purified under aerobic conditions as described in Materials and Methods. The untransformed electrospray mass spectrum of NifL (Fig. 4) indicated that there were two protein populations present in a 1:1 ratio; they differed in mass by a minimum of 124. Quantitative determination of the N-terminal amino acid sequence of the protein sample showed that 50% of the pro-

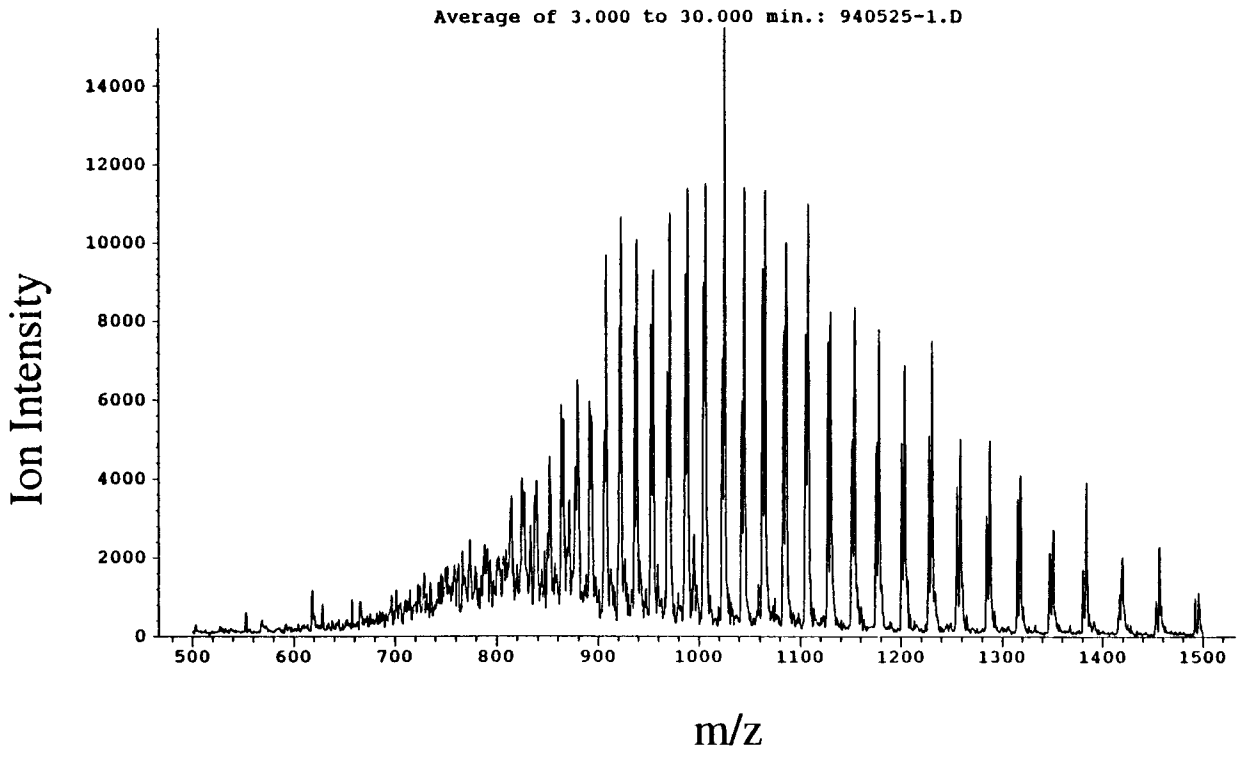

FIG. 4. Electrospray mass spectrum of purified NifL. NifL was overproduced in strain NCM632/pJES283, purified, and extracted from inclusion bodies with aqueous acetonitrile as described in Materials and Methods. The empirical mass was 55,310.85 ± 1.9 kDa (see text), whereas that calculated based on the amino acid sequence was 55,311.72 kDa.

tein had retained the N-terminal methionine residue, whereas 50% had lost it. The empirical mass of NifL including the N-terminal methionine was calculated to be 55,310.85 ± 1.9 kDa. The theoretical mass based on the amino acid sequence of NifL is 55,311.72 kDa. The fact that the experimentally determined mass and the mass calculated from the amino acid sequence are in agreement clearly indicates that NifL synthesized under aerobic conditions in vivo is not covalently modified and does not contain a covalently bound cofactor.

