Regulation of nitrogen fixation in *Klebsiella pneumoniae*: The role of Fnr in oxygen signal-transduction

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Summary

- In the free-living diazotroph *Klebsiella pneumoniae*, a member of the γ-subgroup of Proteobacteria, nitrogen fixation (*nif*) genes are under the control of the *nifLA* operon, the products of which regulate transcription of the *nif* operons. NifA activates *nif* gene transcription by alternative RNA polymerase, σ^{54}-holoenzyme; the negative regulator NifL modulates activity of NifA in response to molecular oxygen and combined nitrogen. Transcriptionally coupled synthesis, immunological studies and complex analysis of both regulators indicate that NifL-mediated inhibition of NifA depends on direct protein-protein interaction.
- The negative regulator NifL is a flavoprotein, which modulates NifA activity depending on the redox state of its N-terminally bound FAD-cofactor. Thus, oxygen might be sensed directly by the redox-sensitive cofactor of NifL or by a global oxygen sensor, for example Fnr (fumarate nitrate reductase regulator), which transduces the oxygen signal towards the NifL-bound cofactor.
- The *fnr* gene of *K. pneumoniae* was cloned, sequenced and biochemically analyzed. The analysis of the deduced amino acid sequence revealed 98 % similarity to the *Escherichia coli* Fnr protein. The conserved cystein residues, which establish the oxygen-sensing [4Fe-4S]-cluster, are located in the N-terminal domain of the *K. pneumoniae* Fnr as it is kown for the *E. coli* protein. Biochemical analysis of the glutathionS-transferase (GST) fusion protein Fnr-GST expressed and purified under aerobic or anaerobic conditions, revealed decreased amounts of iron and acid-labile sulphur in the aerobic protein compared to the anaerobic protein. This indicates that *K. pneumoniae* Fnr sesnes oxygen based on an oxygen-sensitive iron-sulphur cluster.
- Studying the oxygen dependent regulation of *nif* induction in *fnr* mutant backgrounds we obtained strong evidence that in *K. pneumoniae* Fnr is the primary oxygen sensor for the *nif* regulatory system. In the absence of Fnr, NifL did not receive the signal of anaerobiosis under nitrogen and oxygen limited conditions resulting in a decreased NifA activity. Thus, Fnr appears to sense the oxygen status of the cell and presumably transduces the signal of anaerobiosis towards Nifl by activating gene(s), the product(s) of which function to reduce the FAD cofactor of NifL resulting in a non-inhibitory conformation. Attractive candidates for the physiological electron donor for NifL

reduction are components of the anaerobic electron transport chain, which are Fnr-dependent transcribed.

- Localization experiments of NifL in *K. pneumoniae* under different growth conditions revealed that NifL is highly membrane associated under derepressing growth conditions. However, when cells were shifted to ammonium sufficiency or presence of oxygen NifL is located in the cytoplasm. Further studies using *K. pneumoniae* mutant strains showed that under derepressing conditions but in the absence of either Fnr or the nitrogen sensor GlnK NifL was located in the cytoplasm and inhibited NifA activity. Presumably in the absence of Fnr or GlnK NifL does not receive the signal of anaerobiosis or nitrogen limitation. In contrast to NifL, NifA remains in the cytoplasm under all conditions tested. Thus, sequestration of NifL to the membrane under nitrogen and oxygen-limitation is involved in the mechanism of NifA regulation.
- Biochemical analysis of purified NifL showed that NifL-bound FAD-cofactor was reduced by $NADH/H^+$ only in the presence of a redox mediator or inside-out vesicles derived from anaerobically grown *K. pneumoniae* cells. This indicates that *in vivo* NifL is reduced at the cytoplasmic membrane.
- In order to identify the physiological electron donor for NifL reduction, the effect of different oxidoreductase systems on *nif* regulation was studied in the respective mutant backgrounds. Using *K. pneumoniae* mutant strains we observed strong evidence, that in the absence of a functional NADH:ubiquinone oxidoreductase or formate dehydrogenaseN NifL inhibition of NifA was not relieved. The same effect was observed in a heterologous *E. coli* system lacking the alternative NADH dehydrogenase (*ndh*). Further studies of *nif* induction of anaerobically grown cultures on glycerol showed significantly reduced NifA activity when nitrate was added as additional electron acceptor. Taking together these findings indicate that more than one oxidoreductase system appears to be responsible for NifL reduction and that NifL receives electrons from the reduced quinone pool.
- We further demonstrated that reduced dimethylnaphthoquinone (DMN_{red}), a soluble quinone derivative is able to reduce the FAD cofactor of NifL in the absence of a redox mediator. This finding supports our model that the cofactor FAD of the membrane-associated NifL receives electrons from the reduced quinone pools, generated by different oxidoreductase systems.

Chapter 1:

Introduction

Biological nitrogen fixation, the enzymatic reduction of molecular nitrogen (N_2) to ammonia, is strictly limited to prokaryotes. However, within the prokaryotes nitrogen fixation is found in a large number of species belonging to the bacterial domain and in several methanogenic *Archaea* (Dean and Jacobson, 1992; Young, 1992; Lobo and Zinder, 1992; Fischer, 1994; Galagan *et al*., 2002; Deppenmeier *et al*., 2002). The reduction of molecular nitrogen is catalyzed by the nitrogenase enzyme complex with high energy demands. Two ATP molecules are consumed for each electron transferred to the catalytic site (Burgess and Lowe, 1996; Howard and Rees, 1996; Rees and Howard, 1999, Halbleib and Ludden, 2000). Because of the high energy requirement for N_2 fixation, up to 40 % of the ATP is utilized by the nitrogenase in nitrogen fixing cells, resulting in a drop of the energy charge from 0.9 to 0.5 (Daesch and Mortenson, 1972; Upchurch *et al*., 1980). In the presence of molecular oxygen the nitrogenase enzyme complex is irreversibly inactivated. Thus, to avoid unnecessary consumption of energy nitrogen fixing microorganisms tightly control synthesis and activity of nitrogenase in response to nitrogen and oxygen availability. In all diazotrophic proteobacteria examined, the transcriptional activator NifA is required for transcription of the *nitrogen fixation* (*nif*) genes. NifA expression and activity is regulated in response to the environmental signals, molecular oxygen and combined nitrogen. However, the mechanisms of NifA regulation vary in different organisms (Fischer, 1996; Dixon, 1998; Halbleib and Ludden, 2000; Schmitz *et al*., 2002). In free-living and symbiotic diazotrophs belonging to the α-and β-subgroup of the proteobacteria (genera *Rhizobium*, *Bradyrhizobium*, *Azospirillum* and *Herbaspirillum*) NifA activity is directly sensitive to molecular oxygen and in some cases affected in the presence of combined nitrogen (Fischer, 1994; Fischer, 1996; Steenhoudt and Vanderleyden, 2000). In contrast, in *Klebsiella pneumoniae* and *Azotobacter vinelandii*, two free-living diazotrophs, which belong to the γ-proteobacteria, NifA activity is not oxygen sensitive. NifA activity is regulated in response to molecular oxygen and fixed nitrogen by a second regulator NifL, the gene of which forms an operon with *nifA* (Filser, 1983; Dixon, 1998). In *K. pneumoniae* the expression of the *nifLA* operon itself is regulated by the nitrogen status, via the NtrB/NtrC two component regulatory system, whereas in *A. vinelandii nifLA* is constitutively expressed (Drummond and Wootton, 1987; Blanco *et al.,* 1993). Interestingly, it was recently found that nitrogen fixation of the endophytic diazotroph *Azoarcus spec.* - belonging to the β-proteobacteria - is also regulated by the coordinated activities of *nifL* and

nifA gene products in response to environmental signals (Egener and Reinhold-Hurek, unpublished).

NifL modulates NifA transcriptional activity by direct protein-protein interaction. The transcriptional activator NifA is composed of three domains: an amino (N)-terminal domain apparently involved in the regulation, a central catalytic domain, and a carboxy (C)-terminal DNA-binding domain (Drummond *et al.*, 1990; Morett and Segovia, 1993). Transcription of *nif* genes by the alternative RNA polymerase (σ^{54}-RNA polymerase) is generally activated by NifA, which binds to an upstream activation sequence (UAS) (Morrett and Buck, 1988) and contacts promoter-bound σ^{54}-RNA polymerase by means of a DNA loop (Buck *et al.,* 1987). Subsequently NifA catalyzes the isomerization of closed complexes between σ^{54}-holoenzyme and the *nif* promoter to transcriptionally productive open complexes (Morett and Buck, 1989; Hoover *et al.,* 1990). This open complex formation requires hydrolysis of ATP or GTP catalyzed by NifA (Lee *et al*., 1993; Austin *et al.,* 1994). In the presence of molecular oxygen or combined nitrogen, NifL inhibits NifA activity *in vivo* (Merrick *et al.,* 1982; Hill *et al.,* 1981; Dixon, 1998; Schmitz *et al*. 2002). The inhibitory protein NifL is composed of two domains separated by a hydrophilic interdomain linker (Q-linker) (Söderbäck *et al.*, 1998; Drummond and Wootton, 1987). The C-terminal domain of NifL shows homology to a histidine protein kinase (Blanco *et al.,* 1993). However, neither autophosphorylation nor possible phosphor transfer between the two regulatory proteins NifA and NifL has been detected in *K. pneumoniae* or *A. vinelandii* (Lee *et al.*, 1993; Austin *et al.*, 1994; Schmitz *et al.*, 1996). The translationally coupled synthesis of *nifL* and *nifA* and immunological studies imply that the inhibition of NifA activity by NifL apparently occurs via a direct protein-protein interaction (Govantes *et al.,* 1998; Henderson *et al.*, 1989). Recently, complex formation between *A. vinelandii* NifL and NifA has been demonstrated by *in vitro* co-chromatography in the presence of adenosine nucleotides and using the yeast two hybrid system (Money *et al*., 2001 and 1999; Lei *et al.*, 1999). Thus, signal transduction apparently occurs via protein-protein interaction. Interestingly, for *A. vinelandii* it was shown that NifL influences both NifA transcriptional activity and DNA-binding capacity *in vitro* (Barrett *et al.,* 2001). The C-terminal domain of *K. pneumoniae* NifL is sufficient to inhibit transcriptional activation by NifA *in vitro* and *in vivo* (Narberhaus *et al.,* 1995). This indicates that the inhibitory function of NifL protein appears to be located in its C-terminal domain, which presumably interacts with NifA by protein-protein interaction.

Nitrogen signal transduction. In *K. pneumoniae,* a shift from nitrogen limitation to nitrogen sufficiency results in repression of *nif* gene induction upon inhibition of NifA transcriptional activity by NifL (Arnott *et al.* 1989; Blanco *et al.*, 1993). This indicates that NifL either senses the nitrogen availability directly or the nitrogen status is sensed in a NifL independent manner and the signal is subsequently transduced to NifL or the NifL/NifA complex. Interestingly, like in *Escherichia coli* a second PII-like protein, encoded by *glnK*, was recently discovered in *K. pneumoniae. glnK* is organized with *amtB* (encoding for an ammonium transporter) in an operon, which is under transcriptional control of NtrC. Upon the high similarity to the PII-protein, the GlnK-protein is an attractive candidate for sensing changes in the glutamine pool size - reflecting the internal nitrogen status - and mediating the signal of the nitrogen status to the *nif* regulatory system (Atkinson and Ninfa, 1998; Xu *et al.,* 1998; van Heeswijk *et al.,* 1996). Studying *nif* regulation in *glnK* mutant strains strong evidence was obtained, that GlnK is indeed required to release NifL inhibition under nitrogen-limiting growth conditions in *K. pneumoniae* (He *et al*., 1998; Jack *et al*., 1999; Arcondeguy *et al*., 1999). This indicates that changes of the internal nitrogen status are not sensed by NifL directly, but are apparently mediated by GlnK to the NifA/NifL regulatory system. Whereas NifL is a negative regulator, GlnK acts positively to antagonize inhibitory effects of NifL under nitrogen-limiting conditions. The uridylylation status of GlnK is probably not required for relief of NifL inhibition (He *et al*. 1998; Arcondeguy *et al*., 1999). Interestingly, the T-loops of GlnK and PII from *K. pneumoniae*, which are supposed to interact with other components involved in the signal transduction, differ only in three amino acid residues 43, 52 and 54. It has been shown that for regulation of the *nif* system residue 54 is the most important amino acid in the T-loop of GlnK, possibly directly involved in the interaction with NifL/NifA (Arcondeguy *et al*., 2000). Although GlnK function has been clearly demonstrated, the question arises, how GlnK is mediating the nitrogen signal towards the NifL/NifA regulatory system. The nitrogen signal is apparently mediated by direct protein-protein interaction but it has to be elucidated, whether GlnK is interacting directly with NifL or is affecting the NifL/NifA complex formation. For diazotrophs not belonging to the γ-proteobacteria and missing NifL (e.g. *Herbaspirillum seropedicae* and *Azospirillum brasilense*) experimental data indicate that the PII proteins participate in signaling the nitrogen status to the N-terminal domain of NifA (Steenhoudt and Vanderleyden, 2000; Souza *et al.,* 1999; Monteiro *et al.,* 1999, Arsene *et al*., 1999).

A. vinelandii contains only one PII-like protein, encoded in a *glnK/amtB*-operon, which is expressed constitutively (Meletzus *et al.,* 1998). Interestingly, *A. vinelandii* GlnK has a T-

loop structure, which resembles more the 'GlnB-like' T-loop rather than the 'GlnK-like' T-loop (Arcondeguy *et al*., 2000). Recent studies concerning the role of *A. vinelandii* GlnK in nitrogen sensing and transducing the nitrogen status to the *nif* regulatory system showed that GlnK is not required for derepression in *A. vinelandii*. In contrary to *K. pneumoniae*, where GlnK apparently has a positive role in relieving NifL inhibition under nitrogen limiting conditions, *in vitro* experiments suggest that the inhibitory function of *A. vinelandii* NifL is activated under nitrogen excess through interaction with PII-like regulatory proteins (Reyes-Ramirez *et al.,* 2000; Little *et al.,* 2000 and 2002). Recently interactions between NifL and GlnK have been reported for *A. vinelandii* using the yeast two-hybrid system (Rudnick *et al*., 2002) and it was demonstrated *in vitro* that GlnK interacts with the C-terminal domain of NifL (Little *et al*., 2002). Dixon and coworker proposed that interaction with NifL only occurs when GlnK is not uridylylated and activates NifL inhibitory functions under nitrogen sufficiency (Little *et al*., 2002). This suggests that NifA inhibition by NifL is relieved when GlnK is uridylylated, but uridylylated GlnK is not required for this relief. However, very recently Merrick and coworkers showed that in *E. coli* and *A. vinelandii* non-uridylylated GlnK is highly membrane associated after a shift to nitrogen sufficiency upon binding to the ammonium transporter AmtB (Coutts *et al*., 2002) and thus, unmodified GlnK should not be available in the cytoplasm to activate NifL inhibitory functions.

NifL response to molecular oxygen. The N-terminal domain of NifL contains conserved S-motifs of PAS-like domains, which are known for a number of regulators sensing oxygen, redox or light (Zhulin *et al*., 1997; Taylor and Zhulin, 1999). This indicates that the N-terminal domain is involved in signal transduction. Biochemical analyses of purified proteins showed that NifL from *A. vinelandii* and from *K. pneumoniae* is a flavoprotein with an N-terminally bound FAD-cofactor (Hill *et al.*, 1996; Schmitz, 1997; Söderbäck *et al.,* 1998; Klopprogge and Schmitz, 1999). Analysis of the inhibitory function of NifL-holoenzyme and NifL-apoenzyme on NifA activity in *in vitro* transcription assays showed that the FAD-cofactor is not directly required for NifL inhibitory function (Schmitz, 1997). This indicates that FAD acts as a redox-sensitive cofactor, which might be involved in the oxygen signal transduction. The oxidized form of NifL inhibits NifA transcriptional activity *in vitro*, whereas *A. vinelandii* NifL reduced by sodium dithionite or by the flavoheme protein (Hmp) from *E. coli* with NADH/H^+ as electron donor does not antagonize open complex formation by NifA *in vitro* (Macheroux *et al*., 1998). Thus, reduction of the flavin moiety of NifL results in a non-inhibitory form of NifL, however functional and physiological relevance for the

reduction of NifL by Hmp, which is proposed to be a global oxygen sensor (Pool, 1994), has not been demonstrated to date. These findings support the model that NifL acts as a redox-sensitive regulatory protein that modulates NifA activity in response to the redox state of its FAD-cofactor and allows NifA activity only in the absence of oxygen. However, in both organisms the physiological electron donor for NifL is not known.

