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Fnr Is Required for NifL-Dependent Oxygen Control of *nif* Gene Expression in *Klebsiella pneumoniae*

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In *Klebsiella pneumoniae,* NifA-dependent transcription of nitrogen fixation (*nif*) genes is inhibited by NifL in response to molecular oxygen and combined nitrogen. We recently showed that *K. pneumoniae* NifL is a flavoprotein, which apparently senses oxygen through a redox-sensitive, conformational change. We have now studied the oxygen regulation of NifL activity in *Escherichia coli* and *K. pneumoniae* strains by monitoring its inhibition of NifA-mediated expression of *K. pneumoniae* ϕ(*nifH′-′lacZ*) fusions in different genetic backgrounds. Strains of both organisms carrying *fnr* null mutations failed to release NifL inhibition of NifA transcriptional activity under oxygen limitation: *nif* induction was similar to the induction under aerobic conditions. When the transcriptional regulator Fnr was synthesized from a plasmid, it was able to complement, i.e., to relieve NifL inhibition in the *fnr* mutant backgrounds. Hence, Fnr appears to be involved, directly or indirectly, in NifL-dependent oxygen regulation of *nif* gene expression in *K. pneumoniae*. The data indicate that in the absence of Fnr, NifL apparently does not receive the signal for anaerobiosis. We therefore hypothesize that in the absence of oxygen, Fnr, as the primary oxygen sensor, activates transcription of a gene or genes whose product or products function to relieve NifL inhibition by reducing the flavin adenine dinucleotide cofactor under oxygen-limiting conditions.

In diazotrophic proteobacteria, transcription of the nitrogen fixation (*nif*) genes is mediated by the *nif*-specific activator protein NifA, a member of a family of activators that functions with σ^{54} (2, 4). Both the expression and the activity of NifA can be regulated in response to the oxygen and/or combined nitrogen status of the cells; the mechanisms of the regulation differ with the organism. In *Klebsiella pneumoniae* and *Azotobacter vinelandii,* NifA transcriptional activity is regulated by a second regulatory protein, NifL. This negative regulator of the *nif* genes inhibits the transcriptional activation by NifA in response to combined nitrogen and/or external molecular oxygen. The translationally coupled synthesis of the two regulatory proteins, immunological studies, complex analyses, and studies using the two-hybrid system in *Saccharomyces cerivisiae* imply that the inhibition of NifA activity by NifL apparently occurs via direct protein-protein interaction (5, 11, 21, 26). The mechanism by which nitrogen is sensed in *K. pneumoniae* and *A. vinelandii* is currently the subject of extensive studies. Very recently, He et al. (10), and Jack et al. (15) provided evidence that in *K. pneumoniae*, the second PII protein, GlnK, is required for relief of NifL inhibition under nitrogen-limiting conditions. This indicates that GlnK regulates NifL inhibition of NifA in response to the nitrogen status of the cells by interacting with NifL or NifA.

In both organisms, *K. pneumoniae* and *A. vinelandii*, the negative regulator NifL is a flavoprotein with an N-terminally bound flavin adenine dinucleotide (FAD) as a prosthetic group (13, 19, 31). In vitro, the oxidized form of NifL inhibits NifA activity, whereas reduction of the FAD cofactor relieves NifL inhibition (13, 22). This indicates that NifL apparently acts as a redox switch in response to the environmental oxygen status and allows NifA activity, only under oxygen-limiting conditions. We recently showed that in vivo, the presence of iron is required to relieve inhibitory effects of NifL on transcriptional activation by NifA and, additionally, that iron is not present in NifL (31, 32). Therefore, we have postulated that an unidentified iron-containing protein may be the physiological reductant for NifL. This putative iron-containing protein is apparently not *nif* specific, since NifL function is regulated normally in response to cellular nitrogen and oxygen availability in *Escherichia coli* in the absence of *nif* proteins other than NifA (9).

The key question concerning the oxygen signal transduction in *K. pneumoniae* is whether NifL senses oxygen directly via a redox-induced conformational change, or whether oxygen is detected by a more general oxygen-sensing system, which then regulates NifL by inducing the oxidation or reduction of the flavin cofactor. One candidate for a general oxygen sensor is the transcriptional fumarate nitrate reductase regulator (Fnr) (35, 36), which in the case of *E. coli* Fnr, senses oxygen via an oxygen-labile iron-sulfur ($[4Fe\text{-}4S]^{+2}$) cluster and is involved in signal transduction of the cellular redox state (7, 18, 25, 37). Recently we cloned and sequenced the *fnr* gene of *K. pneumoniae* and characterized the protein (6). Because the *K. pneumoniae* Fnr amino acid sequence is 98% identical to the *E. coli* Fnr and contains an iron-sulfur cluster, we have now tested the hypothesis that Fnr transduces the oxygen signal to NifL. We present evidence that in the absence of Fnr, NifL inhibits NifA activity under oxygen limitation, suggesting that Fnr is required for relief of NifL inhibition in *K. pneumoniae* under anaerobic conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. Plasmid DNA was transformed into *E. coli* cells according to the method of Inoue et al. (14) and into *K. pneumoniae* cells by