NifL is not phosphorylated or adenylylated under conditions in which it inhibits NifA activity. As shown above, NifL synthesized aerobically in vivo is not covalently modified. To show rigorously that no covalent modification is required for its inhibitory activity in vitro, we showed that NifL is not modified when it is incubated with the central domain of NifA in the presence of ATP, conditions under which it is inhibitory in a purified transcription system. (The only other components present for open complex formation would be the DNA template and σ^{54}-holoenzyme.) Purified NifL and the central domain of NifA were incubated separately or together at 30°C with either [γ-^{32}P]ATP to monitor phosphorylation or [α-^{32}P]ATP to monitor adenylylation. Samples were taken after 5, 10, and 60 min of incubation, diluted 1:2 with loading buffer, and separated on a 10% polyacrylamide gel in the presence of SDS. As a control, NtrC was incubated with NtrB in the presence of [γ-^{32}P]ATP. Exposure of the gel to X-ray film showed that NtrC had been maximally phosphorylated within 10 min. By contrast, even after 60 min of incubation, neither NifL nor the central domain of NifA had been detectably phosphorylated or adenylylated, whether the two proteins were incubated separately or together (not shown). These results show that NifL does not modify itself using the ATP present in transcription reactions and that it is not modified by the central domain of NifA. Hence, NifL can inhibit the activity of the central domain of NifA without being covalently modified. Moreover, NifL does not appear to modify the central domain of NifA to exert its inhibitory effect.

Further investigation of the phenanthroline effect. Since our finding that Fe was required to relieve NifL inhibition in vivo was in direct conflict with that of Henderson et al. (21), we performed an experiment at the same high levels of *nifLA* expression that they had used (100 μM IPTG). Under these conditions, we did not see a phenanthroline effect on anaerobic, nitrogen-limited cultures, although aerobiosis prevented induction of *nifK-lacZ*, as it had at lower levels of *nifLA* expression (Table 4; experiment performed with the preparations noted in Table 1). Although *nifL* and *nifA* are transcribed from the same promoter and their syntheses are translationally coupled, we suspected that the lack of a phenanthroline effect at high levels of expression was due to the fact that the cells contained more functional NifA than NifL under these conditions. To test this idea, we first established a differential rate of synthesis of β-galactosidase from the *nifHDK* promoter at 100 μM IPTG, which was 1,800 U/ml/OD_{600} (Fig. 5), and then aerated the culture vigorously as described in Materials and Methods. Oxygen had little if any effect on transcription from the *nifHDK* promoter, commensurate with the view that there is an excess of functional NifA over NifL at 100 μM IPTG. We suspect, though we have no direct evidence for it, that this is due to differential solubility of the two proteins. A control experiment indicated that oxygen inhibited transcription from the *nifHDK* promoter markedly at 10 μM IPTG (not shown). We also suspect that there is an excess of functional NifL over NifA at low levels of *nifLA* expression and that the excess NifL

TABLE 4. The iron chelator *o*-phenanthroline fails to inhibit NifA-mediated activation of transcription in a *K. pneumoniae* strain that overproduces NifL and NifA to very high levels[a]

Growth conditions	β-Galactosidase activity (U/ml/OD_{600})	Doubling time (h)
Aerobic	20	2.0
Anaerobic	1,800	4.0
Anaerobic + phenanthroline	1,700	7.5
Anaerobic + phenanthroline + $FeCl_3$	1,850	4.0