Reduction of the FAD-cofactor by the physiological electron donor apparently transduces the signal for anaerobiosis to NifL. As a consequence, components of the oxygen signal transduction are attractive candidates for the electron transfer towards NifL *in vitro*. Thus, the key question concerning the oxygen signal transduction is, whether NifL senses the oxygen status of the cell directly via a redox induced conformational change. Alternatively, oxygen might be detected by a more general oxygen-sensing system, which then regulates NifL by inducing the oxidation or reduction of the flavin cofactor. In this respect it is of interest that in *K. pneumoniae,* iron is specifically required for relief of NifL inhibition under oxygen and nitrogen limitation (Schmitz *et al.,* 1996). The finding that *K. pneumoniae* NifL does not contain non-heme iron or an acid-labile sulphur cluster (Schmitz *et al.,* 1996; Klopprogge and Schmitz 1999), indicates the presence an iron containing protein in the oxygen signal cascade towards NifL. In *E. coli* the transcriptional regulator Fnr (fumarate nitrate reductase regulator) plays an overarching role in sensing the switch from anaerobic to aerobic conditions. The mechanism of oxygen sensing in Fnr is mediated via an [4Fe-4S]-cluster (Green *et al*., 1996; Unden and Shirawski, 1997; Kiley and Beinert, 1998). Interestingly, in *Rhizobium leguminosarum* FnrN, a Fnr homologous protein, regulates nitrogen fixation in an oxygen-dependent manner (Gutierrez *et al*., 1997). Thus, it is attractive to speculate that a Fnr homologous protein is involved in oxygen-dependent regulation of nitrogen fixation in *K. pneumoniae*.

The intention of this thesis was to study the signal transduction of molecular oxygen towards NifL in *K. pneumoniae*. Investigations were performed to study (i) the role of Fnr in the oxygen-sensing mechanism for nitrogen fixation (chapter 2 and 3), (ii) the cellular localization of NifL followed by functional analyses of NifL localization for NifA regulation (chapter 4), and (iii) the effect of membrane-bound oxidoreductase systems concerning oxygen sensing on *nif* regulation (chapter 5).

Chapter 2:

Cloning, sequencing and characterization of Fnr from *Klebsiella pneumoniae*

ABSTRACT

The transcription factor Fnr (fumarate nitrate reductase regulator) globally regulates gene expression in response to oxygen deprivation in *Escherichia coli*. We report here the cloning and sequencing of the *fnr* gene from the facultative anaerobic bacterium *Klebsiella pneumoniae* M5al, another member of the enteric bacteria. The deduced amino acid sequence of *K. pneumoniae fnr* showed very high similarity (98 % amino acid identity) to the Fnr protein from *E. coli* and contained the four essential cysteine residues which are presumed to build the oxygen-sensing $[4Fe4S]^{+2}$ center. Transfer of the *K. pneumoniae* gene to a *fnr* mutant of *E. coli* complemented the mutation and permitted synthesis of nitrate reductase and fumarate reductase during anaerobic growth. A gene fusion between *K. pneumoniae fnr* and glutathione S-transferase was constructed and expressed in *E. coli* under anaerobic conditions in order to make the protein available in preparative amounts. The overproduced protein was purified by glutathione-Sepharose 4B affinity chromatography in the absence of oxygen, and biochemically characterized.

INTRODUCTION:

Many of the oxygen-responsive gene regulators of bacteria are members of the fumarate nitrate reductase / cyclic AMP receptor protein family of transcriptional regulators (Spiro 1994, Gunsalus & Park 1994, Unden et al. 1995). The fumarate nitrate reductase regulator from *Escherichia coli* (Fnr_{Ec}) acts as a redox-responsive transcriptional regulator that activates genes whose products are involved in anaerobic respiration and represses other genes required for aerobic respiration (Spiro 1994, Gunsalus & Park 1994, Unden et al. 1995, Bauer et al. 1999). It contains a cluster of three closely-spaced cysteine residues located near the N-terminus ($^{20}CysX_2CysX_5{}^{29}Cys$) plus an additional cysteine residue, Cys122. These cysteine residues are required for the oxygen-sensing function (Spiro & Guest 1988). Recent data suggest that these residues bind an $[4Fe4S]^{+2}$-cluster and that this cluster apparently

mediates the sensitivity of the transcriptional activator to oxygen (Green et al. 1996; Khoroshilova et al. 1997; Kiley & Beinert 1998). In addition, the presence of the $[4Fe4S]^{+2}$-cluster in the anaerobically-purified form of Fnr is correlated with dimerization and specific DNA binding. Upon addition of oxygen, the $[4Fe4S]^{+2}$-cluster is disrupted, resulting in the conversion of Fnr into an inactive monomeric protein (Lazazzera et al. 1996; Melville & Gunsalus 1996). Homologs of Fnr have been identified in several gram-negative and gram-positive bacteria, some of which differ with respect to the cystein residues and the coordination of the iron-sulphur clusters (reviewed in Spiro 1994; Cruz Ramos et al. 1995; Saunders et al. 1999; Vollack et al. 1999). Recently discovered examples of Fnr homologues, which do not exhibit the structural elements or coordinate the iron-sulphur clusters differently are: (i) Fnr from *Bacillus subtilis* and *B. licheniformis,* for which a C-terminal cluster coordination is found (Cruz Ramos et al. 1995; Klinger et al. 1998); (ii) Fnr homologues from *Lactobacillus casei* and *L. lactis,* that lack two of the four essential cysteine residues and in the case of *L. casei*, Flp redox sensitive switch is operated based on a reversible interconversion of an intramolecular disulphide bridge (Gostick et al. 1998; Scott et al. 2000); and (iii) the Fnr homologues DnrD, DnrE and DnrS of *Pseudomonas stutzeri*, which completely lack the respective cysteine residues and iron-sulphur centres (Vollack et al. 1999).

Adaptation of the facultative anaerobic bacterium *Klebsiella pneumoniae* to anaerobic growth conditions is also accompanied by dramatic changes in metabolic gene expression. In addition, it is only when growing in the absence of molecular oxygen that *K. pneumoniae* is able to use molecular nitrogen as sole nitrogen source under nitrogen limitation (Dixon 1998). In order to make these adaptations, *K. pneumoniae* must sense changes in environmental oxygen availability. In contrast to *E. coli,* little is known about a regulatory oxygen-sensing system in *Klebsiella*. However, there are some evidences suggesting the presence of an Fnr-homologue in *K. pneumoniae*: Fnr is possibly involved in expression of the citrate-specific fermentation genes in *K. pneumoniae* (Bott et al. 1995) and in *K. terrigena* Fnr might act as a repressor of the butanediol (*bud*) operon (Mayer et al. 1995).

In this communication we report on the sequencing and characterization of the regulatory gene *fnr* from *K. pneumoniae*.

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. (1990) or by electroporation using a Gene pulser and Pulse controller (BioRad Laboratories). The *fnr*::Tn*10* allele was transferred from the *fnr*::Tn*10* derivative of M182 (Jayaraman et al. 1988) by P1-mediated transduction into NCM1529 and RM123 as described previously (Silhavy et al. 1984) with selection for tetracycline resistance; the resulting strain designated RAS1 and RAS6 respectively. Strains RAS3, RAS4 and RAS5 contain plasmids pRS120, pRS127 and pRS137, respectively, in RAS1; strain RAS21 contains pRS137 in RAS6. Plasmids pRS120 and pRS137 contain the *E. coli fnr* gene and *K. pneumoniae fnr* gene, respectively, inserted into the *Sal*I and *Eco*RV site of pACYC184 and thereby expressed from the *tet* promoter.

Media and growth conditions.

For cloning, *E. coli* was routinely grown in LB medium at 37 °C (Ausubel et al. 1987). The medium was supplemented with ampicillin at 100 µg/ml or chloramphenicol at 15 µg/ml to maintain recombinant plasmids; additionally, 5 µg/ml tetracycline was added to the growth medium when NCM1529(*fnr*::Tn*10*) or RM123(*fnr*::Tn*10*) were the host strains. For complementation experiments, strains were grown under anaerobic conditions with N_2 as gas phase at 37 °C in minimal medium (100 mM KH_2PO_4, 50 mM $NaHPO_4$, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 µM Na_2SeO_3, 10 µM Na_2MoO_4, 0.3 mM sulfide and 0.002 % resazurine (to monitor anaerobiosis) pH = 6.5), containing 0.8 % glycerol as the C-source and 1 % KNO_3 as the only nitrogen source. Precultures were grown overnight in closed bottles, with N_2 as gas phase, in medium lacking sulfide and resazurine, and additionally supplemented with 4 mM ammonium acetate which was completely utilized after growth of the precultures to saturation. The main cultures (25 ml) were inoculated from saturated precultures and were grown in closed bottles at 37° C without shaking.

Construction of a gene library of *K. pneumoniae* chromosomal DNA.

Chromosomal DNA from *K. pneumoniae* M5a1 was isolated according the method described by Ausubel et al. (1987). Fifty micrograms of DNA was partially digested with *Sau*3AI so that the majority of fragments were in the size range of between 20 and 30 kbp. The purified digested DNA was ligated to 1 µg pWE15, which had been completely digested with *BamH*I and dephosphorylated. The ligation mixture was then packed and transduced

into *E. coli* VCS257 using the Gigapack III Gold (Stratagene, La Jolla, US) packaging extract according the protocol of the manufacturer. Approximately 8000 colonies were collected.

Generation of a 100 bp hybridization probe for the *fnr* gene from *K. pneumoniae*.

A probe for the *fnr* gene was obtained by PCR using genomic DNA from *K. pneumoniae* as template. The oligonucleotides were derived from the *E. coli fnr* sequence: 5' primer (5'ATCAATTACGGATCCAGCAGACCTATGATCCCG3') and 3' primer (5'GTGTGAACG GGATCCAAAGCTGGC3'). Reactions were carried out in 100 µl volumes using Vent polymerase (New England Biolabs, UK) and primers at a concentration of 0.3 µM. The annealing temperature was at 65 °C and synthesis was carried out for 30 s, for 25 cycles. The 100 bp PCR product was purified with Wizard® Plus PCR Purification system (Promega, Heidelberg, Germany) and labeled with the random Dig-labeling kit from Boehringer Mannheim according the protocol of the manufacturer. The specificity of the probe was tested by Southern hybridizations (Sambrook et al. 1989) with *K. pneumoniae* DNA digested completely digested by *Bam*HI and *Eco*RI. Under the conditions employed, the hybridization with the labeled probe resulted in only one hybridization signal in each digest.

TABLE 1: **Bacterial strains and plasmids used in this study**

Strains / plasmids	Relevant genotype and/or characteristic(s)	Reference or description
Strains		
M182(*fnr*::Tn*10*)	M182 but *fnr*::Tn*10*	Jayaraman et al. 1998
NCM1529	*araD139Δ(argF-lacU)169 fth D5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1 trpDC700putPA1303::[*Kan^r*-(nifH'-'lacZ)]*	He et al. 1997
RAS1	NCM1529 but *fnr*::Tn*10*	

		See Materials and Methods
RAS3	RAS1/pRS120	See Materials and Methods
RAS4	RAS1/pRS127	See Materials and Methods
RAS5	RAS1/pRS137	See Materials and Methods
Plasmids	Relevant genotype and / or characteristic(s)	Reference or description
pWE15	cosmid vector	Stratagene, La Jolla, US
pBluescript SK+	cloning vector	Stratagene, La Jolla, US
pACYC184	low copy vector	New England Biolabs (UK)
pGEX-2T	Expression vector, expression in fusion with glutathione-S transferase	Pharmacia, Freiburg Germany
pRS120	*E. coli fnr* controlled by the *tet* promoter on pACYC184	See Materials and Methods
pRS127	2.1 kbp fragment in pBluescript SK+ containing *K. pneumoniae fnr*	See Materials and Methods

pRS131	*K. pneumoniae fnr* cloned into pGEX-2T under the control of the *tac* promoter, coding for glutathione-S transferase fused to Fnr	See Materials and Methods
pRS137	*K. pneumoniae fnr* controlled by the *tet* promoter on pACYC184	See Materials and Methods

Cloning and sequencing of *K. pneumoniae fnr* gene.

Heterologous cosmids from the gene library of *K. pneumoniae* chromosomal DNA was completely digested by *Bam*H1 and *Eco*RI. After blotting onto Nylon membrane Hybond-N (Amersham) and Southern hybridization (Sambrock et al. 1989) using the 100 bp probe, the digested cosmids were screened for positives using the luminescent detection kit for nucleic acids from Boehringer Mannheim. Three positive cosmids were obtained and subcloned into pSK^+ Bluescript (Stratagene, La Jolla, US) resulting in plasmid pRS127, containing a 2.1 kbp *Eco*RI/*Bam*HI fragment which hybridized with the *fnr* probe. DNA sequences of both strands were determined independently and completely by commercial sequencing by MWG Biotech (Ebersberg, Germany). Sequence analysis was performed with the Genetics Computer Group (GCG) program package (Devereux et al. 1984).

Enzyme activities.

To determine synthesis of fumarate reductase by measuring fumarate reductase activity cells were grown in minimal medium (Schmitz et al. 1996) supplemented with 10 mM ammonium, 1 % glucose and 50 mM fumarate. Cell extracts were prepared from anaerobically grown cells at an $O.D._{600}$ = 0.6. Cells were disrupted under anaerobic conditions in breakage buffer (50 mM Tris/HCl buffer pH = 7.6 containing 4 mM dithiothreithol and 10 % glycerol) using a French pressure cell followed by centrifugation at 20,000 x g. Fumarate reductase was assayed in 1.5 ml glass cuvettes with N_2 as gas phase at 37 °C. The 0.8 ml standard assay mixture contained 50 mM Tris /HCl buffer pH = 7.4, 4 mM dithiothreitol, 5 mM $MgCl_2$, 250 µM reduced methyl viologen, 1 mM fumarate and 50 to 400 µg cell extract protein. The reactions were started by the addition of 1 mM fumarate and the reduction of fumarate was monitored by following the decrease in absorbance at 604 nm (ε = 26.8 mM^{-1} cm^{-1} per 2

electron transfer). One unit (U) is the amount catalysing the reduction of 1 µmol fumarate per minute at concentrations of 250 µM methyl viologen and 1 mM fumarate

Expression of glutathione S-transferase (GST) fused to *K. pneumoniae fnr* in *E. coli* NCM1529.