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or characteristic(s)	Source or reference
Strains		
E. coli		
NCM1529	*araD139*Δ(*argF-lacU*)*169 fthD5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1 trpDC700putPA1303*::[Kan^r-(*nifH′-′lacZ*)] (wild type)	10
NCM1528	NCM1529/pNH3	10
NCM1527	NCM1529/pJES851	10
RAS1	NCM1529, but *fnr*::Tn*10*	6
RAS2	NCM1529/pRS107	This study
RAS6	RAS1/pRS107	This study
RAS7	RAS1/pNH3	This study
RAS8	RAS1/pJES851	This study
RAS9	RAS1/pNH3 and pRS79	This study
RAS10	RAS1/pNH3 and pRS120	This study
RAS11	RAS1/pNH3 and pMCL210	This study
RAS12	RAS1/pNH3 and pACYC184	This study
RM101	MC4100, but Δ*fnr*	30
RAS13	RM101, but [Kan^r-(*nifH′-′lacZ*)]	This study
RAS21	MC4100, but [Kan^r-(*nifH′-′lacZ*)]	This study
RAS22	RAS21/pNH3	This study
RAS23	RAS21/pJES851	This study
RAS24	RAS21/pRS107	This study
RAS14	RAS13/pNH3	This study
EAS15	RAS13/pJES851	This study
RAS25	RAS13/pRS107	This study
RAS16	RAS13/pNH3 and pRS120	This study
RAS17	RAS13/pNH3 and pACYC184	This study
K. pneumoniae		
M5al	Wild type	
UN4495	ϕ(*nifK-lacZ*)*5935* Δ*lac-4001 his D4226* Gal^r	23
RAS18	ϕ(*nifK-lacZ*)*5935* Δ*lac-4001 his D4226* Gal^r *fnr*::Ω	This study
RAS19	RAS18/pRS137	This study
RAS20	RAS18/pACYC184	This study
RAS26	UN4495/pRS159	This study
RAS27	RAS18/pRS159	This study
RAS28	UN4495/pJES839	9
RAS29	RAS18/pJES839	This study
RAS30	UN4995Δ(*nifLA*)*6293*::Km/pJES839	32; this study
Plasmids		
pNH3	*K. pneumoniae nifLA* controlled by the *tac* promoter	11
pJES839	pNH3, but additional tetracycline resistance cassette	32
pJES851	*K. pneumoniae nifA* controlled by the *tac* promoter	32
pRS79	*E. coli fnr* controlled by the *lac* promoter on pMCL210	This study
pRS107	*K. pneumoniae nifL*$^{C184S/C187S}$*nifA* controlled by the *tac* promoter	This study
pRS159	*K. pneumoniae nifL*$^{C184S/C187S}$*nifA* controlled by the *tac* promoter	This study
pRS120	*E. coli fnr* controlled by the *tet* promoter on pACYC184	6
pRS127	2.1-kbp fragment in pBluescript SK$^+$containing *K. pneumoniae fnr*	6
pRS137	*K. pneumoniae fnr* controlled by the *tet* promoter on pACYC184	6
pACYC184	Low-copy vector	New England Biolabs
pMCL210	Low-copy vector	27
pBluescript SK$^+$	Cloning vector	Stratagene, La Jolla, Calif.

electroporation. Transduction by phage P1 was performed as described previously (33).

(i) *E. coli* strains. *E. coli* NCM1529, which contains a ø(*nifH′-′lacZ*) fusion (9), and derivatives of NCM1529 were chosen to study NifA/NifL regulation in *E. coli*. The *fnr*::Tn*10* allele was transferred from the *fnr*::Tn*10* derivative of M182 (16) into NCM1529 by P1-mediated transduction with selection for tetracycline resistance, resulting in RAS1 (6). Strains RAS6, RAS7, RAS8, RAS9, RAS10, RAS11, and RAS12 contain plasmids pRS107, pNH3, pJES851, pNH3 plus pRS79, pNH3 plus pRS120, pNH3 plus pMCL210, and pNH3 plus pACYC184, respectively, in RAS1. To construct an independent second *fnr* null mutant, the [Kan^r-(*nifH′-′lacZ*)] allele was transferred from strain NCM1529 by P1-mediated transduction into the independent *fnr* mutant strain RM101 (30) and into the parental strain MC4100 with selection for kanamycin resistance, resulting in RAS13 and RAS21, respectively. Strains RAS25, RAS14, RAS15, RAS16 and RAS17 contain plasmids pRS107, pNH3, pJES851, pNH3 plus pRS120, and pNH3 plus pACYC184, respectively, in RAS13.

(ii) *Klebsiella* strains. *K. pneumoniae* strains M5al (wild type) and UN4495 [ø(*nifK-lacZ*)*5935* Δ*lac-4001 his D4226* Gal^r] (23) were provided by Gary Roberts.

Construction of an *fnr*::Ω mutation. Strain RAS18 was obtained by insertion of a kanamycin resistance cassette (28) into the *fnr* gene of *K. pneumoniae* UN4495 as detailed in the following steps. (i) The 2.1-kbp *Eco*RI-*Bam*HI fragment, which carries the *ogt-fnr-ydaA′*- region of *K. pneumoniae,* was subcloned into pBluescript SK$^+$ to produce pRS127. (ii) A 2.1-kb *Hin*dIII cassette containing an Ω interposon fragment with a kanamycin resistance gene derived from plasmid pHP45Ω (28) was cloned into the *Hin*dIII site of *fnr* in pRS127 to yield

plasmid pRS142. (iii) A 2.9-kb PCR fragment carrying *fnr*::Ω was generated with pRS142 as a template and a set of primers which were homologues to the *fnr* flanking 5′ and 3′ regions, with additional *Bam*HI synthetic restriction recognition sites (underlined) (5′ATATCAATGGATCCCTGAGCAGACTTATGAT CC3′, sense primer; 5′CTTATATGGATCCAATGAAACAGGGGAGGA3′, antisense primer). The 2.9-kb PCR product was cloned into the *Bam*HI site of the *sacB*-containing vector pKNG101 (17), creating plasmid pRS144. The correct insertion was analyzed by sequencing. (iv) pRS144 was transformed into *K. pneumoniae* UN4495, and recombinant strains (generated by means of a double crossover) were identified by the ability to grow on Luria-Bertani (LB) medium supplemented with 5% sucrose and resistance to kanamycin. The *fnr*::Ω mutation in strain RAS18 was confirmed by Southern blot analysis (29) and by PCR.

Strains RAS26 and RAS28 contain pRS159 and pJES839, respectively, in *K. pneumoniae* UN4495 and strains RAS19, RAS27 and RAS29 contain pRS137, pRS159 and pJES839, respectively, in RAS18.

(iii) Construction of plasmids. Plasmid pRS107 contains the *K. pneumoniae* *nifL*$^{C184S/C187S}$*nifA* operon under the control of the *tac* promoter, in which the Cys^{184} and Cys^{187} of *nifL* are changed to serine (Ser^{184}-Ala-Asp-Ser^{187}). It was constructed from pNH3 (11) by introducing the double mutation into *nifL* by site-directed mutagenesis. Site-directed mutagenesis was performed with the GeneEditor System (Promega) according to the protocol of the manufacturer. The double mutation was confirmed by sequencing. Plasmid pRS159 was constructed by inserting a tetracycline resistance cassette (32) into the *Sca*I site of plasmid pRS107. Plasmid pRS79 contains the *E. coli fnr* gene inserted into the *Bam*HI-*Pst*I site of pMCL210 (27) under the control of the *lac* promoter. pRS120 and pRS137 contain the *E. coli fnr* gene and *K. pneumoniae fnr* gene, respectively, inserted into the *Sal*I-*Bam*HI site of pACYC184 and thereby expressed from the *tet* promoter (6).