[a] The strain and conditions were the same as for Table 1 except that 100 μM IPTG was used to induce expression of NifL and NifA from the *tac* promoter.

inhibits the activity of NifA synthesized from the chromosome (Table 1 [0, 0.1, and 1.0 μM IPTG] compared with Fig. 1 and 2A). Whereas an increase in the ratio of functional NifA to NifL at higher levels of expression can account for the decreasing effect of phenanthroline and of aeration of the cultures at late times, it is difficult to reconcile with the observation that aerating the cultures from the time IPTG is added prevents transcription from the *nifHDK* promoter at all levels of IPTG. Although interpretation of the effects of phenanthroline is complicated by effects of different levels of expression of *nifLA* from a plasmid, our fundamental conclusion, that Fe is required to relieve NifL inhibition under anaerobic, nitrogen-limiting conditions, does not depend on such interpretation. It rests on the effects of phenanthroline and phenanthroline plus Fe on a strain of *K. pneumoniae* with a single chromosomal copy of *nifLA*.

DISCUSSION

Fe is required to relieve the inhibition of nitrogen fixation by NifL. In an attempt to extend the findings of Henderson et al. (21), we studied the effects of Fe depletion on NifL function in a strain of *K. pneumoniae* with a single chromosomal copy of *nifLA*. Contrary to findings in *E. coli* when *nifLA* was expressed at high levels from a multicopy plasmid, we found that Fe is required to relieve NifL inhibition of NifA activity under anaerobic, nitrogen-limiting conditions (Fig. 1 and 2; Table 1) and that it is not required to achieve such inhibition under aerobic conditions (see Results). Apart from expressing *nifLA* from the chromosome of *K. pneumoniae* or using lower levels of induction from a plasmid (see Results), some other salient differences between our experiments and those of Henderson et al. (21) were that (i) we maintained strictly anaerobic conditions and (ii) we measured differential rates of β-galactosidase expression from a chromosomal *nifK-lacZ* fusion. By contrast, Henderson et al. achieved anaerobiosis by closing cultures and allowing them to deplete oxygen and determined endpoint synthesis of β-galactosidase from a second plasmid carrying a *nifH-lacZ* fusion. Both groups are agreed that Fe and Fe chelators have no effect on NifA activation in the absence of NifL (Table 2) and hence that Fe involvement in *nif* expression is mediated by NifL.

NifL does not appear to contain Fe or an Fe-S cluster. We obtained no evidence that NifL contains Fe or an Fe-S cluster. Mn-SOD from *E. coli* failed to relieve NifL inhibition under aerobic, nitrogen-limiting conditions in vivo (Table 3), whereas increasing the superoxide dismutase activity of *Rhodobacter capsulatus* only twofold did relieve oxygen inhibition of transcription of the *puf* photosynthesis operon (51). Superoxide dismutase is known to protect superoxide-sensitive Fe-S clusters from destruction (16, 29). Neither anaerobiosis nor anaerobiosis plus Fe or Mn-SOD allowed synthesis of a noninhibitory form of NifL in a coupled transcription-translation system (Fig. 3). NifL overproduced aerobically in vivo inhibits NifA function in vitro (5, 28). The mass of this NifL is that of the primary translation product (Fig. 4), indicating that it contains no covalently bound cofactor and is not covalently modified. Moreover, this NifL is not subsequently modified by phosphorylation or adenylylation under conditions in which it inhibits NifA activity in a purified transcription system (not shown). Incubation of NifL with the NifS protein of *A. vinelandii*, which is known to catalyze the formation of Fe-S clusters, did not reverse its inhibition of NifA activity (not shown).