The recombinant pRS131 containing the *fnr* gene of *K. pneumoniae* fused at the 5' end to the 3' end of the gene for GST was constructed by cloning the PCR amplified *fnr* into the *Bam*HI and *Eco*RI restriction recognition sites of pGEX-2T (Pharmacia, Freiburg, Germany). *K. pneumoniae fnr* was amplified from chromosomal DNA using a set of primers with synthetic restriction recognition sites (underlined): a sense primer with an additional *Bam*HI restriction recognition site 5' of the start codon (5'ATATCAATGGATCCCTGAGCAGACTTATGATCC3') and an antisense primer with a *Eco*RI restriction recognition site downstream of the stop codcon (5'CGATCCGGCCGAATTCAGAGGGACT ATCAG3'). The PCR product was purified as described above, digested with *Bam*HI and *Eco*RI and ligated into pGEX-2T, which had been linearized with the corresponding enzymes, resulting in plasmid pRS131. The PCR product cloned into pGEX-2T was sequenced, revealing no mutation of *fnr* and correct insertion. From the sequence, the GST-Fnr fusion protein is predicted to have a molecular mass of 58 kDa and a recognition site for thrombin between GST and Fnr. pRS131 was transformed into *E. coli* NCM1529, which grows well under anaerobic conditions. For expression of the GST-Fnr fusion protein, *E. coli* NCM1529/pRS131 was grown aerobically or anaerobically with N_2 as gas phase in minimal medium (modified K-medium, Schmitz et al. 1996) with 0.8 % glucose as the C-source and 10 mM ammonium as the nitrogen source. Expression of the fusion protein was induced with 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) when cultures reached an $O.D._{600}$ = 0.6. Cell extract was prepared by disruption of the cells in breakage buffer (50 mM Tris/HCl buffer pH = 7.6 containing 10 % glycerol) using a French pressure cell followed by centrifugation at 20,000 x g. Fusion proteins were purified from the supernatant by affinity chromatography with glutathione-Sepharose 4B (Pharmacia) according the instruction protocol of the manufacturer. In the case of anaerobic purification all steps described were performed under a nitrogen atmosphere in an anaerobic chamber and the buffers employed contained 2.0 mM dithiothreitol.

Determination of non-hem iron, acid-labile sulfur, and protein.

Non-hem iron was determined colorimetrically as described by Fish (1988). Acid-labile sulfur was analyzed using methylene blue (Cline 1969). Protein was determined via the method of Bradford (1976) with the BioRad protein assay using bovine serum albumin as standard.

SDS-PAGE Analyses.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli using 12.8% acrylamide (Laemmli 1970). Gels were stained for protein with Coomassie Brilliant Blue.

RESULTS AND DISCUSSION

The present work was designed to characterize the oxygen-sensing system in *K. pneumoniae* by cloning the *fnr* homologue. We expressed the Fnr protein from *K. pneumoniae* in fusion to the glutathion-S transferase and analyzed purified protein for iron-sulfur clusters.

Cloning and nucleotide sequence of *K. pneumoniae fnr*. A 100-bp fragment encoding part of *K. pneumoniae fnr* was amplified by PCR using *K. pneumoniae* chromosomal DNA as template and using primers based on the N-terminal sequence of the *E. coli fnr* gene. This fragment was labeled with digoxigenin-dUTP and used as a hybridization probe to screen a cosmid library of *K. pneumoniae* chromosomal DNA as described in Materials and Methods.

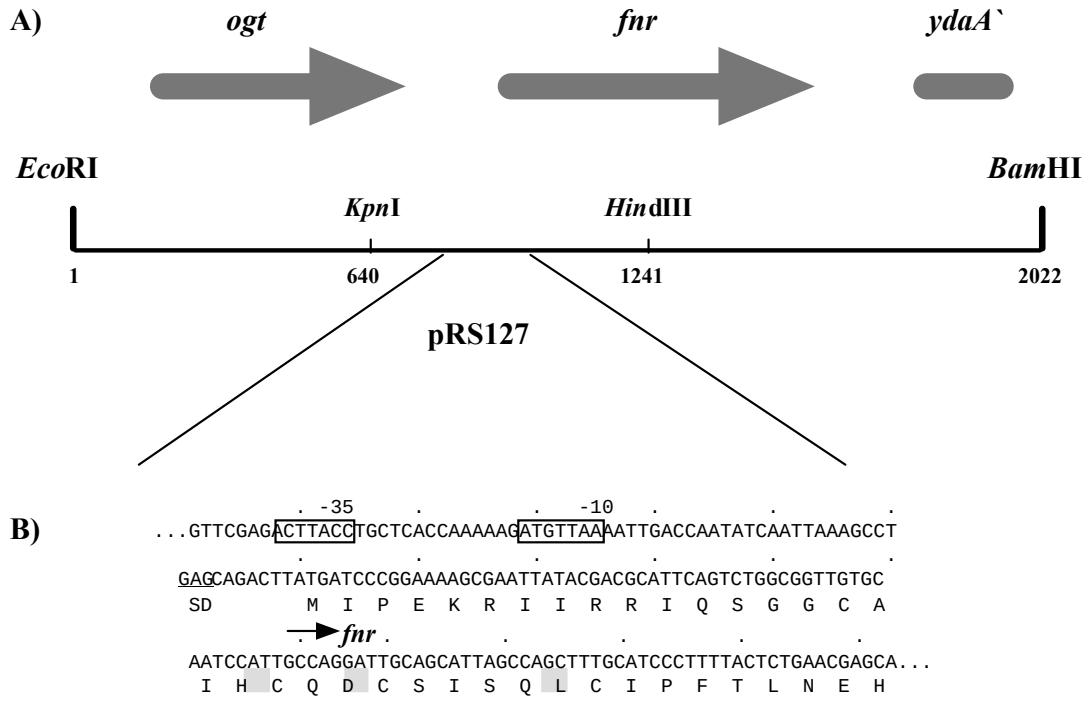

Figure 1: Organization of the cloned region of *K. pneumoniae* (A) and the sequence for the promoter and N-terminal region of *fnr* (B). The deduced amino acid sequence is given in capital letters; the amino acid symbols (one letter code) are written below the first nucleotide of the corresponding codon. Three of the four cystein residues near the N-terminus, which are apparently required for the [4Fe-4S]-cluster ligation, are marled in grey. A potential ribosome binding site (SD) is underlined and a putative s70-dependent promoter sequence is boxed. The sequence of the cloned region has been submitted to GenBank under accession number AF220669.

K. pneumoniae fnr was identified on a 2.1 kbp *Eco*RI/*Bam*HI fragment. This fragment was subcloned into pSK$^+$ Bluescript and the resulting plasmid designated pRS127. The insert of pRS127 was entirely sequenced in both directions. Analysis of the sequence revealed two open reading frames, *orfA* and *orfB,* and part of a third putative open reading frame (*orfC'*) as shown in Fig. 1. *orfB* showed high similarities to *fnr* from *E. coli* and was therefore designated as *fnr.* The open reading frame upstream of *fnr* was identified as *ogt* by homology to the equivalent *E. coli* gene and *orfC'* downstream of *fnr* shows homology to *ydaA'* of *E. coli*. The *fnr* gene of *K. pneumoniae* is preceded by a weak ribosomal binding site, appropriately spaced from the start codon; in addition, a sequence for a putative σ^{70}-dependent promoter is located upstream of *fnr* in position -61 to -32 (Fig. 1). The *fnr* gene (753 bp) codes for a polypeptide of 250 amino acids with a predicted molecular mass of 27939 Da, which shows 98 % amino acid identity to Fnr of *E. coli (*Shaw & Guest 1982). In addition Fnr of *K. pneumoniae* (Fnr_{Kp}) contained all four essential cysteine residues (Cys20, Cys22, Cys29 and Cys122) which are presumed to comprise the oxygen-sensing $[4Fe4S]^{2+}$-center in *E. coli* Fnr (Fnr_{Ec}) (Spiro & Guest 1988).

Function of *K. pneumoniae* Fnr as an oxygen-sensitive transcriptional regulator. Based on high similarity, *K. pneumoniae* Fnr (Fnr_{Kp}) is presumed to function as a transcriptional activator of nitrate metabolism under anaerobic conditions in the same manner as *E. coli* Fnr. We therefore studied growth on glycerol and nitrate under anaerobic conditions of an *E. coli* strain with a chromosomal *fnr* deletion (RAS1). This mutant strain is not able to grow on nitrate and glycerol in the absence of oxygen (Fig. 2). A plasmid-bound copy of the *fnr* gene of *K. pneumoniae* under the control of the tetracycline resistance promoter (pRS137), was able to completely complement the mutation, and allow growth on glycerol and nitrate (RAS5) as it is the case for a plasmid born copy of the native *fnr* gene of *E. coli* (pRS120) (see Fig. 2).

To obtain additional evidence we studied expression of another Fnr-dependent gene in *E. coli* strains with a chromosomal *fnr* deletion. Synthesis of fumarate reductase under anaerobic growth conditions was determined by measuring the activity of fumarate reductase in a *fnr* deletion strain and the same strain containing *K. pneumoniae fnr* on a plasmid.

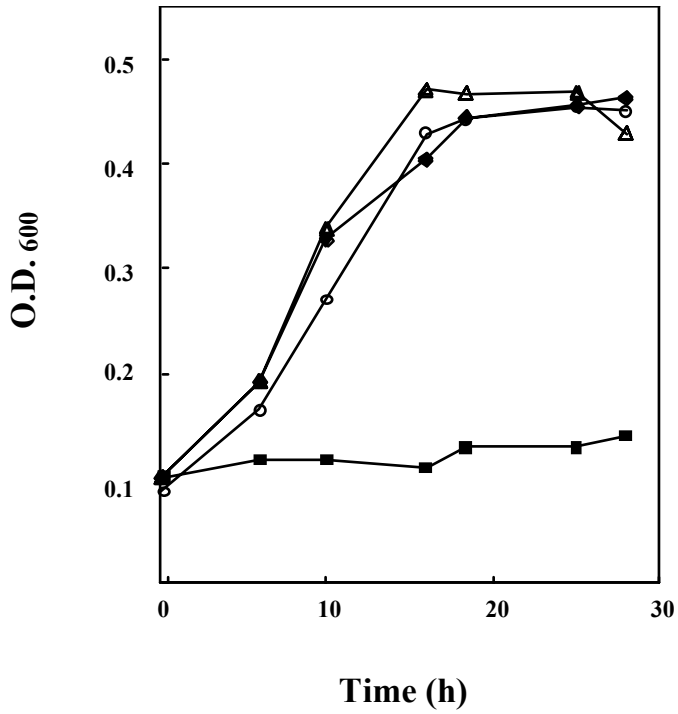

Figure 2: Growth of *E. coli* under anaerobic conditions in minimal medium supplemented with 0.8% glycerol as the sole C-source and 1% KNO3 as the sole nitrogen source (see Materials and methods). (closed diamonds), NCM1529 (parental strain); (closed squares), RAS1 (NCM1529 but *fnr*::Tn*10*); (open circles), RAS1 transformed with pRS120 (*E.coli fnr* controlled by the *tet* promoter); (open triangles), RAS1 transformed with pRS137 (*K. pneumoniae fnr* controlled by the *tet* promoter).

Fumarate reductase activity in the *fnr* deletion strain (RAS1) was determined to be 23 mU / mg cell extract protein, which was equivalent to 10 % of the activity in the parental strain NCM1529 (231 mU / mg cell extract protein). When the *fnr* gene of *K. pneumoniae* was expressed *in trans* under the control of the *tet* promoter (RAS5) it was able to complement the *fnr* mutation in *E. coli* and allow significant higher fumarate reductase activity under anaerobic conditions (174.5 mU / mg cell extract protein). These results indicate that Fnr_{Kp} is functional in *E. coli* and apparently acts as an oxygen-sensing transcriptional regulator.

Purification and characterization of heterologous expressed Fnr_{Kp}. In order to characterize the iron-sulfur clusters we fused Fnr_{Kp} to the glutathione S-transferase (GST) by cloning the *fnr* gene into pGEX-2T (see Materials and Methods). The resulting plasmid, which contains the *gst*-gene *C*-terminally fused to *K. pneumoniae fnr* under the control of the *tac* promoter, was designated pRS131. After transforming pRS131 into *E. coli* NCM1529, which grows well under anaerobic conditions (He et al. 1997), the fusion protein was synthesized in minimal medium under aerobic and anaerobic growth conditions as described in Materials and Methods with ammonium as nitrogen source. Under both growth conditions, induction of the fusion protein at an optical density of $O.D._{600}$ = 0.6 resulted in a retarded growth. The overexpressed fusion protein fractions were purified in the presence and absence of molecular oxygen, respectively, by Glutathione-Sepharose 4B affinity chromatography and cleaved with the site-specific protease thrombin. In both cases, homogeneous Fnr_{Kp} preparations were obtained, as revealed by sodium dodecylsulfate/polyacrylamide gel electrophoresis. The apparent molecular mass of Fnr_{Kp} was determined to be 28 kDa (see Fig. 3). After purification, the cofactors of both protein fractions were determined. The aerobic Fnr preparations were found to contain less than 0.1 mol of acid-labile sulfur and 1.0 mol iron per mol Fnr. For the anaerobic Fnr preparations, 2.6 mol iron and 2.2 mol acid-labile sulfur was found per mol Fnr, indicating the presence of an [3Fe3S]-cluster or an [4Fe4S]-cluster in the anaerobic protein. Synthesis and purification under aerobic conditions apparently resulted in the disruption of the iron-sulfur cluster and loss of the iron. These results indicate that Fnr_{Kp} apparently contains an iron-sulfur center responsible for oxygen sensing, as it is the case for Fnr_{Ec}, which is disrupted in the presence of molecular oxygen (Green et al. 1996, Khoroshilova et al. 1997, Kiley & Beinert 1998).

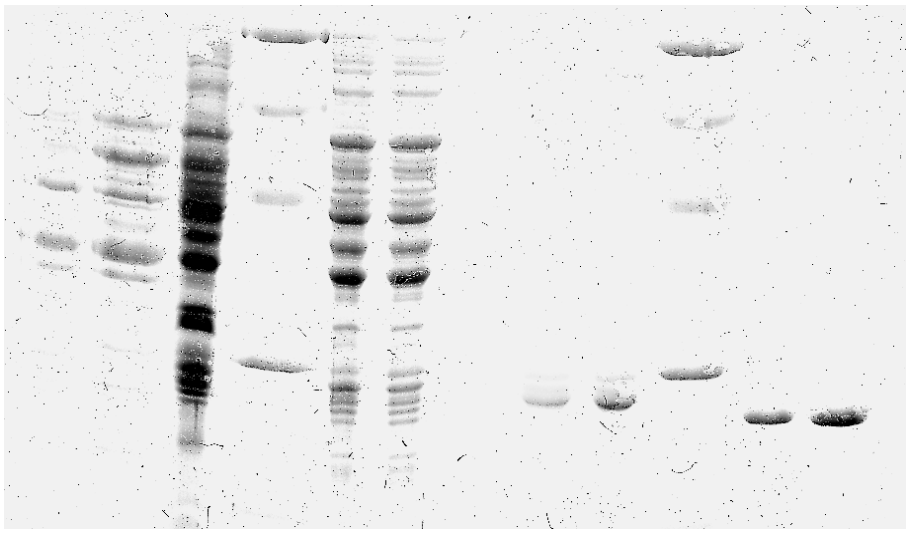

Figure 3: Purification of *K. pneumoniae* Fnr fused to glutathion S-transferase (GST) and synthesized under anaerobic conditions. Various stages in the purification are seperated by SDS-Page (12.8%). Lanes: 1 and 2, whole cell extract before and after IPTG induction, respectively; 3, low-speed supernatant from cell extract; 4 and 10. low molecular mass marker (Pharmacia); 5, 6 and 7, wash fractions of GST-Fnr bound to Glutathione Sepharose 4B; 8 and 9, flow through fractions following thrombin digest of GST-Fnr bound to Glutathione Sepharose 4B; 11 and 12, GST fraction eluted with glutathion supplemented buffer. The gel was stained with coomassie Brillant Blue R250.

In order to further analyse the iron-sulfur center of Fnr_{Kp} we studied the spectroscopic properties of the anaerobic Fnr_{Kp} protein fraction. (i) UV-visible spectroscopy of the anaerobic Fnr_{Kp} protein showed no detectable absorption in the range of 400 to 420 nm. (ii) Using Low Temperature EPR analyses, we revealed no signal typical for an iron sulfur cluster for the anaerobic Fnr fraction (data not shown). This might be due to the low protein concentrations we observed from the anaerobic protein purification (approximately 0.5 mg/ml) or due to disruption of the iron-sulfur clusters during the purification procedure even when performed under anaerobic conditions.