Growth. *K. pneumoniae* and *E. coli* strains were grown under anaerobic conditions with N_2 as the gas phase at 30°C in minimal medium (32) supplemented with 4 mM glutamine, 10 mM Na_2CO_3, 0.3 mM sulfide and 0.002% resazurin to monitor anaerobiosis. The medium was further supplemented with 0.004% histidine and with 0.4% sucrose as the sole carbon source for *K. pneumoniae* strains. For *E. coli* strains, the medium was supplemented with 0.1 mM tryptophan and 0.8% glucose as the carbon source. Precultures were grown overnight in closed bottles with N_2 as the gas phase, in medium lacking sulfide and resazurin, but supplemented with 4 mM ammonium acetate in addition to glutamine; both ammonium and glutamine were completely utilized during growth of precultures. The cultures (25 ml) were grown in closed bottles with N_2 as the gas phase at 30°C under strictly anaerobic conditions without shaking. Samples for monitoring growth at 600 nm and determining β-galactosidase activity were taken anaerobically. In *E. coli* strains carrying a plasmid encoding NifL and NifA (pNH3) (11) or NifL$^{C184S/C187S}$ and NifA (pRS107) or a plasmid encoding NifA alone (pJES851) (32), expression of *nifLA*, *nifL*$^{C184S/C187S}$*nifA*, or *nifA* was induced from the *tac* promoter with 10 μM IPTG (isopropyl-β-D-thiogalactopyranoside).

Fnr phenotypes of RAS1, RAS13, and RAS18 and the respective complemented strains RAS9, RAS10, RAS16, and RAS19 were tested anaerobically by using glycerol and nitrate (0.5%) as the sole carbon and nitrogen sources, respectively, in minimal medium.

β-Galactosidase assay. NifA-mediated activation of transcription from the *nifHDK* promoter in *K. pneumoniae* UN4495 and *E. coli* strains was monitored by measuring the differential rate of β-galactosidase synthesis during exponential growth (units per milliliter per optical density unit at 600 nm [OD_{600}]) (32). Inhibitory effects of NifL on NifA activity were assessed by virtue of a decrease in *nifH* expression.

Western blot analysis. Cells were grown anaerobically in minimal medium with glutamine as the nitrogen source; when the culture reached a turbidity of 0.4 to 0.7 at 660 nm, 1-ml samples of the exponentially growing cultures were harvested and concentrated 20-fold into sodium dodecyl sulfate (SDS) gel loading buffer (20). Samples were separated by SDS-polyacrylamide (12%) gel electrophoresis and transferred to nitrocellulose membranes as described previously (29). Membranes were exposed to polyclonal rabbit antisera directed against the NifL or NifA proteins of *K. pneumoniae*, and protein bands were detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (Bio-Rad Laboratories). Purified NifA and NifL from *K. pneumoniae* and prestained protein markers (New England Biolabs, Frankfurt, Germany) were used as standards.

Nucleotide sequence accession number. The sequence of *K. pneumoniae fnr* has been submitted to GenBank under accession no. AF220669.

RESULTS

We recently showed that in vivo iron is specifically required for *nif* induction in *K. pneumoniae,* and additionally, that iron is not present in NifL (31, 32). In order to examine whether oxygen is detected by a more general system rather than by NifL directly, we chose to examine the possible influence of Fnr on the *nif* induction in a heterologous *E. coli* system. We performed all experiments under nitrogen-limiting growth conditions to exclude NifA inhibition by NifL in response to the presence of ammonium. If Fnr is indeed the primary oxygen sensor, which transduces the oxygen signal to NifL, the iron requirement for the *nif* induction under oxygen-limiting conditions may be based on the iron requirement for the assembly of iron sulfur clusters of Fnr.

Studying the effect of Fnr on the *nif* induction in a heterologous *E. coli* system. In order to study the effect of Fnr on *nif* regulation in response to oxygen, we chose a heterologous *E. coli* system. Strain NCM1529 carrying a chromosomal *nifH′-′lacZ* fusion was used as parental strain (9). NifL and NifA were induced independent of the Ntr system from plasmids which carried the *K. pneumoniae nifLA* (pNH3) and *nifA* (pJES851) genes under the control of the *tac* promoter. The two regulatory proteins were induced with 10 μM IPTG to levels at which NifL function is regulated normally in response to oxygen and combined nitrogen in *E. coli* in the absence of *nif* proteins other than NifA (9). To study the effect of an *fnr* null mutation on the regulation of NifL activity in response to oxygen, an *fnr* null allele (*fnr*::Tn*10*) was introduced by P1 transduction into the parental strain NCM1529 carrying the ø(*nifH′-′lacZ*) fusion as described in Materials and Methods, resulting in strain RAS1. After introducing *nifLA* and *nifA* on plasmids, the resulting strains were generally grown in mineral medium with glucose as the sole carbon source and under nitrogen limitation to exclude NifA inhibition by NifL in response to combined nitrogen. Determination of the doubling times of the different strains under anaerobic and aerobic conditions revealed no significant difference in growth rates for *fnr* mutant strains compared to the respective parental strains (Table 2). NifA-mediated activation of transcription from the *nifH′* promoter in the different backgrounds was monitored by determining the differential rate of β-galactosidase synthesis during exponential growth. Inhibitory effects of NifL on NifA activity in strain RAS7 carrying the *fnr* null allele and carrying *nifLA* on a plasmid are detectable; they result in a decrease in *nifH* expression. Interestingly, under oxygen-limiting conditions, strain RAS7 showed a β-galactosidase synthesis rate from the *nifH′* promoter of only 100 ± 10 U/ml/OD_{600} unit when *nifLA* was induced with 10 μM IPTG. This is in the range of the synthesis rate under aerobic conditions in the parental strain NCM1528 (60 ± 5 U/ml/OD_{600} unit) and equivalent to 3% of the synthesis rate under anaerobic conditions in NCM1528 (3,000 ± 100 U/ml/OD_{600} unit) (Table 2).