An unidentified Fe-containing protein may be involved in sensing of molecular oxygen and/or combined nitrogen by NifL. Although we cannot rule out the possibility that NifL itself contains Fe or an Fe-S cluster, we were unable to obtain evidence for this. Hence, we propose as a working hypothesis that another Fe-containing protein is involved in oxygen-sensing by NifL (Fig. 6). The fact that the iron chelator phenanthroline prevents relief of NifL inhibition at concentrations that decreased anaerobic growth rates by <30% indicates that this putative additional protein is not essential. Moreover, since NifL function is regulated normally by oxygen and combined nitrogen in *E. coli* in the absence of *nif* proteins other than NifA (20), the putative Fe-containing protein is apparently not *nif* specific. One particularly attractive candidate is a flavoheme protein from *E. coli* studied by Poole (40), which is believed to function as an oxygen sensor and is known to be dispensable by virtue of the fact that the gene encoding it can be deleted. Studies of the potential involvement of this protein in controlling NifL function are under way.

Models for NifL function. It is clear that the primary translation product of the *nifL* gene of *K. pneumoniae* can inhibit NifA function in vitro (references 5 and 28 and Fig. 1 and 3). If the primary translation product (NifL [Fig. 6]) is the physiologically inhibitory form in vivo, we must postulate that the noninhibitory form (NifL*) is conformationally altered, carries a cofactor, or is covalently modified. As indicated above, we have obtained no evidence that NifL contains Fe or an Fe-S cofactor, but Fe or an unidentified Fe-containing protein would presumably be required for the conformational change in NifL or acquisition of the required cofactor or modification. If the physiologically inhibitory form of NifL is not the primary

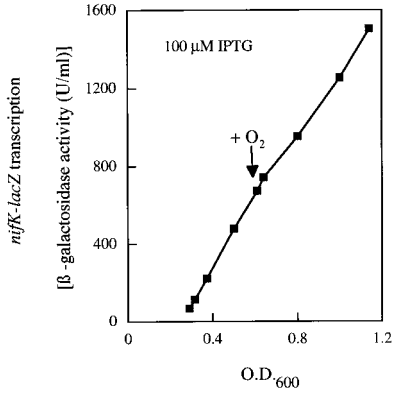

FIG. 5. Effect of molecular oxygen on NifA activity in a culture with high levels of *nifLA* expression. Transcription of *nifLA* was induced from the *tac* promoter of plasmid pJES839 in *K. pneumoniae* UN4495/pJES839 with 100 μM IPTG, and the activity of β-galactosidase was plotted as a function of OD_{600}. The 25-ml culture was grown anaerobically at 30°C with an initial sulfide concentration of 30 μM and 4 mM glutamine as the nitrogen source. At an OD_{600} of 0.6 the culture was shifted to aerobiosis (+ O_2) by incubating it aerobically in a 500-ml flask with rapid shaking (250 rpm).

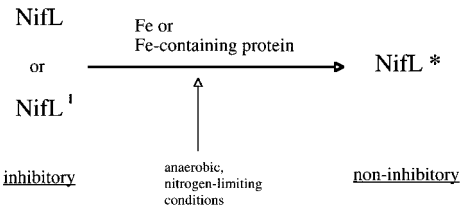

FIG. 6. Models for NifL function. NifL, primary translation product; NifL*, noninhibitory form of NifL; NifL′, modified primary translation product.

translation product but rather one that carries a cofactor or is covalently modified (NifL′), Fe or the Fe-containing protein would presumably be involved in altering this cofactor or changing the covalent modification of NifL under anaerobic, nitrogen-limiting conditions to yield a noninhibitory form (NifL*).

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ADDENDUM IN PROOF

After this manuscript was submitted, it was reported that the NifL protein of *A. vinelandii* contains FAD and that reduction of this FAD by dithionite relieves NifL inhibition in vitro (S. Hill, S. Austin, T. Eydmann, T. Jones, and R. Dixon, Proc. Natl. Acad. Sci. USA **93:**2143–2148, 1996). Since the NifL protein of *K. pneumoniae* also contains FAD (R. Schmitz, unpublished data), we hypothesize that the unidentified Fe-containing protein discussed in this paper is required to reduce the FAD cofactor of NifL in vivo.

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