In summary. In order to characterize the oxygen-sensing system in *K. pneumoniae* we have cloned and characterized the *fnr* gene of *K. pneumoniae*. Analyses of the *K. pneumoniae fnr* gene showed high similarities to the *E. coli fnr* gene (98 % amino acid identity, Shaw & Guest 1982). The ability of fnr_{Kp} to functionally complement fnr_{Ec} was shown in vivo by restoration of growth on glycerol plus nitrate, and expression of Fnr-dependent genes (*frd*ABCD) in an *E. coli fnr* deletion strain transformed with a plasmid-bound copy of Fnr_{Kp}.

These results indicate that Fnr_{Kp} activates transcription of genes in a similar way like *E. coli* Fnr. They further suggest a similarity in the oxygen-sensing mechanism of the two organisms. In addition, characterization of purified protein indicated the presence of an oxygen sensitive $[4Fe4S]^{2+}$-center in Fnr_{Kp}: (i) The deduced amino acid sequence of *K. pneumoniae fnr* contained all four essential cysteine residues near the N-terminus, which are required for the oxygen-sensing function (Spiro & Guest 1988, Khoroshilova et al. 1997). (ii) Determination of iron and acid-labile sulfur in aerobic- and anaerobic-purified protein fractions suggested the presence of an iron-sulfur cluster, which is apparently disrupted upon the influence of oxygen.

Chapter 3:

Fnr is required for NifL-dependent oxygen control of *nif* gene expression in *Klebsiella pneumoniae*

Abstract

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* ø(*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*$^-$-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(s) whose product(s) function to relieve NifL inhibition by reducing the FAD cofactor under oxygen-limiting conditions.

Introduction

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} (Dixon, 1998, Fischer, 1994). 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 (Govantes et al., 1998, Henderson et al., 1989; Lei et al., 1999; Money et al., 1999). 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.* (He et al., 1998), and Jack *et al.* (1999) 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 as a prosthetic group (Hill et al., 1996; Klopprogge and Schmitz, 1999: Schmitz, 1997). In vitro, the oxidized form of NifL inhibits NifA activity, whereas reduction of the FAD cofactor relieves NifL inhibition (Hill et al., 1996; Macheroux et al., 1999). 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 (Schmitz, 1997; Schmitz et al., 1996). 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 (He et al., 1998).

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 regulator (Fnr) (Spiro, 1994; Spiro and Guest, 1990), 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 (Green et al., 1996; Khoroshilova et al., 1997; Melville and Gunsalus, 1990; Unden and Schirawski, 1997). Recently we cloned and sequenced the *fnr* gene of *K. pneumoniae* and characterized the protein (Grabbe et al., 2000). As 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 2. Plasmid DNA was transformed into *E. coli* cells according to the method of Inoue *et al.* (1990) and into *K. pneumoniae* cells by electroporation. Transduction by phage P1 was performed as described previously (Silhavy et al., 1984).

***E. coli* strains.** *E. coli* NCM1529, which contains a ø(*nifH'-'lacZ*) fusion (He et al. 1997), 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 (Jayaraman et al., 1988) into NCM1529 by P1-mediated transduction with selection for tetracycline resistance,

resulting in RAS1 (Grabbe et al., 2001a). 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 (Sawers and Suppman, 1992) 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.

***Klebsiella* strains.** *K. pneumoniae* strains M5al (wild type) and UN4495 (ø(*nifK-lacZ)5935* Δ*lac-4001 his D4226* Gal^r) (McNeil et al., 1981) were provided by Gary Roberts.

Construction of a *fnr*::Ω mutation: Strain RAS18 was obtained by insertion of a kanamycin resistance cassette (Prentki et al., 1984) into the *fnr* gene of *K. pneumoniae* UN4495 as achieved 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 *Hind*III cassette containing an Ω interposon fragment with a kanamycin resistance gene derived from plasmid pHP45Ω (Prentki et al., 1984) was cloned into the *Hind*III site of *fnr* in pRS127 to yield plasmid pRS142. (iii) A 2.9 kb PCR fragment carrying *fnr*::Ω was generated using pRS142 as template and a set of primers which were homologue to the *fnr* flanking 5´- and 3´-regions with additional *Bam*HI synthetic restriction recognition sites (underlined) (5'ATATCAATGGATCCCTGAGCAGACTTA TGATCC3', 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 (18), 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 cross over) were identified by the ability to grow on LB supplemented with 5%

sucrose and resistance to kanamycin. The *fnr*::Ω mutation in strain RAS18 was confirmed by southern blot analysis (Sambrook et al., 1989) 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.

Table 2: Bacterial strains and Plasmids used in this study.

Strains / plasmids	Relevant genotype and/or characteristic(s)	Reference or description
E. coli strains		
NCM1529	*araD139*Δ(*argF-lacU)169 fth D5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1 trpDC700putPA1303*::[Kan^r-(*nifH'-'lacZ*)] (wild type)	He *et al*. 1997
NCM1528	NCM1529/pNH3	He *et al*. 1997
NCM1527	NCM1529/pJES851	He *et al*. 1997
RAS1	NCM1529 but *fnr*::Tn*10*	Grabbe *et al*. 2001a
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*	Schmitz 1997
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
RAS15	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 strains		
M5al	Wild type	
UN4495	ø(*nifK-lacZ)5935* Δ*lac-4001 his D4226* Gal^r	MacNeil *et al*. 1981
RAS18	ø (*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	He *et al*. 1997
RAS29	RAS18/pJES839	This study
RAS30	UN4495Δ(*nifLA*)*6293::*Km / pJES839	Schmitz *et al*. 1996 and this study
Plasmids		
pNH3	*K. pneumoniae nifLA* controlled by the *tac* promoter	Henderson *et al*. 1989
pJES839	pNH3 but additional tetracycline resistance cassette	Schmitz *et al*. 1996
pJES851	*K. pneumoniae nifA* controlled by the *tac* promoter	Schmitz *et al*. 1996
pRS79	*E. coli fnr* controlled by the *lac* promoter on pMCL210	This study
pRS107	*K. pneumoniae nifL*$^{C184S/C187S}$*nifA* controlled b the *tac* promoter	This study
pRS159	*K. pneumoniae nifL*$^{C184SC/187S}$*nifA* controlled b the *tac* promoter	This study
pRS120	*E. coli fnr* controlled by the *tet* promoter on pACYC184	Grabbe *et al*. 2001a

pRS127	2.1 kbp fragment in pBluescript SK^+ containing *K. pneumoniae fnr*	Grabbe *et al*. 2001a
pRS137	*K. pneumoniae fnr* controlled by the*tet* promoter on pACYC184	Grabbe *et al*. 2001a
pACYC184	Low copy vector	New England Biolabs, UK
pMCL210	Low copy vector	Nakano *et al*. 1995
pBluescript SK^+	Cloning vector	Stratagene, La Jolla, US

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 (Henderson et al., 1989) by introducing the double mutation into *nifL* by site directed mutagenesis. Site directed mutagenesis was performed using 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 (Schmitz et al., 1996) into the *Sca*I site of plasmid pRS107. Plasmid pRS79 contains the *E. coli fnr* gene inserted into the *Bam*HI and *Pst*I site of pMCL210 (Nakano et al., 1995) under the control of the *lac* promoter. pRS120 and pRS137 contain *E. coli fnr* gene and *K. pneumoniae fnr* gene, respectively, inserted into the *Sal*I and *Bam*HI site of pACYC184 and thereby expressed from the *tet* promoter (Grabbe et al., 2001a).

Growth. *K. pneumoniae* and *E. coli* strains were grown under anaerobic conditions with N_2 as gas phase at 30° C in minimal medium (Schmitz et al., 1996) supplemented with 4 mM glutamine, 10 mM Na_2CO_3, 0.3 mM sulfide and 0.002 % resazurine to monitor anaerobiosis. The medium was further supplemented with 0.004% histidine and with 0.4% sucrose as sole

carbon source for *K. pneumoniae* strains. For *E. coli* strains, the medium was supplemented with 0.1 mM tryptophane and 0.8 % glucose as the carbon source. Precultures were grown overnight in closed bottles with N_2 as gas phase, in medium lacking sulfide and resazurine 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 gas phase at 30° C under strictly anaerobic conditions without shaking. Samples for monitoring growth at 600 nm and determining ß-galactosidase activity were taken anaerobically. In *E. coli* strains carrying a plasmid encoding NifL and NifA (pNH3 (12)), $NifL^{C184S/C187S}$ and NifA (pRS107) or a plasmid encoding NifA alone (pJES851 (Schmitz et al., 1996)) expression of *nifLA*, *nifL*C184SC/187S*nifA* or *nifA* was induced from the *tac* promoter with 10 μM IPTG (isopropyl-ß-D-thiogalactopyranoside).
Fnr phenotypes of RAS1, RAS13, RAS18 and the respective complemented strains RAS9, RAS10, RAS16 and RAS19 were tested anaerobically using glycerol and nitrate (0.5%) as sole carbon and nitrogen source in minimal medium.

ß-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 ß-galactosidase synthesis during exponential growth (units per milliliter per OD_{600}) (Schmitz et al., 1996). 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 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 (Laemmli, 1970). Samples were separated by SDS/polyacrylamide (12%) gel electrophoresis and transferred to nitrocellulose membranes as described previously (Sambrook et al., 1989). Membranes were exposed to polyclonal rabbit antisera directed against the NifL or NifA proteins of *K. pneumoniae*, protein bands were

detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (BioRad Laboratories). Purified NifA and NifL from *K. pneumoniae* and prestained protein markers (New England Biolabs, UK) were used as standards.

Data deposition. *K. pneumoniae fnr* sequence has been submitted to GenBank under accession number 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 (Schmitz, 1997; Schmitz et al., 1996). 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 of ammonium presence. 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 (He et al., 1997). 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 (He et al., 1997). 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 sole carbon source and under nitrogen-limitation to exclude NifA inhibition by NifL in response to combined nitrogen. Determining the doubling times of the different strains under anaerobic and aerobic conditions revealed no significant difference in growth rates for *fnr*⁻ strains compared to the respective parental strains (Table 3). NifA-mediated activation of transcription from the *nifH'*-promoter in the different backgrounds was monitored by determining the differential rate of ß-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 ß-galactosidase synthesis rate from the *nifH'*-promoter of only 100 ± 10 U/ml OD_{600} when *nifLA* was induced with 10 µM IPTG. This is in the range of synthesis rate under aerobic conditions in the parental strain NCM1528 (60 ± 5 U/ml OD_{600}) and equivalent to 3 % of the synthesis rate under anaerobic conditions in NCM1528 (3000 ± 100 U/ml OD_{600}) (Table 3).

Table 3: **Effects of an *fnr* null allele on activity of the *K. pneumoniae* NifL protein in different *E. coli* backgrounds.**

Strain	**Relevant genotype**	**Presence of oxygen**	**Expression of *nifH'-'lacZ'* (U/ml · O.D.$_{600}$)** [a]	**Doubling time (h)**
NCM1528	Wild type/*Ptac-nifLA*	-	3000 ± 100	5.0
NCM1528	Wild type/*Ptac-nifLA*	+	60 ± 5	2.0
NCM1527	Wild type/*Ptac-nifA*	-	5300 ± 200	4.8

NCM1527	Wild type/*Ptac-nifA*	+	5118 [d]	2.1
RAS2	Wild type/*Ptac-nifL⁻ nifA*	-	2950 ± 120	5.2
RAS2	Wild type/*Ptac-nifL⁻ nifA*	+	2900 ± 50	2.0
RAS8 [b]	*fnr⁻*/*Ptac-nifA*	-	4800 ± 100	4.9
RAS8 [b]	*fnr⁻*/*Ptac-nifA*	+	5200 ± 200	2.2
RAS6 [b]	*fnr⁻*/*Ptac-nifL⁻ nifA*	-	2800 ± 100	5.0
RAS6 [b]	*fnr⁻*/*Ptac-nifL⁻ nifA*	+	3000 ± 200	2.0
RAS7 [b]	*fnr⁻*/*Ptac-nifLA*	-	100 ± 10	5.0
RAS7 [b]	*fnr⁻*/*Ptac-nifLA*	+	30 ± 3	2.0
RAS9 [b]	*fnr⁻*/*Ptac-nifLA*/*Plac fnr*	-	3000 ± 100	5.2
RAS10 [b]	*fnr⁻*/*Ptac-nifLA*/*Ptet fnr*	-	2870 ± 70	5.2
RAS11 [b]	*fnr⁻*/*Ptac-nifLA*/pMCL210	-	66 ± 5	5.5
RAS12 [b]	*fnr⁻*/*Ptac-nifLA*/pACYC184	-	70 ± 6	5.5
RAS22	Wild type/*Ptac-nifLA*	-	3500 ± 80	5.0
RAS22	Wild type/*Ptac-nifLA*	+	70 ± 5	2.2
RAS23	Wild type/*Ptac-nifA*	-	5900 ± 250	5.1
RAS23	Wild type/*tac-nifA*	+	5725 ± 150	2.2
RAS24	Wild type/*Ptac-nifL⁻ nifA*	-	3400 ± 200	4.9
RAS24	Wild type/*Ptac-nifL⁻ nifA*	+	2800 ± 150	2.1

RAS15[c]	*fnr*⁻/*Ptac-nifA*	-	5300± 200	5.6
RAS15[c]	*fnr*⁻/*Ptac-nifA*	+	5130± 150	2.1
RAS25[c]	*fnr*⁻/*Ptac-nifL*⁻ *nifA*	-	3200 ± 200	5.0
RAS25[c]	*fnr*⁻/*Ptac-nifL*⁻ *nifA*	+	3400 ± 100	2.2
RAS14[c]	*fnr*⁻/*Ptac-nifLA*	-	160 ± 10	5.3
RAS14[c]	*fnr*⁻/*Ptac-nifLA*	+	40 ± 5	2.0
RAS16[c]	*fnr*⁻/*Ptac-nifLA*/*Ptet-fnr*	-	3200 ± 100	5.2
RAS17[c]	*fnr*⁻/*Ptac-nifLA*/pACYC184	-	190 ± 10	5.4

[a], data presented present mean values of three independent experiments

[b], Strains contain the *fnr* null allele from M182 *(fnr*::Tn*10*) (Jayaramann et al., 1988)

[c], Strains contain the *fnr* null allele from RM101 (Sawers and Suppmann, 1992)

[d], Determined by He *et al.* (1997)

nifL⁻ *nifA*, *nifL*$^{C184S/C187S}$*nifA* (see Materials & Methods); *Plac*, *Ptac* or *Ptet*, under the control of the *lac*, *tac* or *tet* promoter, respectively.

In the case of NifA synthesis in the *fnr*⁻ strain in the absence of NifL (RAS8), however, the ß-galactosidase synthesis rate under anaerobic conditions was not significantly altered compared to the parental strain NCM1527 (4800 ± 100 U/ml OD_{600} and 5300 ± 200 U/ml OD_{600}, respectively) and was not affected by oxygen (Table 3). This indicates that the observed Fnr effect is mediated by NifL towards NifA in RAS7. However, *nif* expression under anaerobic conditions by NifA induced from the *tac* promoter in the absence of NifL synthesis using pJES851 (NCM1527) is significantly higher than using plasmid pNH3 (NCM1528), in which NifA expression depends on NifL synthesis based on translational coupling in the *nifLA* operon (Govantes et al., 1998). In addition western blot analysis showed that under our experimental conditions NifA amounts synthesized in NCM1527 were

approximately 30 - 40 % higher compared to NifA amounts 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 NCM1528 (data not shown) and expression of NifL$^{C184S/C187S}$, which completely lost its nitrogen and oxygen regulatory function (Klopprogge and Schmitz, unpublished). Determination of ß-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 3). These findings indicate that the *fnr* null allele is not affecting 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* and on plasmids, the respective strains RAS14, RAS15 and RAS25 were grown under oxygen-limitation. By determining the ß-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}, when *nifLA* was expressed under anaerobic conditions. This *nif*-induction is again significantly lower than in the parental strain RAS22 (3500 ± 80 U/ml OD_{600}) and is in the range of aerobic *nif*-induction in the parental strain (70 ± 5 U/ml OD_{600}) (Table 3). Similar to RAS8 and RAS6 the ß-galactosidase synthesis rate in the case of NifA synthesis in the absence of a functional NifL was not affected by the *fnr*$^{-}$ mutation (RAS15 compared to RAS23 and RAS25 compared to RAS24).