In the case of NifA synthesis in the *fnr* mutant strain in the absence of NifL (RAS8), however, the β-galactosidase synthesis rate under anaerobic conditions was not significantly altered compared to the parental strain NCM1527 (4,800 ± 100 and 5,300 ± 200 U/ml/OD_{600} unit, respectively) and was not affected by oxygen (Table 2). This indicates that the observed Fnr effect is mediated by NifL towards NifA in RAS7. How-

TABLE 2. Effects of an *fnr* null allele on activity of the *K. pneumoniae* NifL protein in different *E. coli* backgrounds

Strain	Relevant genotype[a]	Presence of oxygen	Expression of *nifH'-'lacZ* (U/ml/OD_{600} U)[b]	Doubling time (h)
NCM1528	Wild type/*Ptac-nifLA*	−	3,000 ± 100	5.0
NCM1528	Wild type/*Ptac-nifLA*	+	60 ± 5	2.0
NCM1527	Wild type/*Ptac-nifA*	−	5,300 ± 200	4.8
NCM1527	Wild type/*Ptac-nifA*	+	5,118[c]	2.1
RAS2	Wild type/*Ptac-nifL* mutant *nifA*	−	2,950 ± 120	5.2
RAS2	Wild type/*Ptac-nifL* mutant *nifA*	+	2,900 ± 50	2.0
RAS8[d]	*fnr* mutant/*Ptac-nifA*	−	4,800 ± 100	4.9
RAS8[d]	*fnr* mutant/*Ptac-nifA*	+	5,200 ± 200	2.2
RAS6[d]	*fnr* mutant/*Ptac-nifL* mutant *nifA*	−	2,800 ± 100	5.0
RAS6[d]	*fnr* mutant/*Ptac-nifL* mutant *nifA*	+	3,000 ± 200	2.0
RAS7[d]	*fnr* mutant/*Ptac-nifLA*	−	100 ± 10	5.0
RAS7[d]	*fnr* mutant/*Ptac-nifLA*	+	30 ± 3	2.0
RAS9[d]	*fnr* mutant/*Ptac-nifLA*/*Plac fnr*	−	3,000 ± 100	5.2
RAS10[d]	*fnr* mutant/*Ptac-nifLA*/*Ptet fnr*	−	2,870 ± 70	5.2
RAS11[d]	*fnr* mutant/*Ptac-nifLA*/pMCL210	−	66 ± 5	5.5
RAS12[d]	*fnr* mutant/*Ptac-nifLA*/pACYC184	−	70 ± 6	5.5
RAS22	Wild type/*Ptac-nifLA*	−	3,500 ± 80	5.0
RAS22	Wild type/*Ptac-nifLA*	+	70 ± 5	2.2
RAS23	Wild type/*Ptac-nifA*	−	5,900 ± 250	5.1
RAS23	Wild type/*Ptac-nifA*	+	5,725 ± 150	2.2
RAS24	Wild type/*Ptac-nifL* mutant *nifA*	−	3,400 ± 200	4.9
RAS24	Wild type/*Ptac-nifL* mutant *nifA*	+	2,800 ± 150	2.1
RAS15[e]	*fnr* mutant/*Ptac-nifA*	−	5,300 ± 200	5.6
RAS15[e]	*fnr* mutant/*Ptac-nifA*	+	5,130 ± 150	2.1
RAS25[e]	*fnr* mutant/*Ptac-nifL* mutant *nifA*	−	3,200 ± 200	5.0
RAS25[e]	*fnr* mutant/*Ptac-nifL* mutant *nifA*	+	3,400 ± 100	2.2
RAS14[e]	*fnr* mutant/*Ptac-nifLA*	−	160 ± 10	5.3
RAS14[e]	*fnr* mutant/*Ptac-nifLA*	+	40 ± 5	2.0
RAS16[e]	*fnr* mutant/*Ptac-nifLA*/*Ptet-fnr*	−	3,200 ± 100	5.2
RAS17[e]	*fnr* mutant/*Ptac-nifLA*/pACYC184	−	190 ± 10	5.4

[a] *nifL* mutant *nifA*, *nifL*$^{C184S/C187S}$*nifA* (see Materials and Methods); *Plac*, *Ptac*, or *Ptet*, under the control of the *lac*, *tac*, or *tet* promoter, respectively.
[b] Data are presented as mean values (± standard errors) of three independent experiments.
[c] Determined by He et al. (9).
[d] Strain contains the *fnr* null allele from M182 (*fnr*::Tn*10*) (16).
[e] Strain contains the *fnr* null allele from RM101 (30).

ever, *nif* expression under anaerobic conditions by NifA induced from the *tac* promoter in the absence of NifL synthesis by using pJES851 (NCM1527) is significantly higher than that with plasmid pNH3 (NCM1528), in which NifA expression depends on NifL synthesis based on translational coupling in the *nifLA* operon (5). In addition, Western blot analysis showed that under our experimental conditions, the amounts of NifA synthesized in NCM1527 were approximately 30 to 40% higher than those synthesized in NCM1528 (data not shown). To rule out that *nif* expression in the *fnr* mutant using pJES851 (RAS8) is not due to this increase in NifA expression, we additionally constructed pRS107 containing *nifL*$^{C184S/C187S}$*nifA* translationally coupled under the control of the *tac* promoter (see Materials and Methods). IPTG induction in NCM1529 containing pRS107 (RAS2) resulted in NifA expression comparable to that in NCM1528 (data not shown) and expression of NifL$^{C184S/C187S}$, which completely lost its nitrogen and oxygen regulatory function (K. Klopprogge and R. A. Schmitz, unpublished observations). Determination of β-galactosidase synthesis rates showed that *nif* induction by NifA expressed from pRS107 in the absence of a functional NifL protein was again not affected by the *fnr* mutation (compare RAS2 with RAS6) and was in the range of *nif* induction in NCM1528 under anaerobic conditions (Table 2). These findings indicate that the *fnr* null allele does not affect NifA activity directly in the absence of functional NifL. In the presence of both regulatory proteins, however, NifL inhibits NifA activity under oxygen-limiting conditions when Fnr is absent, suggesting that the Fnr effect is mediated through NifL to NifA.