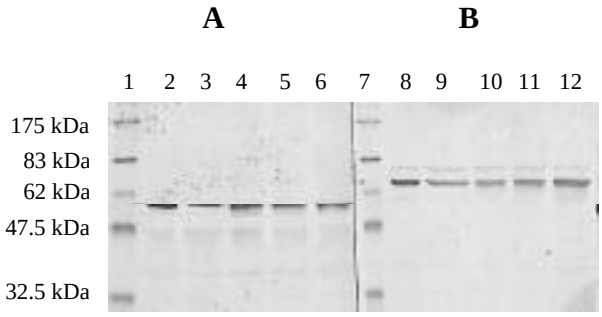

Figure 4: Amounts of NifA and NifL in wild type and *fnr*⁻ strains of *E. coli*. Cultures were grown at 30° C in minimal medium under anaerobic conditions with 4 mM glutamine as limiting nitrogen source. The strains carried *K. pneumoniae* NifL and NifA under the control of the *tac* promoter on pNH3. Expression of NifL and NifA was induced with 10 µM IPTG in 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). Amounts of NifL (**A**) and NifA (**B**) were determined by Western blotting. Prestained protein marker broad range (lanes 1 and 6) was purchased from New England Biolabs (UK).

The *fnr* null alleles are not affecting 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 Figure 4 we observed no obvious differences in the amounts of the regulatory proteins of *K. pneumoniae* in the different *fnr* ⁻ backgrounds compared to 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) which is displayed graphically in Figure 5. 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 3). 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.

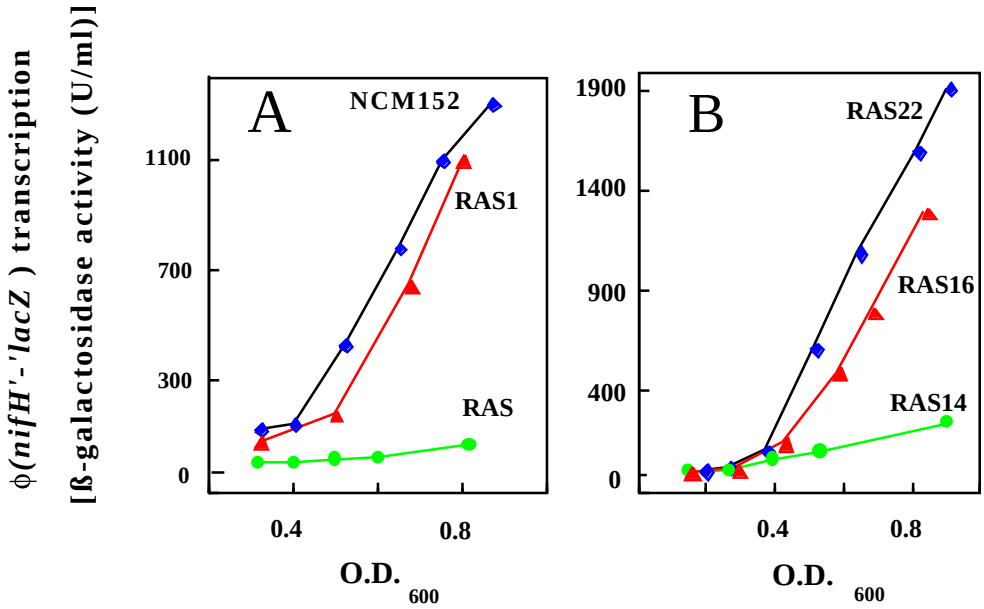

Figure 5: Effects of *fnr* null alleles on expression of a ø(*nifH'-'lacZ*) fusion in heterologous *E. coli* strains carrying *K. pneumonia nifLA* on a plasmid. The activity of ß-galactosidase was plotted as a function of OD_{600} for cultures grown at 30° C in minimal medium under anaerobic conditions with 4 mM glutamine as 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 (He et al. 1997) 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), complemented respective *fnr* mutant by constitutive expression of *E. coli fnr* on pACYC184 (RAS10) (triangles). **B** *fnr* null allele from RM101: Wild type RAS22 (diamonds), the respective *fnr*

null allele in RAS22 (RAS14) (circles), complemented respective *fnr* mutant by constitutive expression of *E. coli fnr* on pACYC184 (RAS16) (triangles).

The wild type strain (NCM1528) grown in the presence of 10 mM ammonium showed *nif* inductions of approximately 3 ± 1 U/ml OD_{600} independent of the oxygen availability (data not shown). This induction level is significantly lower than the *nif*-induction observed in the *fnr*$^-$ mutant strains (RAS7 and RAS14) under oxygen- and nitrogen-limiting growth conditions (100 ± 10 U/ml OD_{600} and 160 ± 10 U/ml OD_{600}, 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.

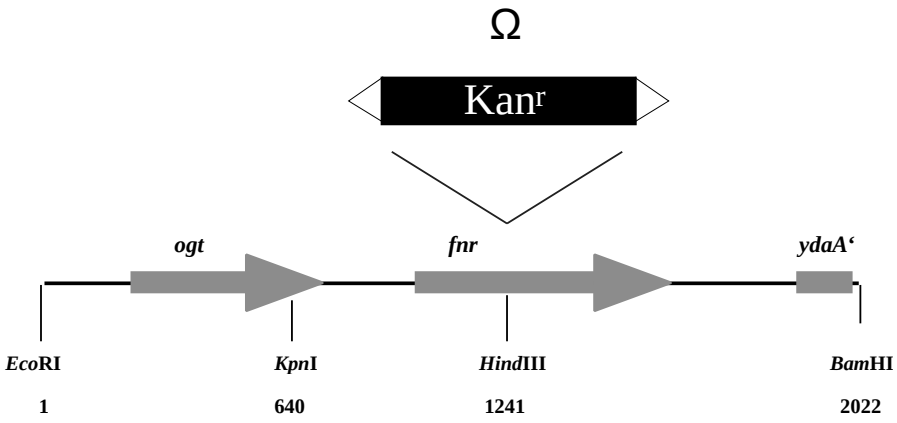

Figure 6: Map of the cloned *Eco*RI-*Bam*HI fragment (pRS127) showing the side of insertion of the Ω interposon fragment with a kanamycin resistance gene derived from plasmid pHP45Ω (Prentki and Kirsch 1984) in *K. pneumoniae fnr*. The Ω interposon fragment is flanked by short inverted repeats including strong transcription termination signals. The sequence of the *Eco*RI-*Bam*HI fragment has been submitted to GenBank under accession number AF220669.

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 ß-galactosidase synthesis (Schmitz et al., 1996). The *fnr* deletion was constructed on a plasmid by inserting an Ω interposon fragment with a kanamycin resistance gene into *K. pneumoniae fnr* (Figure 6), which was than 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}) 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}) (Table 4). Determination of NifA and NifL proteins in the *fnr*⁻ mutant strain revealed no differences in the amount of the regulatory proteins compared to the parental strain (data not shown), indicating that the failure to express *nifH* could not be accounted for by a decrease of 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 (Figure 7). *nif* induction in the complemented mutant (RAS19) was determined to be 3800 ± 50 U / ml OD_{600}, 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.

Table 4: **Effects of a *fnr*::Ω mutation on NifL activity in *K. pneumoniae* UN4495.**

Strain	Relevant genotype	Nitrogen source	Presence of oxygen	Expression of *nifH´-´lacZ‘* (U/ml · O.D.$_{600}$) [a]	Doubling time (h)
UN 4495	Wild type	glutamine	-	4400 ± 100	3.5
UN 4495	Wild type	glutamine	+	220 ± 10	2.0
RAS18	*fnr*$^-$	glutamine	-	400 ± 20	4.0
RAS18	*fnr*$^-$	glutamine	+	100 ± 10	2.2
RAS19	*fnr*$^-$ / *Ptet-fnr*[b]	glutamine	-	3800 ± 50	3.8
RAS20	*fnr*$^-$ / pACYC184	glutamine	-	660 ± 30	4.2
RAS26	Wild type / *Ptac-nifL*$^-$ *nifA*	ammonium[c]	-	2350 ± 100	3.7
RAS26	Wild type / *Ptac-nifL*$^-$ *nifA*	ammonium[c]	+	2100 ± 100	1.7
RAS27	*fnr*$^-$ / *Ptac-nifL*$^-$ *nifA*	ammonium[c]	-	2200 ± 50	4.1
RAS27	*fnr*$^-$ / *Ptac-nifL*$^-$ *nifA*	ammonium[c]	+	2150 ± 150	1.6
RAS28	Wild type / *Ptac-nifLA*	glutamine	-	2400 ± 30	4.0
RAS28	Wild type / *Ptac-nifLA*	glutamine	+	160 ± 5	1.6
RAS29	*fnr*$^-$ / *Ptac-nifLA*	glutamine	-	430 ± 30	3.6
RAS29	*fnr*$^-$ / *Ptac-nifLA*	glutamine	+	310 ± 30	1.6
RAS30	4495Δ*nifLA* / *Ptac-nifLA*	glutamine	-	2450 ± 30	4.1

[a], data presented represent mean values of three independent experiments

[b], *K. pneumoniae fnr* is expressed under the control of the *tet* promoter (*Ptet*)

[c], grown in the presence of 10 mM ammonium to repress chromosomal *nifLA* induction

nifL$^-$ *nifA*, *nifL*$^{C184S/C187S}$*nifA* (see Materials & Methods); *Ptac*, under the control of the *tac* promoter.

In order to confirm our finding observed 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. As growth in minimal medium in the presence of 10 mM ammonium results in repression of the chromosomal *nifLA* operon, under nitrogen sufficieny only *nifL*$^{C184S/C187S}$*nifA* from pRS159 is induced, resulting in the synthesis of NifA and a non-functional NifL protein (see above). Determination of ß-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 is not affecting NifA activity in the absence of a functional NifL protein (2200 ± 50 U / ml OD_{600} and 2350 ± 100 U / ml OD_{600}, respectively) (Table 4). 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.

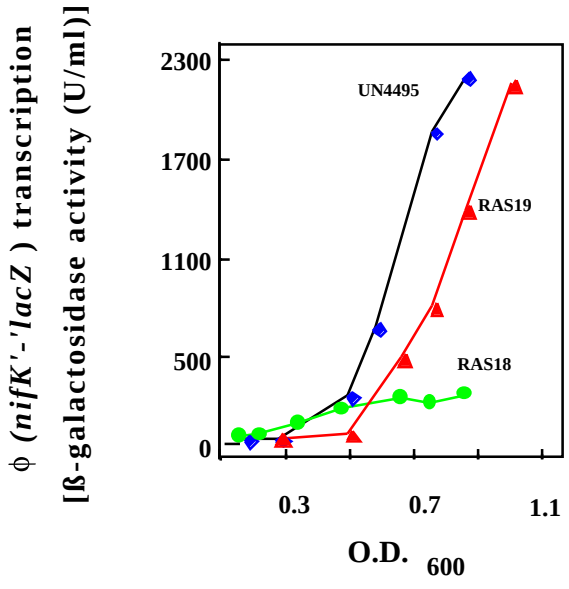

Figure 7: Effects of an *fnr* null allele on expression of a *nifK-LacZ* fusion in *K. pneumoniae* strain UN4495.

The activity of ß-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 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), complemented respective *fnr* mutant by constitutive expression of *K. pneumoniae fnr* on pACYC184 (RAS19) (triangles).

Discussion

Our goal is to determine how *K. pneumoniae* NifL perceives the oxygen status of the cells in order to regulate NifA activity 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 (Hill et al., 1996; Macheroux et al., 1998; Schmitz 1996). We recently showed that iron is specifically required for *nif*-induction, but is not present in NifL (Schmitz, 1997; Schmitz et al., 1996). 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 effects 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 (Figures 6 and 8). 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 (Schmitz et al.,1996). 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 (Fischer, 1994 and therein cited papers, Guiterrez et al., 1997), 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 (1985), 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*$^{-}$ strains carrying the Nif+ plasmid pRD1 under derepressing conditions. Also, as plasmid pRD1 contains in addition to the *nif* genes non-identified *K. pneumoniae* genes (Dixon et al., 1976) 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*.

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 wilde type strain than observed in *fnr* mutant strains under nitrogen- and oxygen-limitation. Both 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,* it still remains to be explained how the oxygen signal is transmitted to NifL. Fnr is either transducing the oxygen signal by directly interacting with NifL in the absence of oxygen or under anaerobic conditions Fnr is activating the transcription of gene(s) whose product(s) mediate the signal to NifL. As Fnr is a transcriptional activator and can be excluded as the physiological electron donor for NifL reduction it is more reasonably that under anaerobic conditions Fnr transduces the signal by transcriptional activation.

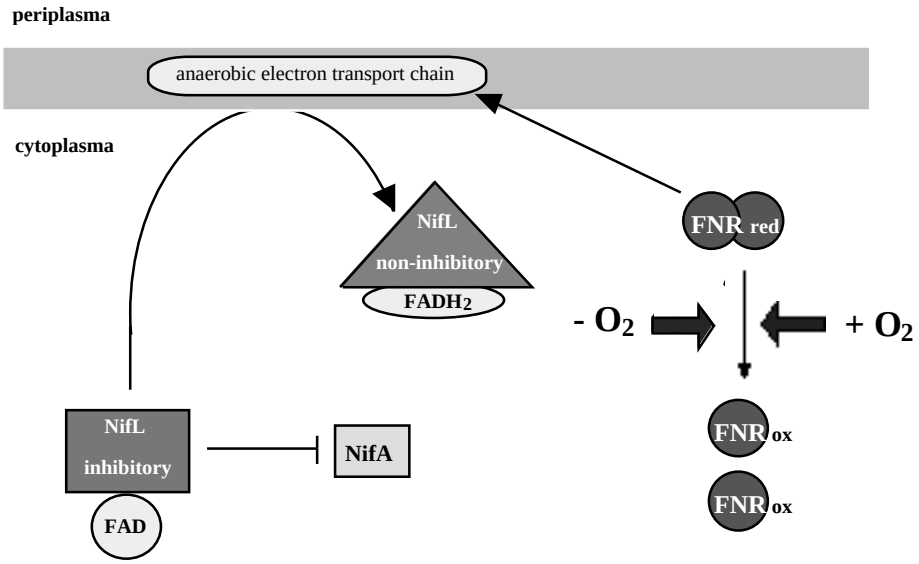

Figure 8: Hypothetical model of oxygen signal transduction in *K. pneumoniae*.

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 (Klopprogge and Schmitz, 1999, Macheroux et al., 1998). 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. As 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 gene(s) whose product(s) 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. 8), particularly the electron transport system to fumarate, whose transcription under anaerobic conditions is directly dependent on Fnr activation (Ackrell, 2000; Manodori et al., 1992; Skotnicki and Rolfe, 1979; Van Hellemond and Tielens, 1994).

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.

Chapter 4:

Membrane association of *Klebsiella pneumoniae* NifL is affected by molecular oxygen and combined nitrogen

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Abstract

In the diazotroph *Klebsiella pneumoniae,* NifL and NifA regulate transcription of the nitrogen fixation genes in response to molecular oxygen and combined nitrogen. We recently showed that Fnr is the primary oxygen sensor, which transduces the oxygen signal towards the negative regulator NifL by activating genes whose products reduce the FAD moiety of NifL under anaerobic conditions. Potentially, these Fnr-dependent gene products could be membrane-bound components of the anaerobic electron transport chain; consequently, in this study we now examine the localization of NifL within the cell under various growth conditions. In *K. pneumoniae* grown under oxygen- and nitrogen-limited conditions, approximately 55 % of the total NifL protein were found in the membrane fraction. However, when the cells were grown aerobically or shifted to nitrogen sufficiency, less than 10 % of total NifL was membrane-associated. In contrast to NifL, NifA was located in the cytoplasm under all growth conditions tested. Further studies using *K. pneumoniae* mutant strains showed that under derepressing conditions but in the absence of either the primary oxygen sensor Fnr or the primary nitrogen sensor GlnK and the ammonium transporter AmtB, NifL was located in the cytoplasm and inhibited NifA activity. These findings suggest that under nitrogen- and oxygen-limitation, a significant higher membrane affinity of NifL may create a spatial gap between NifL and its cytoplasmic target protein NifA thereby impairing inhibition of NifA by NifL. Localization of GlnK further showed that under nitrogen-limited conditions, but independent of oxygen presence, 15 - 20 % of the total GlnK is membrane associated.

Introduction

The free-living diazotroph *Klebsiella pneumoniae* is able to fix molecular nitrogen under anaerobic and nitrogen-limited growth conditions. To avoid unnecessary consumption of energy synthesis of nitrogenase is tightly controlled by the regulatory nitrogen fixation operon *nifLA* (for review see Dixon, 1998; Schmitz et al., 2001). Products of the *nifLA* operon regulate transcription of the other *nif* genes in response to environmental signals. NifA is the

transcriptional activator of all of the *nif* operons except the *nifLA* operon, which is under control of the global nitrogen regulatory system, *ntr* (Drummond and Wootton, 1987; Blanco et al., 1993); NifL antagonizes the transcriptional activity of NifA in response to combined nitrogen and molecular oxygen by direct protein-protein interaction with NifA (Merrick et al., 1982; Hill et al., 1981; Money et al., 1999; Lei et al., 1999; Money et al., 2001; Little et al., 2000, Barrett et al., 2001).

External nitrogen availability is apparently perceived by *K. pneumoniae* through changes in the internal glutamine pool (Schmitz, 2000), which are subsequently mediated towards the *nif* regulatory system. Recent evidence strongly suggests that the nitrogen status of the cells is transduced towards the NifL/NifA regulatory system in *K. pneumoniae* and *Azotobacter vinelandii* by the GlnK protein, a paralogue PII-protein (He et al., 1998; Jack et al., 1999; Arcondéguy et al., 1999 and 2000; Little et al., 2000; Reyes-Ramirez et al., 2001). The effect of GlnK, which apparently interacts with NifL or affects the NifL/NifA-complex via direct protein-protein interaction, appears to be contradictory in *K. pneumoniae* and *A. vinelandii* (He et al., 1998; Jack et al., 1999; Little et al., 2000; Reyes-Ramirez et al., 2001). The oxygen signal is received by NifL, which contains an *N*-terminally bound flavin adenine dinucleotide (FAD) as a prosthetic group. Recent work has shown that the flavoprotein NifL acts as a redox-sensitive regulatory protein, which modulates NifA activity in response to the redox state of its FAD cofactor and allows NifA activity only in the absence of oxygen (Hill et al., 1996; Schmitz, 1997; Macheroux et al., 1998; Little et al., 2000). Thus, under anaerobic conditions in the absence of combined nitrogen, reduction of the flavin moiety of NifL results in a non-inhibitory conformation of the NifL protein. We have recently shown that in *K. pneumoniae,* the global transcriptional regulator Fnr is required to mediate the signal of anaerobiosis to NifL (Grabbe et al., 2001b). Thus, we proposed that in the absence of oxygen the primary oxygen sensor Fnr activates transcription of a gene or genes whose product or products reduce the FAD cofactor of NifL, resulting in a non-inhibitory conformation of the protein, assuming the absence of a sufficient nitrogen source. Candidates for the physiological electron donor for NifL reduction include those components of the anaerobic electron transport system with Fnr-dependent synthesis (Grabbe et al., 2001b). This model implies that under anaerobic conditions, NifL will contact the cytoplasmic membrane during the reduction of its flavin cofactor. If NifL reduction indeed occurs by a membrane-associated electron donor, this provides a potential mechanism for the signal transduction of anaerobiosis that is similar to the signal transduction of oxygen presence proposed for the *Escherichia coli* FAD-containing aerotaxis protein Aer (Bibikov *et al*. 1997; Rebbapragada et al.,

1997).

Our goal is to analyze the reduction process of NifL-bound FAD and the subsequent conformational change of the protein. We therefore localized of NifL protein in *K. pneumoniae* and *K. pneumoniae* mutant strains grown under various conditions. We report here that under derepressing conditions, NifL shows a significantly higher association with the cytoplasmic membrane than in the presence of either molecular oxygen or combined nitrogen. As the presence of molecular oxygen or combined nitrogen results in *nif* gene repression, these findings imply that a spatial separation of cytoplasmic NifA and its antagonist NifL may be responsible for *nif* gene induction under oxygen- and nitrogen-limited conditions.

Materials and methods

Bacterial strains and plasmids

K. pneumoniae strains M5al (wild-type), M5al containing plasmid pNH3, and *K. pneumoniae* UN4495 (ø(*nifK-lacZ)5935* Δ*lac-4001 hisD4226* Gal^r) (MacNeil et al., 1981) and mutant derivatives (*K. pneumoniae* UN4495 *fnr*::Ω (RAS18, Grabbe et al., 2001b), *K. pneumoniae* UN4495 *glnK*::KIXX) were used in this study. The *glnK*::KIXX allele was transferred from *K. pneumoniae* UNF3433 (Jack et al., 1999) into *K. pneumoniae* UN4495 by P1-mediated transduction with selection for kanamycin resistance, resulting in RAS36. Plasmid pNH3 carries the *K. pneumoniae nifLA* operon under the control of the *tac* promoter (Henderson et al., 1989).

Growth conditions

K. pneumoniae strains were grown anaerobically with molecular nitrogen (N_2) as gas phase at 30°C in minimal medium supplemented with either 2 mM glutamine (nitrogen limitation) or 10 mM ammonium (nitrogen sufficiency) as the sole nitrogen source, 0.004 % histidine, 10 mM Na_2CO_3, 0.3 mM sulfide and 1 % sucrose as the sole carbon source (Schmitz et al., 1996). To monitor anaerobiosis, the medium was further supplemented with 0.002 % resazurin. Precultures were grown overnight in closed bottles with N_2 as gas phase in the same medium but lacking sulfide and resazurin. The 1-l main cultures were inoculated from precultures and incubated in closed bottles with molecular nitrogen as gas phase under strictly anaerobic conditions without shaking. Samples were taken anaerobically for monitoring the optical density at 600 nm and determining ß-galactosidase activity. Aerobic 1-l cultures were incubated in 2-l flasks with vigorous shaking (130 rpm) using the same medium and culture

supplements as described for the anaerobic growth but lacking Na_2CO_3, sulfide and resazurin. For ammonium shift experiments, 1-l cultures growing under nitrogen limitation in the presence of 2 mM glutamine were shifted to nitrogen sufficiency by the addition of 10 mM NH_4Cl during mid-exponential growth; the shifted cultures were further incubated for 2 h before the cells were harvested.

β-Galactosidase assay

NifA-mediated activation of transcription from the *nifHDK* promoter in *K. pneumoniae* UN4495 and mutant derivatives (UN4495 *fnr*::Ω and UN4495 *glnK*::KIXX) was monitored by measuring the differential rate of β-galactosidase synthesis during exponential growth (U ml^{-1} $(OD_{600})^{-1}$) as described by Schmitz *et al*. (1996). Inhibitory effects of NifL on NifA activity were assessed by a decrease in *nifH* expression.

Electron microscopy

For electron microscopy, *K. pneumoniae* strain M5al carrying a plasmid-borne *nifLA*-operon under the control of the *tac* promoter was used (pNH3, Henderson et al., 1989) in addition to the chromosomal *nifLA* operon. Cultures (50 ml) were incubated anaerobically in minimal medium supplemented with either 2 mM glutamine or 10 mM NH_4Cl as the sole nitrogen source, as described above. During growth additional NifL and NifA synthesis from the plasmid was induced by the addition of 10 µM IPTG. When cultures reached an OD_{600} of 0.8, cells were harvested by centrifugation at 10.000 x g under anaerobic conditions. The resulting cell pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.3, and cells were chemically fixed in 0.2 % (w/v) formaldehyde and 0.3 % (w/v) glutardialdehyde solution for 90 min at 0 °C in a closed reaction cup under anaerobic conditions. Finally, the cells were dehydrated in a graded methanol series and embedded in Lowicryl K4M resin under air (Roth et al., 1981; Hoppert and Holzenburg, 1998). Resin sections of 80 - 100 nm in thickness were cut with glass knives. NifL was localized in resin sections using specific polyclonal antisera directed against NifL and goat-anti-rabbit-IgG linked to colloidal gold (10 nm in diameter, BBI, Cardiff, UK), essentially as described by Roth et al. (1978) with some modifications (Hoppert and Holzenburg, 1998). Electron micrographs were taken, at calibrated magnifications, with a Philips EM 301 (Philips, Eindhoven, The Netherlands).

Membrane preparation

To localize of NifL synthesized from the single chromosomal copy of the *nifL* gene, cytoplasmic and membrane fractions of *K. pneumoniae* UN4495 and mutant derivatives were separated by several centrifugation steps. Membranes from anaerobically grown cells were prepared under strictly anaerobic conditions in the presence of 2 mM dithiothreitol under a nitrogen atmosphere; aerobic membranes were prepared under aerobic conditions in the absence of dithiothreitol. To separate the membrane and cytoplasmic fractions, exponentially growing 1-l cultures were harvested by centrifugation, resuspended in 30 ml B buffer (2 mM Epps (*N*-[2-hydroxyethyl]piperazine-*N*'-3-propanesulfonic acid), 25 mM potassium glutamate, 5 % glycerol, pH 8.0) and disrupted using a French pressure cell. Cell debris were sedimented by centrifugation twice at 20,000 x g for 30 min each time. The resulting cell-free cell extract was centrifuged twice at 120,000 x g for 2 h to sediment the membrane fraction. The membrane fraction was subsequently washed two times with 10 ml B-buffer followed by centrifugation at 120,000 x g for 2 h. The resulting supernatants of all ultracentrifugation steps were combined (a totat volume 50 ml), designated the cytoplasmic fraction and stored at 4 °C for further studies. The resulting hydrophobic pellets were resuspended in 10 ml B-buffer containing 3 mM Triton X-100. The membrane-bound and membrane-associated proteins were solubilized out of the membrane fraction by incubating the resuspended membrane pellet for 30 min at 4 °C under vigorous shaking. After this solubilization step, the phospholipids were subsequently separated from the solubilized protein by centrifugation at 120,000 x g for 2 h. The supernatant of a total volume of 10 ml containing the solubilized proteins was designated the membrane fraction and stored at 4 °C for further studies. Protein concentration of the membrane and cytoplasmic fraction was determined via the method of Bradford (1976) with the BioRad protein assay using bovine serum albumin as standard.

The quality of the membrane preparations was evaluated by determination of the malate dehydrogenase activity in both the membrane and the cytoplasmic fraction, according to Bergmayer (1983). The oxidation of NADH was measured at room temperature in 1-ml test assays containing 100 mM HEPES pH 7.4, 0.44 mM NADH, and 100 µl of the respective samples. The reactions were started by the addition of 1.8 mM oxaloacetate. The oxidation of the NADH was monitored at 365 nm using a Jasco V550 UV/Vis-spectrophotometer. In addition, quinoproteins were specifically detected by a redox-cycle stain assay to detect leakage of membrane proteins into the cytoplasmic fraction. Aliquots (5 µl) of membrane and cytoplasmic fractions were spotted on a nitrocellulose membrane and stained using 0.24 mM nitroblue tetrazolium in 2 M potassium glycinate (pH 10) as described by Flückiger et al. (1995). The nitrocellulose membrane was immersed in the nitroblue tetrazolium/glycinate

solution in the dark for 45 min, resulting in a blue-purple stain of quinoproteins. Subsequently protein was stained red with Ponceau S (0.1 % in 5 % acetic acid); the already-stained quinoproteins remained blue-purple.

Western blot analysis and quantification of NifL, NifA and GlnK in membrane and cytoplasmic fractions

Samples of the membrane and cytoplasmic fractions were diluted 1:1 with gel-loading buffer containing or lacking SDS and subsequently separated by SDS-polyacrylamide (12 %) gel electrophoresis (Laemmli, 1970) or native polyacrylamide (12.5 %) gel electrophoresis (Atkinson et al., 1994), respectively. Prestained protein markers (New England Biolabs, UK) were used as molecular mass standards. After separation, proteins were transferred to nitrocellulose membranes as described previously (Sambrook et al., 1989). Membranes were exposed to specific polyclonal rabbit antisera directed against the NifL, NifA or GlnK proteins of *K. pneumoniae*. Polyclonal antibodies directed against NifL, NifA and GlnK from *K. pneumoniae* were specific for the *K. pneumoniae* proteins NifL, NifA and GlnK, respectively. Polyclonal GlnK antibody was used in a very high dilution range, conditions under which cross-reaction with GlnB was approximately negligible as confirmed by separating purified GlnB and GlnK by isoeletric focusing and western blot analysis.

Protein bands were detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (BioRad Laboratories). The bands were visualized using the ECLplus system (Amersham Pharmacia) with a fluoroimager (Storm, Molecular Dynamics). The protein bands were quantified for each growth condition in three independent membrane preparations using the ImageQuant v1.2 software (Molecular Dynamics) and known amounts of the respective purified proteins. The calibration with purified *K. pneumoniae* proteins showed that quantification of NifL and NifA was linear within absolute amounts of 0.5 to 10 µg per lane and GlnK within 0.5 to 5 µg; all quantifications of those proteins in *K. pneumoniae* cell fractions have been performed within this linear range of the detection system. For calculation of the protein amounts in the fractions the quantifications were normalized to the actual volume for both membrane and cytoplasmic fractions. This was done either by initially applying 20 µl of the cytoplasmic and 4 µl of the membrane fraction onto the gels or applying equal amounts of the fractions onto the gel and considering the higher total volume of the cytoplasmic fraction in the calculation. Relative amounts of protein in the respective fraction to total amount were calculated by setting the absolute amounts in both the cytoplasmic and membrane fraction of a membrane preparation as 100 %.

Analysis of GlnK uridylylation by native gel electrophoresis

For the analysis of GlnK modification, the different mobilities of the uridylylated and unmodified protein in non-denaturating polyacrylamide gels was investigated (Forchhammer and Hedler, 1997). Portein samples were separated by native gel electrohoresis using 12.5 % polyacrylamide gels (29:1, acrylamide:bisacrylamide) with 5% stacking gels. The buffer for the running gels was 187.5 mM Tris/HCl, pH 8.9, the buffer for the stacking gels was 62.5 mM Tris/HCl, pH 7.5, and the running buffer was 82.6 mM Tris/HCl, pH 9.4, containing 33 mM glycine. After gelelectrophoresis using a BioRad Miniprotein I electrophoresis apparatus and proteins were subsequently transferred on nitrocellulose membranes for western blot analysis. In general, uridylylated forms of GlnK proteins show higher mobilities in non-denaturing polyacrylamide gels resulting in a protein band with an apparent lower molecular mass than the respective non-modified protein.

Results

In our current working model for the oxygen signal transduction in *K. pneumoniae,* we hypothesize that under anaerobic conditions, the FAD moiety of NifL is reduced by a component of the anaerobic electron transport chain, which is transcriptionally controlled by Fnr. If the reduction of NifL indeed occurs by a membrane-bound electron donor, then NifL must contact the cell membrane. We therefore localized NifL in *K. pneumoniae* and mutant strains growing under various conditions.