The finding that in the absence of Fnr NifL inhibits NifA activity under oxygen-limiting conditions to the same amount as under aerobic growth conditions indicates that NifL apparently does not receive the signal of anaerobiosis when Fnr is absent. To confirm this observation, we analyzed the *nif* induction under anaerobic conditions in a different *fnr* mutant strain (RAS13). After introduction of *nifLA*, *nifA*, and *nifL*$^{C184S/C187S}$*nifA* on plasmids, the respective strains RAS14, RAS15, and RAS25 were grown under oxygen limitation. By determining the β-galactosidase synthesis rates from the *nifH'* promoter in RAS14, we observed that in this independent *fnr* mutant strain, the *nif* induction was 160 ± 10 U/ml/OD_{600} unit, when *nifLA* was expressed under anaerobic conditions. This *nif* induction is again significantly lower than in the parental strain RAS22 (3,500 ± 80 U/ml/OD_{600} unit) and is in the range of aerobic *nif* induction in the parental strain (70 ± 5 U/ml/OD_{600} unit) (Table 2). Similar to RAS8 and RAS6, the β-galactosidase synthesis rate in the case of NifA synthesis in the absence of a functional NifL protein was not affected by the *fnr* mutation (RAS15 compared to RAS23 and RAS25 compared to RAS24).

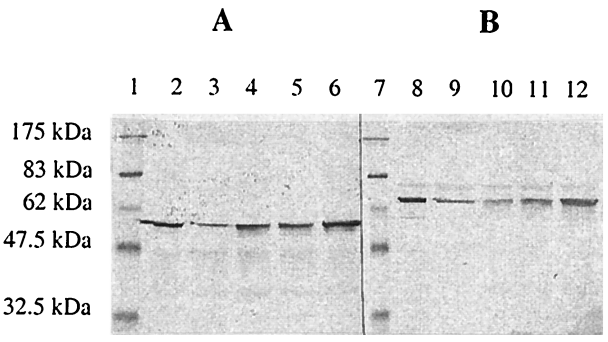

FIG. 1. Amounts of NifA and NifL in wild-type and *fnr* mutant strains of *E. coli*. Cultures were grown at 30°C in minimal medium under anaerobic conditions with 4 mM glutamine as a limiting nitrogen source. The strains carried *K. pneumoniae* NifL and NifA under the control of the *tac* promoter on pNH3. Expression of NifL and NifA was induced with 10 μM IPTG in the wild-type strain (lanes 2 and 8), in *fnr* null allele strains RAS7 (lanes 3 and 9) and RAS14 (lanes 5 and 11), and in complemented strains RAS10 (lanes 4 and 10) and RAS16 (lanes 6 and 12). The amounts of NifL (A) and NifA (B) were determined by Western blotting. Prestained broad-range protein markers (lanes 1 and 7) were purchased from New England Biolabs.

The *fnr* null alleles do not affect the synthesis of NifL and NifA. To demonstrate that the failure of the *fnr* mutant strains to express *nifH* under anaerobic conditions could not be accounted for by a decreased amount of NifA protein, we determined the amounts of NifA and NifL protein in the wild-type and *fnr* mutant strains by immunological means. As shown in Fig. 1, we observed no obvious differences in the amounts of the regulatory proteins of *K. pneumoniae* in the different *fnr* mutant backgrounds compared to those in the parental strains.

Fnr is required for release of NifL inhibition of NifA activity under anaerobic conditions in the heterologous *E. coli* system. To determine if constitutive expression of *fnr* is able to restore *nif* induction in the *fnr* mutant strains, we expressed *E. coli fnr* from the *tet* promoter (pRS120) or the *lac* promoter (pRS79) in addition to the *nifLA* operon. Expression of Fnr in *trans* from either promoter resulted in complementation with a restoration of anaerobic growth on nitrate and glycerol (data not shown). It further resulted in relief of NifL inhibition of NifA activity under oxygen-limiting conditions. This restoration of *nif* induction was achieved in both strains carrying independent chromosomal *fnr* null alleles (RAS10 and RAS16, respectively) and is displayed graphically in Fig. 2. The *nif* induction under anaerobic conditions in both mutant strains was restored to the induction level of the parental strains (NCM1528 and RAS22, respectively) by expressing *E. coli fnr* from promoter P*tet* on pACYC184 or promoter P*lac* on pMCL210, whereas the vectors pACYC184 and pMCL210 alone did not restore *nif* induction (Table 2). These results and the finding that Fnr affects NifA only in the presence of NifL (see above) strongly indicate that in the heterologous *E. coli* system, Fnr is required for release of NifL inhibition of NifA activity under anaerobic conditions.

The wild-type strain (NCM1528) grown in the presence of 10 mM ammonium showed *nif* inductions of approximately 3 ± 1 U/ml/OD_{600} unit independent of oxygen availability (data not shown). This induction level is significantly lower than the *nif*