Localization of NifL in *K. pneumoniae* cells by electron microscopy. We localized NifL in *K. pneumoniae* strain M5a1 grown anaerobically under nitrogen limitation or nitrogen-sufficient conditions in the presence of 2 mM glutamine or 10 mM ammonium, respectively. The detection of NifL synthezised from the chromosomal *nifL* gene could not be analyzed statistically by electron microscopy as the level of expression was too low (data not shown). We therefore induced additional NifL expression from the plasmid pNH3 in *K. pneumoniae* M5a1 with 10 µM IPTG to levels at which NifL function is regulated normally in response to oxygen and combined nitrogen in *K. pneumoniae* (Schmitz et al., 1996). Cells in mid exponential phase grown anaerobically were harvested in the absence of oxygen, and prepared for electron microscopy under a nitrogen atmosphere in a glove box, as described in Materials and Methods. Immunogold detection by electron microscopy analysis of the overexpressed protein in 50 independent cells showed that approximately 76.4 % of total NifL were found in

close proximity to the cell membrane, when cells were grown under nitrogen-limiting conditions indicating that NifL is membrane associated (Fig. 9 A_1 to A_4). In contrast, in cells grown under nitrogen-sufficient conditions, the NifL protein was, in general, not attached to the cell membrane but was found mainly within the lumen of the cell (up to 80 % of total NifL, Fig. 9 B_1 to B_4). These findings indicate that NifL is apparently membrane associated when synthesized under oxygen- and nitrogen-limitation, but is localized in the cytoplasm when grown in the presence of sufficient nitrogen source.

NifL synthesized from the chromosomal *nifL* gene is highly membrane associated under derepressing conditions. Localization of overproduced NifL by electron microscopy indicated that NifL is membrane associated in *K. pneumoniae* when cells are grown anaerobically under nitrogen limitation. As the amount of NifL synthezised from the chromosomal *nifL* gene was too insignificant for localization by immunogold labelling, we used immunological means for the detection and quantification of NifL synthezised from the chromosomal *nifL* gene in cytoplasmic and membrane fractions of *K. pneumoniae* cells grown under various conditions. In case of cell extract preparation and separation of membrane and cytoplasmic fraction of anaerobic grown cells all steps were performed in the presence of 2.0 mM dithiothreitol and under a nitrogen atmosphere.

K. pneumoniae strain UN4495 carrying a chromosomal *nifK-lacZ* fusion was used for the NifL localization experiments, in order to be able to monitor NifA activity during growth. The cells were grown under nitrogen limitation to induce chromosomal expression of NifL and NifA in the absence or presence of molecular oxygen. In order to control NifL regulation of NifA activity in the respective cultures, we analyzed NifA activity by determining β-galactosidase activity. In general, anaerobically growing cultures exhibited a β-galactosidase synthesis rate of approximately 4000 U $ml^{-1} \cdot OD_{600}^{-1}$, whereas the synthesis rate of aerobic cultures was determined to be approximately 200 U $ml^{-1} \cdot OD_{600}^{-1}$. This indicated that the *nif* genes were fully induced under nitrogen- and oxygen-limiting conditions, and repressed in the presence of oxygen.

In order to localize NifL in these cells, membranes were prepared under anaerobic or aerobic conditions, and the membrane and cytoplasmic fractions were separated, as described in Materials and Methods. The quality of the membrane preparations was evaluated using malate dehydrogenase as a marker for the cytoplasmic fraction and quinones as a marker for the membrane fraction (see Materials and methods). For the various membrane preparations, we found that, in general, approximately 99 % of the malate dehydrogenase activity was located in the cytoplasmic fraction, and quinones were detectable only in the membrane fraction

(Table 5). The solubilized proteins of the various membrane and the cytoplasmic fractions were analyzed by gel electrophoresis, and subsequent detection of NifL protein by immunological means. The NifL protein in the different fractions was quantified for each growth condition in three independent membrane preparations using the fluoroimager and the ImageQuant software (Molecular Dynamics). Relative amounts of NifL in the respective fractions relative to total amount were calculated as described in Materials and Methods.

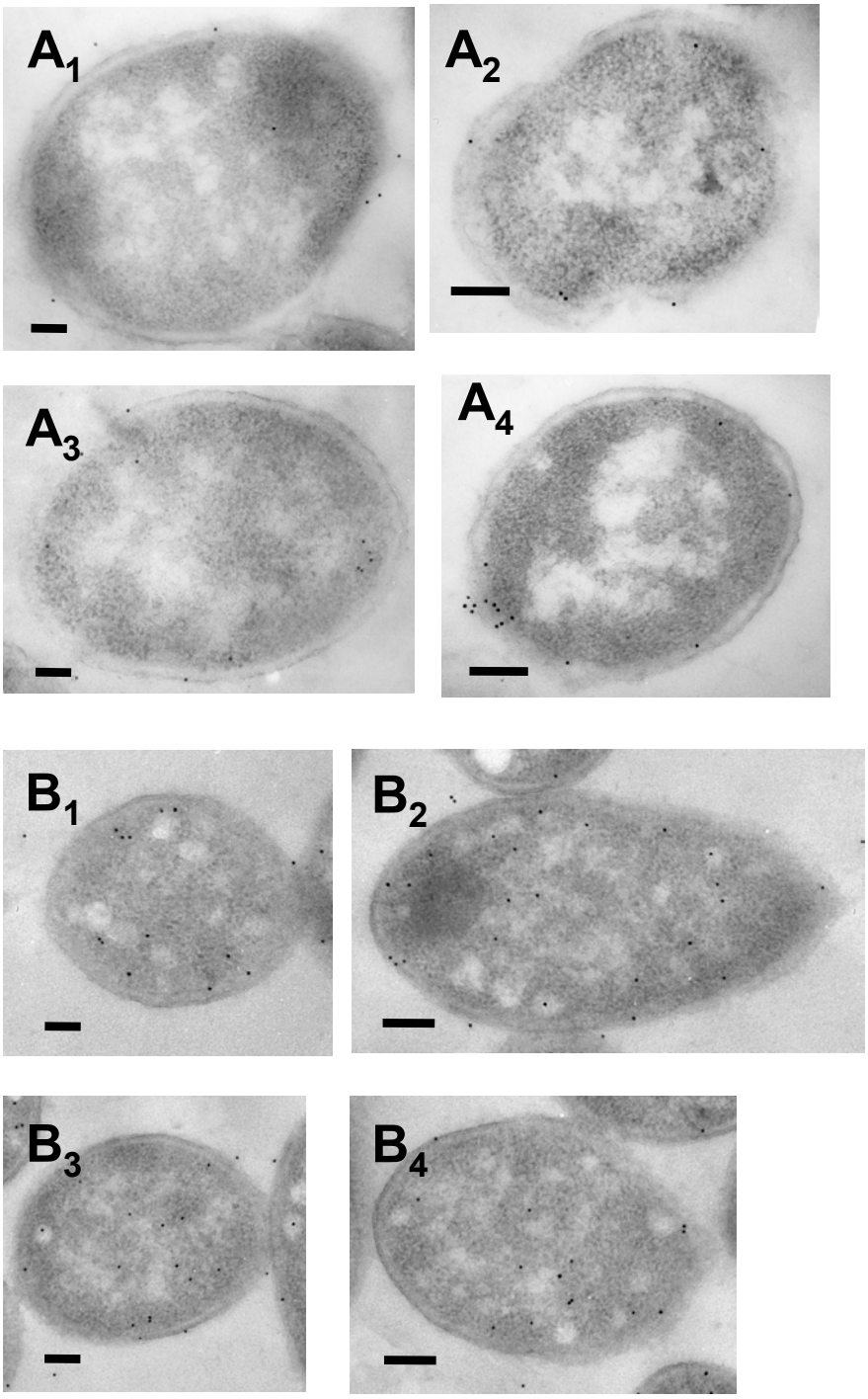


Fig. 9: Effect of ammonium on NifL localization in *K. pneumoniae.* NifL was overexpressed from the *tac* promoter by 10 µM IPTG in *K. pneumoniae* growing anaerobically under nitrogen-limited (A_1 to A_4) and nitrogen excess (B_1 to B_4) conditions. Cells were harvested in mid exponential growth and prepared for electron microscopy as described in Materials and Methods. NifL identified by immunogold labelling appears as dark spots (colloidal gold particles). Horizontal bars equal 0.1 µM.

Table 5: Quality of membrane preparations. The quality of the membrane preparations of *K. pneumoniae* UN4495 cells grown under the respective conditions was evaluated by determination of malate dehydrogenase activity according to Bergmayer (1983) and quinoprotein analysis by redox-cycle staining as described by Flückiger et al. (1995) in the respective fractions.

Growth condition	Cell fraction	Malate dehydrogenase activity (U x fraction^{-1})	Redox cycle stain (presence of quinoproteins)
Glutamine, aerobic	membrane	0.08	+
	cytoplasm	22	-
Glutamine, anaerobic	membrane	0.1	+
	cytoplasm	16	-

Initial experiments concentrated on the localization of NifL under nitrogen-limiting conditions, in both the absence and presence of oxygen. Under anaerobic growth conditions, approximately 55 % of total NifL were found in the membrane fraction (Fig. 10, lanes 1 and 2). In contrast, 6 % or less of total NifL synthesized under aerobic growth conditions was found in the membrane fraction (Fig. 10, lanes 5 and 6). The total amount of NifL synthesized under aerobic conditions was in the same range as the total amount synthesized under anaerobic conditions. This indicates that under anaerobic conditions, NifL is membrane-associated, whereas in the presence of oxygen, membrane association of NifL significantly decreases. Next we analyzed anaerobically growing cultures which were shifted from nitrogen-limited growth to nitrogen-excess conditions and grown for an additional 2 h when examined. Interestingly, although the cells were grown anaerobically, also only approximately 10 % of total NifL was found in the membrane fraction (Fig. 10, lanes 3 and 4). The total amount of NifL, however, did not significantly decrease after the shift to nitrogen sufficiency. Within the 2 h incubation in the presence of ammonium, no synthesis of NifL can occur because of repression of NifL synthesis by the nitrogen regulatory system (Drummond and Wootton, 1987). Thus, the presence of ammonium apparently resulted in a significant dissociation of NifL from the cytoplasmic membrane.

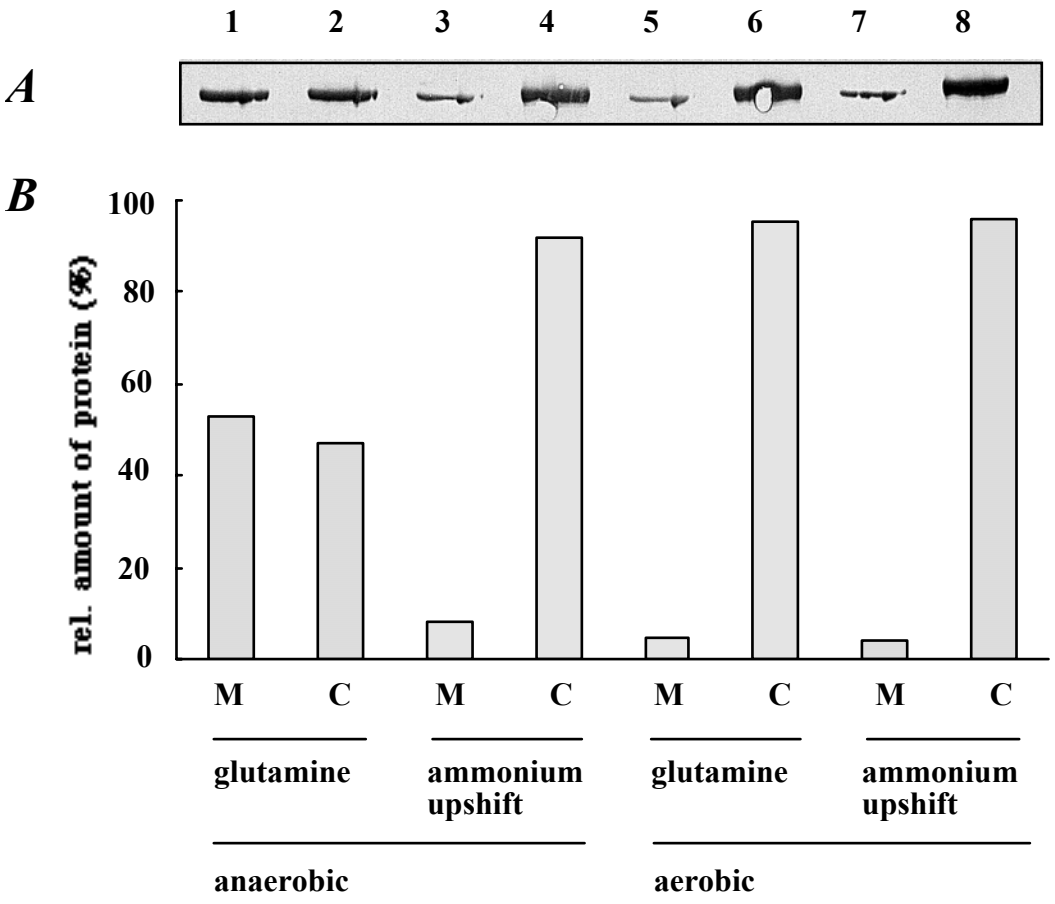


Fig. 10: **Localization of NifL synthezised from the chromosomal *nifL* gene in *K. pneumoniae* UN4495 grown under different conditions.** Cells of *K. pneumoniae* UN4495 were grown aerobically or anaerobically in minimal medium containing 2 mM glutamine as the sole nitrogen source. Exponentially growing cells were split and one half was shifted to ammonium excess (10 mM), as described in Materials and Methods. After an additional 2 h incubation, the cells were harvested and separated into membrane and cytoplasmic fractions. Aliquots of the observed membrane and cytoplasmic fractions (4 µl and 20 µl, respectively) were subjected to SDS-PAGE, and subsequently analyzed by westernblotting. Polyclonal NifL antibodies were used to detect NifL in the fractions. NifL found in the membrane and cytoplasmic fractions was quantified with a fluoroimager (Molecular Dynamics storm, ImageQuant software) as described in Material and Methods. **A**, original western blot. Lanes 1 and 2, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation; lanes 3 and 4, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation but shifted to nitrogen sufficiency and incubated for additional 2 hours; lanes 5 and 6, membrane and cytoplasmic fraction of cells grown aerobically under nitrogen limitation; lanes 7 and 8, membrane and cytoplasmic fraction of cells grown aerobically under nitrogen limitation but shifted to nitrogen sufficiency and incubated for additional 2 hours. **B**, quantity of NifL in the cytoplasmic and membrane fractions, as relative to total NifL; setting the absolute amounts in both fractions of the respective membrane preparation as 100 %.

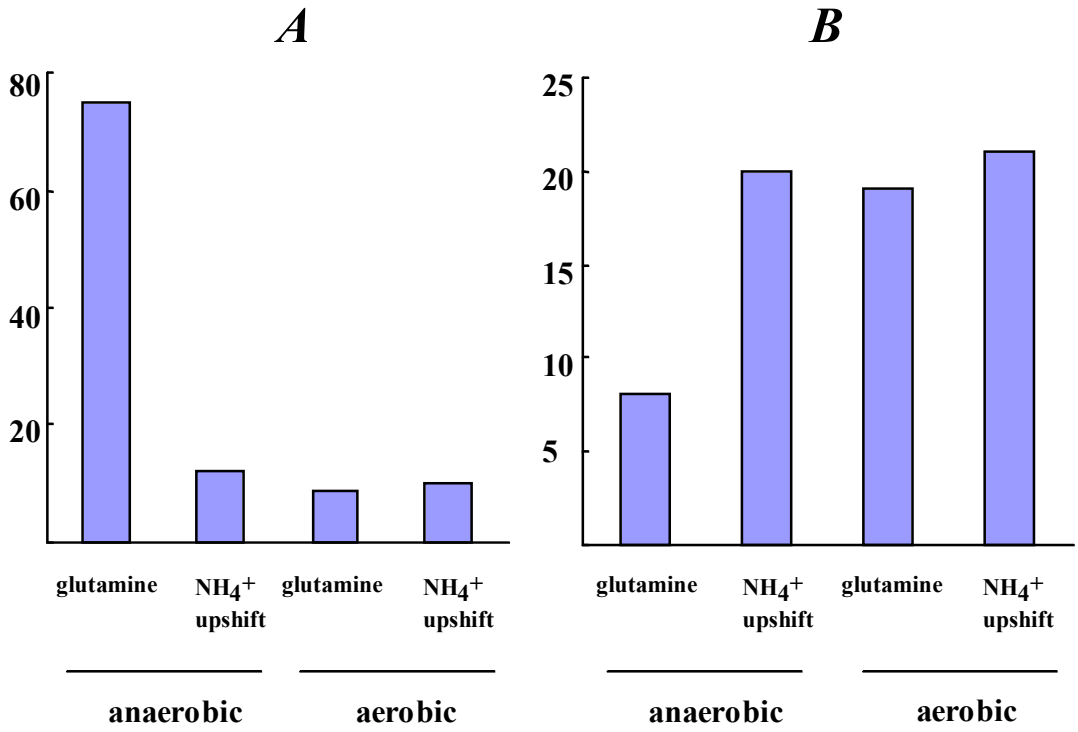


Fig. 11: **Comparison of relative amounts of NifL in the membrane fraction (A) and the cytoplasmic fraction (B) of *K. pneumoniae* UN4495 cells grown under different conditions.** Total amount of NifL in the respective cell fractions described in Fig. 2 were calculated using a fluoroimager (Molecular Dynamics storm, ImageQuant software) and known amounts of purified NifL-protein. Total amounts of NifL in the membrane (A) and the cytoplasmic fraction (B) under different growth conditions are plotted as relative to total protein in the respective fraction.