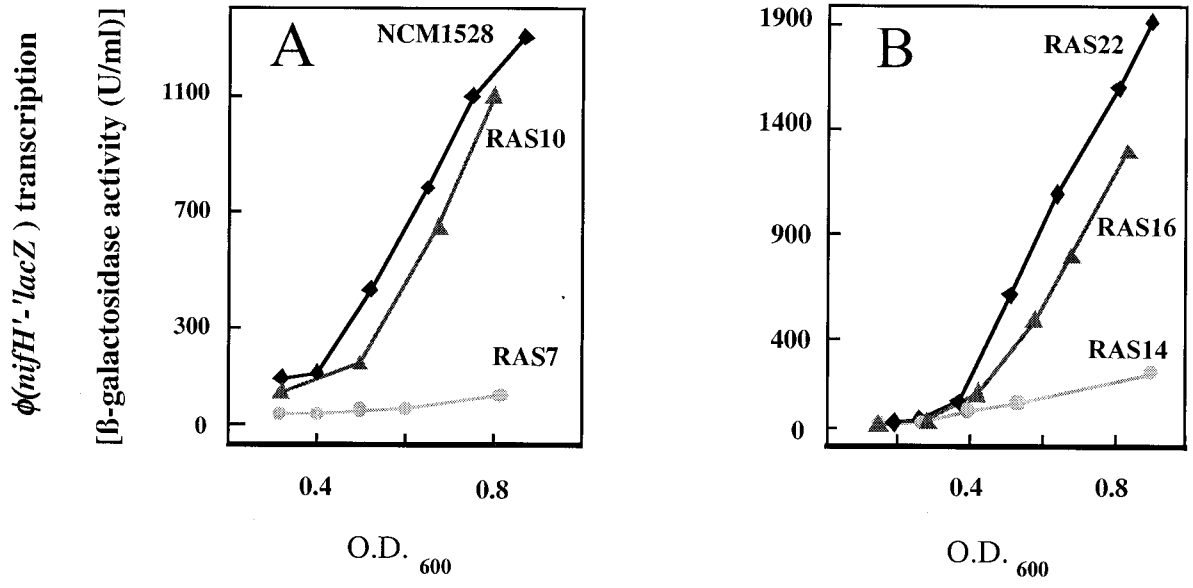

FIG. 2. Effects of *fnr* null alleles on expression of a ø(*nifH'-'lacZ*) fusion in heterologous *E. coli* strains carrying *K. pneumoniae nifLA* on a plasmid. The activity of β-galactosidase was plotted as a function of OD_{600} for cultures grown at 30°C in minimal medium under anaerobic conditions with 4 mM glutamine as a limiting nitrogen source. Differential rates of transcription from the *nifH* promoter, which reflect NifA activity, were determined from the slopes of these plots. All strains carried a single copy of a ø(*nifH'-'lacZ*) fusion at the *trp* locus (9) and plasmid pNH3 encoding NifL and NifA under the control of the *tac* promoter. (A) *fnr* null allele transduced from M182 (*fnr*::Tn*10*): wild-type NCM1528 (diamonds), the respective *fnr* null allele in NCM1528 (RAS7) (circles), and the complemented respective *fnr* mutant by constitutive expression of *E. coli fnr* on pACYC184 (RAS10) (triangles) are shown. (B) *fnr* null allele from RM101: wild-type RAS22 (diamonds), the respective *fnr* null allele in RAS22 (RAS14) (circles), and the complemented respective *fnr* mutant by constitutive expression of *E. coli fnr* on pACYC184 (RAS16) (triangles) are shown.

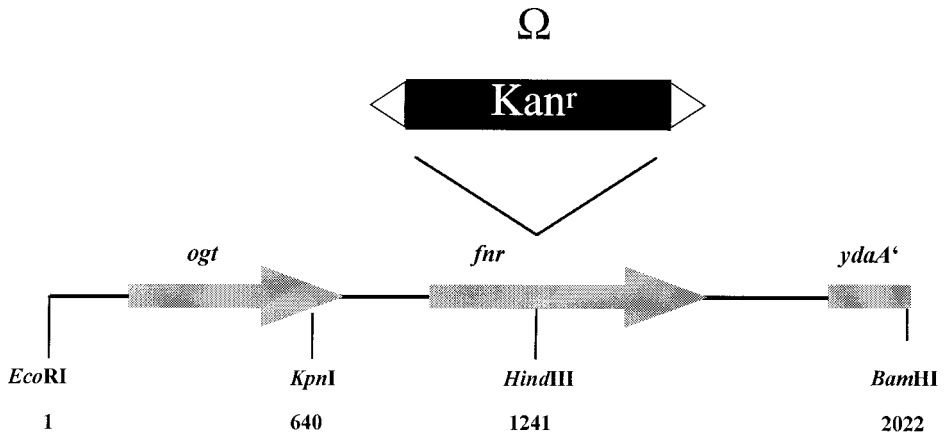

FIG. 3. Map of the cloned *Eco*RI-*Bam*HI fragment (pRS127) showing the site of insertion of the Ω interposon fragment with a kanamycin resistance gene derived from plasmid pHP45Ω (28) in *K. pneumoniae fnr*. The Ω interposon fragment is flanked by short inverted repeats, including strong transcription termination signals. The sequence of the *Eco*RI-*Bam*HI fragment has been submitted to GenBank under accession no. F220669.

induction observed in the *fnr* mutant strains (RAS7 and RAS14) under oxygen- and nitrogen-limiting growth conditions (100 ± 10 and 160 ± 10 U/ml/OD_{600} unit, respectively). These data suggest that Fnr is required for the oxygen signal transduction to NifL rather than for the ammonium signal transduction. They further indicate that in the absence of Fnr, NifL apparently does not receive the signal for absence of oxygen and therefore inhibits NifA activity under anaerobic conditions.

Studying the effect of Fnr on the *nif* induction in *K. pneumoniae*. In order to confirm the requirement of Fnr for relief of NifL inhibition under anaerobic conditions in the heterologous *E. coli* system, we constructed a chromosomal *fnr* null allele in *K. pneumoniae*. We used *K. pneumoniae* strain UN4495 carrying *nifLA* and a *nifK-lacZ* fusion on the chromosome, which allows monitoring of NifA-mediated transcription from the *nifHDK* promoter by measuring the differential rate of β-galactosidase synthesis (32). The *fnr* deletion was constructed on a plasmid by inserting an Ω interposon fragment with a kanamycin resistance gene into *K. pneumoniae fnr* (Fig. 3), which was then introduced into the chromosome by marker exchange using the *sac* system (see Materials and Methods). The disruption of the *fnr* gene was confirmed by PCR and Southern blot analysis (data not shown).

Klebsiella strains with the exception of RAS26 and RAS27 were generally grown in minimal medium under nitrogen limitation to exclude NifA inhibition by NifL in response to ammonium. The *fnr*::Ω mutation in *K. pneumoniae* UN4495 did not result in a significant growth rate reduction, but did reduce the *nif* induction under oxygen-limiting conditions to 10% of the *nif* induction in the parental strain. The observed induction level of the *K. pneumoniae fnr* mutant strain (RAS18) under anaerobic conditions (400 ± 20 U/ml/OD_{600} unit) again is in the same range as the *nif* induction in the presence of oxygen in the parental *K. pneumoniae* strain (220 ± 20 U/ml/OD_{600} unit) (Table 3). Determination of NifA and NifL proteins in the *fnr* mutant strain revealed no differences in the amount of the regulatory proteins compared to those of the parental strain (data not shown), indicating that the failure to express *nifH* could not be accounted for by a decrease in NifA expression. Normal NifL/NifA-dependent regulation was restored by introduction of the *K. pneumoniae fnr* gene expressed from the *tet* promoter on pRS137 into the *fnr* mutant (Fig. 4). *nif* induction in the complemented mutant (RAS19) was determined to be 3,800 ± 50 U/ml/OD_{600} unit, whereas the low-copy vector pACYC184 alone did not result in complementation (RAS20). These findings in the native background again suggest that Fnr is required for *nif* expression in *K. pneumoniae* under anaerobic conditions.

In order to confirm our finding in the heterologous *E. coli* system that Fnr is required to relieve NifL inhibition of NifA activity under anaerobic conditions, we studied the effect of the *fnr* null allele on NifA in *Klebsiella*. Plasmid pRS159 carrying $nifL^{C184S/C187S}nifA$ translationally coupled under the control of the *tac* promoter was introduced into *K. pneumoniae* UN4495 and the corresponding *fnr* mutant strain RAS18. Because growth in minimal medium in the presence of 10 mM ammonium results in repression of the chromosomal *nifLA* operon, under nitrogen sufficiency, only $nifL^{C184S/C187S}nifA$ from pRS159 is induced, resulting in the synthesis of NifA and a nonfunctional NifL protein (see above). Determination of β-galactosidase synthesis rates under those conditions in the *fnr* mutant strain (RAS27) and the parental strain (RAS26) showed that the absence of Fnr under anaerobic conditions does not affect NifA activity in the absence of a functional NifL protein (2,200 ± 50 and 2,350 ± 100 U/ml/OD_{600} unit, respectively) (Table 3). These results indicate that the Fnr effect on *nif* regulation observed in the native background is based on the Fnr requirement for relief of NifL inhibition under oxygen-limiting growth conditions. Based on our findings, we hypothesize that in *K. pneumoniae,* Fnr is the primary oxygen sensor for the *nif* regulation, which transduces the signal directly or indirectly to NifL.

DISCUSSION

Our goal is to determine how *K. pneumoniae* NifL perceives the oxygen status of the cells in order to regulate NifA activity

TABLE 3. Effects of an *fnr*::Ω mutation on NifL activity in *K. pneumoniae* UN4495

Strain	Relevant genotype[a]	Nitrogen source	Presence of oxygen	Expression of *nifH'-'lacZ* (U/ml/OD_{600} U)[b]	Doubling time (h)
UN 4495	Wild type	Glutamine	−	4,400 ± 100	3.5
UN 4495	Wild type	Glutamine	+	220 ± 10	2.0
RAS18	*fnr* mutant	Glutamine	−	400 ± 20	4.0
RAS18	*fnr* mutant	Glutamine	+	100 ± 10	2.2
RAS19	*fnr* mutant/*Ptet-fnr*[c]	Glutamine	−	3,800 ± 50	3.8
RAS20	*fnr* mutant/pACYC184	Glutamine	−	660 ± 30	4.2
RAS26	Wild type/*Ptac-nifL* mutant *nifA*	Ammonium[d]	−	2,350 ± 100	3.7
RAS26	Wild type/*Ptac-nifL* mutant *nifA*	Ammonium[d]	+	2,100 ± 100	1.7
RAS27	*fnr* mutant/*Ptac-nifL* mutant *nifA*	Ammonium[d]	−	2,200 ± 50	4.1
RAS27	*fnr* mutant/*Ptac-nifL* mutant *nifA*	Ammonium[d]	+	2,150 ± 150	1.6
RAS28	Wild type/*Ptac-nifLA*	Glutamine	−	2,400 ± 30	4.0
RAS28	Wild type/*Ptac-nifLA*	Glutamine	+	160 ± 5	1.6
RAS29	*fnr* mutant/*Ptac-nifLA*	Glutamine	−	430 ± 30	3.6
RAS29	*fnr* mutant/*Ptac-nifLA*	Glutamine	+	310 ± 30	1.6
RAS30	4495Δ*nifLA*/*Ptac-nifLA*	Glutamine	−	2,450 ± 30	4.1

[a] *nifL* mutant *nifA*, *nifL*$^{C184S/C187S}$ *nifA* (see Materials and Methods); *Ptac*, under the control of the *tac* promoter.
[b] Data are presented as mean values (± standard errors) of three independent experiments.
[c] *K. pneumoniae fnr* is expressed under the control of the *tet* promoter (*Ptet*).
[d] Grown in the presence of 10 mM ammonium to repress chromosomal *nifLA* induction.

in response to environmental oxygen. The main question concerning the oxygen signal transduction is whether NifL senses oxygen directly via a redox-induced conformational change, or whether oxygen is detected by a more general system. After receiving the oxygen signal, directly or indirectly, the redox state of the flavoprotein NifL is thought to influence the ability of NifL to modulate the NifA activity in response to environmental oxygen and to allow NifA activity only in the absence of oxygen (13, 22, 31). We recently showed that iron is specifically required for *nif* induction, but is not present in NifL (31, 32). To determine whether this iron requirement for *nif* induction could be accounted for by the role of Fnr in transducing the oxygen signal to NifL, we determined the effect of an *fnr* null allele on *nif* regulation. Using different genetic backgrounds and independent *fnr* null alleles, we were able to show that the absence of Fnr affects the *nif* regulation dramatically. The *nif* induction in the absence of Fnr was low, similar to the *nif* induction under aerobic conditions, even though cells were growing under oxygen limitation. Normal *nif* regulation was achieved in the mutant strains by introduction of a low-copy vector expressing *fnr* constitutively (Fig. 2 and 4). These data indicate that Fnr is required to relieve NifL inhibition of NifA activity under anaerobic conditions, and this appears to account for the iron requirement of *nif* induction (32). Therefore, in addition to the rhizobial homologous Fnr proteins, FnrN and FixK, which are known to be involved in regulation of nitrogen fixation in the symbiotic bacteria (8; see reference 4 and references cited therein), in *K. pneumoniae,* the transcriptional activator Fnr is apparently also involved in regulation of nitrogen fixation. These results are in contrast to the report of Hill (12), that redox regulation of *nif* expression in a heterologous *E. coli* strain is independent of the *E. coli fnr* gene product. This discrepancy may be due to experimental differences. We determined NifA-mediated transcriptional activation by measuring differential rates of β-galactosidase expression from a chromosomal *nifK-lacZ* fusion in order to monitor *nif* induction. In contrast, Hill determined acetylene reduction by nitrogenase after growing heterologous *E. coli fnr* mutant strains carrying the Nif$^+$ plasmid pRD1 under derepressing conditions. Also, because plasmid pRD1 contains in addition to the *nif* genes nonidentified *K. pneumoniae* genes (3), we cannot completely rule out that *K. pneumoniae fnr* is encoded on the plasmid. Apart from these experimental differences concerning the heterologous *E. coli* systems, we confirmed the Fnr requirement for the *nif* regulation in the native-genetic-background *K. pneumoniae*.

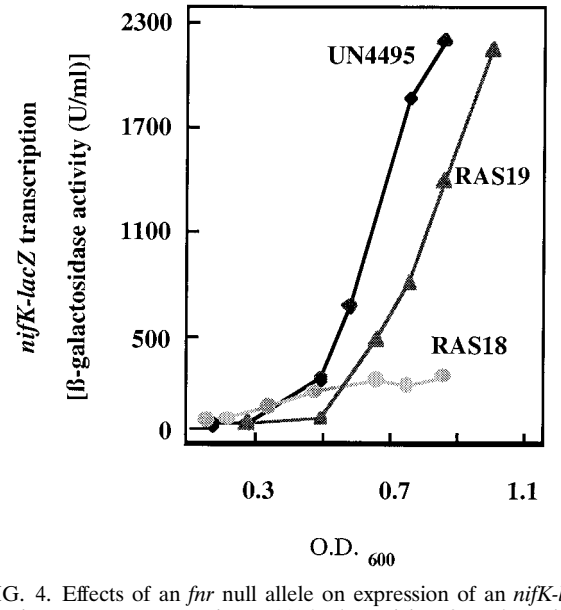

FIG. 4. Effects of an *fnr* null allele on expression of an *nifK-lacZ* fusion in *K. pneumoniae* strain UN4495. The activity of β-galactosidase was plotted as a function of the OD_{600} for cultures grown at 30°C in minimal medium under anaerobic conditions with 4 mM glutamine as a limiting nitrogen source. Differential rates of transcription from the *nifHDK* promoter were determined from the slopes of these plots. Wild-type UN4495 (diamonds), the *fnr* mutant strain of UN4495 (RAS18) (circles), and the complemented respective *fnr* mutant by constitutive expression of *K. pneumoniae fnr* on pACYC184 (RAS19) (triangles) are shown.

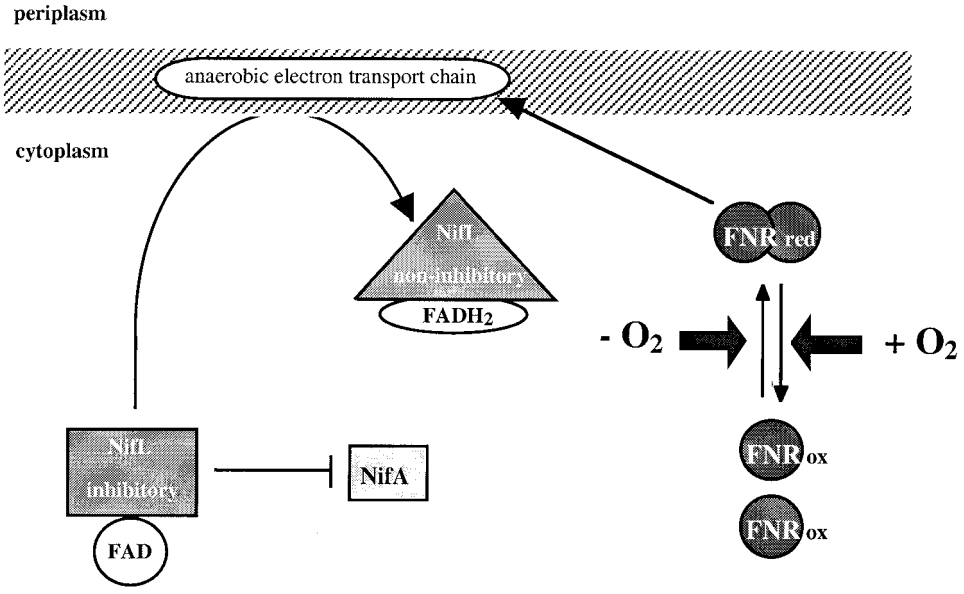

FIG. 5. Hypothetical model of oxygen signal transduction in *K. pneumoniae*. red, reduced; ox, oxidized.

We further showed that the general oxygen sensor Fnr is required for relief of NifL inhibition under anaerobic growth conditions and that the presence of ammonium results in significantly lower *nif* inductions in the wild-type strain than those observed in *fnr* mutant strains under nitrogen and oxygen limitation. Both of these findings suggest that the oxygen signal is not detected by NifL directly but by Fnr, which transduces the signal—directly or indirectly—to NifL. However, at this state of experimental data, we cannot completely rule out that the Fnr requirement might be due to some Fnr-dependent metabolic signals not directly related to the lack of oxygen. If Fnr is indeed the primary oxygen sensor for the *nif* regulation in *K. pneumoniae,* how the oxygen signal is transmitted to NifL remains to be explained. Fnr either is transducing the oxygen signal by directly interacting with NifL in the absence of oxygen, or under anaerobic conditions, Fnr is activating the transcription of a gene or genes whose product or products mediate the signal to NifL. Because Fnr is a transcriptional activator and can be excluded as the physiological electron donor for NifL reduction, it is more reasonable that under anaerobic conditions, Fnr transduces the signal by transcriptional activation.

Hypothetical model for oxygen signal transduction. In *K. pneumoniae,* as in *A. vinelandii,* the redox state of the flavoprotein NifL is thought to influence its ability to modulate the NifA activity in response to the oxygen levels. However, the physiological electron donors for NifL have not yet been identified (19, 22). If the redox state of the flavoproteins is indeed responsible for mediating the oxygen signal to NifA, one could postulate that by reducing the cofactor of NifL, the physiological electron donor is transducing the oxygen signal to NifL. Thus, the physiological electron donor for the NifL reduction may be a component of the oxygen signal transduction. Because one can exclude Fnr as the physiological electron donor for NifL reduction in the absence of oxygen, one has to postulate another downstream signal transductant following Fnr. We therefore hypothesize that in the absence of oxygen, Fnr activates transcription of a gene or genes whose product or products function to relieve NifL inhibition by reducing the FAD cofactor of NifL. Attractive hypothetical candidates for the physiological electron donor for NifL are components of the anaerobic electron transport system (Fig. 5), particularly the electron transport system to fumarate, whose transcription under anaerobic conditions is directly dependent on Fnr activation (1, 24, 34, 38). Preliminary data, which indicate that *K. pneumoniae* NifL under anaerobic conditions is membrane associated, whereas in the presence of oxygen NifL is in the cytosolic fraction, support this model (Klopprogge and Schmitz, unpublished). Studies of the anaerobic electron transport system components as potential physiological electron donors for NifL are in process.

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