This was confirmed by plotting relative amounts of NifL in both the membrane and cytoplasmic fraction as relative to total protein in the respective fraction under the growth conditions tested (Fig. 11). These findings which are consistent with the results obtained by electron microscopy for overproduced NifL (Fig. 9) indicate that NifL is membrane associated only when cells are growing under derepressing nitrogen-fixation conditions. However, both individual signals, molecular oxygen or nitrogen sufficiency appear to result in a significant decrease in the membrane association of NifL to 10 % or less of total NifL. This suggests that the observed spatial separation of membrane-associated NifL and cytoplasmic NifA under anaerobic and nitrogen-limited growth conditions may be responsible for *nif* gene induction.

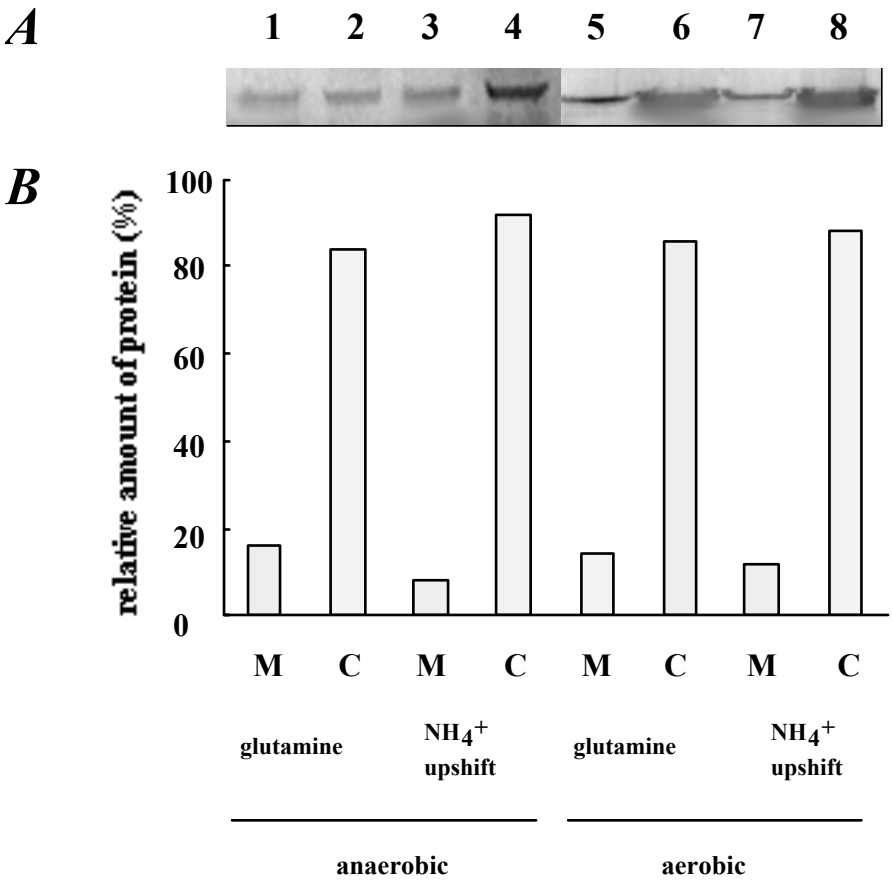


Fig. 12: Localization of NifA synthezised from the chromosomal *nifA* gene in *K. pneumoniae* UN4495 grown under different conditions. Membrane preparations of *K. pneumoniae* UN4495 cells grown under various conditions were performed as described in the legend of Fig. 2. Aliquots of the observed membrane and cytoplasmic fractions (4 µl and 20 µl, respectively) were subjected to SDS-PAGE, and subsequently analyzed by westernblotting. Polyclonal NifA antibodies were used to detect NifA in the fractions. NifA found in the membrane and cytoplasmic fractions was quantified with a fluoroimager (Molecular Dynamics storm, ImageQuant software) as described in Material and Methods. **A**, original western blot. Lanes 1 and 2, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation; lanes 3 and 4, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation but shifted to nitrogen sufficiency and incubated for additional 2 hours; lanes 5 and 6, membrane and cytoplasmic fraction of cells grown aerobically under nitrogen limitation; lanes 7 and 8, membrane and cytoplasmic fraction of cells grown aerobically under nitrogen limitation but shifted to nitrogen sufficiency and incubated for additional 2 hours. **B**, quantity of NifA in the cytoplasmic and membrane fractions, as relative to total NifA; setting the absolute amounts in both fractions of the respective membrane preparation as 100 %.

NifA is located in the cytoplasm under all conditions. In the presence of molecular oxygen or combined nitrogen, NifL inhibits NifA-dependent transcriptional activity by direct protein-protein interaction. In order to prove the hypothesis of a spatial separation of NifL and its target NifA under oxygen- and nitrogen-limited conditions, we localized NifA synthesized

from the chromosomal *nifLA* operon using the same membrane and cytoplasmic fractions in which we had localized NifL. We found that approximately 12 ± 3 % of total NifA are located in the membrane fraction under all growth conditions tested (Fig. 12). As shown for NifL, no difference in total NifA protein was detected under the various growth conditions. Taking into account that (i) NifA has to be localized in the cytoplasm to activate *nif* transcription, and (ii) the NifA membrane-associated fraction is under all conditions in the same range as the membrane association of NifL in the presence of oxygen or ammonium, a membrane association in the range of 10 % may be based on non-specific binding of hydrophobic regions of the two proteins to the membrane.

Under derepressing conditions but in the absence of either Fnr or GlnK ant AmtB, NifL is located in the cytoplasm. In order to observe additional evidence that both individual signals, molecular oxygen and nitrogen sufficiency, result in a significant decrease in the membrane association of NifL, we localized NifL synthesized from the chromosomal *nifL* gene under derepressing conditions in the absence of either the oxygen sensory protein Fnr or the nitrogen sensory protein GlnK and AmtB. *K. pneumonaie* UN4495 carrying an *fnr* null-allele (*K. pneumoniae* UN4495 *fnr*::Ω, RAS18) and *K. pneumoniae* UN4495 carrying an *glnKamtB* null-allele (*K. pneumoniae* UN4495 *glnK*::KIXX, RAS36) were grown under nitrogen- and oxygen-limited conditions. During growth NifA activity was analyzed by determining β-galactosidase synthesis rates.

As expected from previous studies (Grabbe et al., 2001b; He et al., 1998; Jack et al., 1999), in the absence of the primary oxygen sensor Fnr (RAS18) or in the absence the primary nitrogen sensor GlnK (RAS36), NifL inhibited NifA activity (Table 6). No obvious differences in the total amounts of NifA or NifL in the mutant backgrounds compared to the parental strain were detected, indicating that the failure of the mutant strains to express *nifH* under derepressed conditions could not be accounted for by a decreased amount of NifA protein. Localization of NifL in the two mutant strains under nitrogen and oxygen limitation in three independent membrane praparations showed that in both, the *fnr* mutant and the *glnKamtB* mutant, approximately 90 % of NifL is localized in the cytoplasmic fraction (Table 6).

Additional shifts to nitrogen excess did not change the NifL location significantly. Thus, in the absence of Fnr or GlnK plus the ammonium transporter AmtB, NifL does not receive the signal for oxygen- or nitrogen limitation, respectively, resulting in a NifL protein which is located in the cytoplasm and inhibits NifA activity. These findings strongly support our model that under derepressing conditions NifL is membrane associated, however either signal,

molecular oxygen or nitrogen sufficiency, result in a significant decrease of membrane association of NifL.

Table 6: Localization of NifL synthezised from the chromosomal *nifL* gene in *K. pneumniae* UN4495 and derived mutants under oxygen and nitrogen limitation and after a shift to nitrogen sufficiency. *nif* induction was monitored by measuring the differential rates of β-galactosidase synthesis as described by Schmitz et al. (1996). Membrane and cytoplasmic fractions were separated and NifL was immunological quantified in the cytoplasmic and membrane fractions by western-blot analysis using a fluoroimager (Molecular Dynamics storm, ImageQuant software) and purified proteins as desribed in Methods. Cells were grown under nitrogen-limitation in the presence of 4 mM glutamine (nitrogen -limitation) or were shifted to ammonium excess und further incubated for 2 h (ammonium upshift). The relative amount of NifL in the respective fraction is presented in% of total NifL and the absolute amount of NifL in µg NifL per mg protein of the respective cell fraction. Data presented represent mean values of three independent experiments.

Strain	UN4495 (ø(*nifK-lacZ*)*5935*Δ*lac-4001 hisD4226* Gal^r)		UN4495 *fnr*::Ω (RAS18)		UN4495*glnK*::KIXX (RAS36)	
Expression of *nifH'-'lacZ* ($U \cdot ml^{-1} \cdot OD_{600}^{-1}$)	4000 ± 100		250 ± 20		≤ 10	
cell fraction	**membrane**	**cytoplasm**	**membrane**	**cytoplasm**	**membrane**	**cytoplasm**
Nitrogen-limitation (%)	55 ± 5	45 ± 5	10 ± 3	90 ± 3	13 ± 2	87 ± 2
Nitrogen-limitation (µg NifL/mg fraction protein)	75 ± 4	8 ± 1	25 ± 3	22 ± 3	27 ± 3	23 ± 3
Ammonium-upshift (%)	7 ± 3	93 ± 3	6 ± 2	94 ± 3	10 ± 3	90 ± 3
Ammonium-upshift (µg NifL/mg fraction protein)	18 ± 2	22 ± 3	24 ± 2	21 ± 2	25 ± 3	22 ± 2

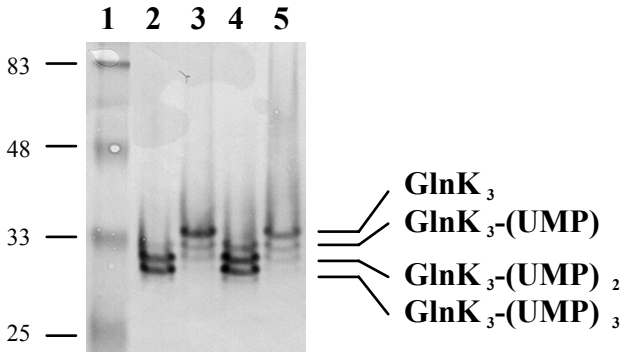


Fig. 13: Uridylylation states of GlnK upon an ammonium upshift. *K. pneumoniae* wild type cultures were grown aerobically and anaerobically in the presence of 2 mM glutamine as sole nitrogen source as described in Materials and Methods. During mid-exponential growth, cultures were split and one part was shifted to nitrogen-excess conditions by the addition of 10 mM ammonium. After an additional incubation of 2 hours the cells were harvested, broken by French Press and analyzed by native-PAGE. Subsequent western-blotting using polyclonal GlnK antibodies was used to detect uridylylated and unmodified GlnK synthesized from the chromosomal *glnK* gene. Lane 1, broad range prestained marker (New England Biolabs); lane 2, cell extract of anaerobically grown cells in the presence of 2 mM glutamine; lane 3, cell extract of anaerobically grown cells after an ammonium upshift with 10 mM ammonium; lane 4, cell extract of aerobically grown cells in the presence of 2 mM glutamine, and lane 5, cell extract of aerobically grown cells after an ammonium upshift with 10 mM ammonium.

Under nitrogen limitation GlnK is partially membrane associated independent of the oxygen status. NifL is membrane associated under oxygen- and nitrogen-limited conditions and dissociates from the membrane upon a shift to nitrogen sufficiency (Figs. 10 and 11). Thus the question arises, how does the NifL/NifA regulatory system receive the nitrogen signal when an upshift to nitrogen sufficiency occurs. As the GlnK protein apparently senses the nitrogen status of the cell and transduces the nitrogen signal to the NifL/NifA regulatory system, GlnK was localized under nitrogen-limiting conditions and after a shift to excess nitrogen.

In *K. pneumoniae,* the *glnK* gene, a *glnB*-like gene, is under the control of the general nitrogen regulatory system, and therefore only expressed under nitrogen starvation as is *E. coli glnK* (van Heeswjik *et al*. 1996; Jack et al., 1999; Arcondéguy et al., 2001). In response to nitrogen limitation, the trimeric *E. coli* GlnK protein is covalently modified by uridylylation at the conserved tyrosine residue (Y51) by the GlnD enzyme. In the presence of ammonium, however, GlnD removes the uridylylation (Atkinson and Ninfa, 1999; Jiang et al., 1998). In *K. pneumoniae* cells grown either anaerobically or aerobicically under nitrogen-

limiting conditions, GlnK trimers were up to 80 % uridylylated ($GlnK_3$-$(UMP)_3$) as detected by native gel electrophoresis and subsequent western blot analysis (Fig. 13, lanes 2 and 4); the uridylylation apparently changes the overall charge of the trimers resulting in a faster migration of the uridylylated forms compared to the non-modified trimers. Two hours after an ammonium upshift, the same cultures showed fully deuridylylated GlnK trimers (Fig. 13, compare lanes 2 and 4 with lanes 3 and 5). These findings show that the uridylylation state of *K. pneumoniae* GlnK, like that of *E. coli* GlnK, is dependent on the nitrogen status of the cell. In order to analyze and localize GlnK trimers after a shift to nitrogen sufficiency, we performed ammonium upshift experiments on *K. pneumoniae* cells grown under nitrogen-limited conditions in the presence or absence of oxygen. Exponentially growing cultures were split and one part was shifted to nitrogen sufficiency by the addition of 10 mM ammonium and further incubated for 2 h. The *glnKamtB* operon is subject to nitrogen control at the transcriptional level mediated by NtrC (Jack et al., 1999), thus within the 2 h incubation in the presence of ammonium, no expression of *glnK* can occur. The membrane and cytoplasmic fractions before and after the ammonium upshift were subjected to native PAGE and subsequent western blot analysis to separate and quantify the GlnK trimers in the different fractions. In the cell-free extracts under nitrogen-limiting conditions approximately 80 % GlnK was found in its completely uridylylated form ($GlnK_3$-$(UMP)_3$). 15 to 20 % of the total GlnK protein was found in the membrane fraction, in both the anaerobic and the aerobic preparation (Fig. 14B, lanes 1 and 2, lanes 5 and 6). The membrane-bound GlnK and cytoplasmic GlnK, however, showed no difference in the uridylylation pattern, indicating that membrane association is not dependent on a defined uridylylation state of GlnK (Fig. 14A, lanes 1 and 2, lanes 5 and 6). This observed membrane association of GlnK under nitrogen limitation is of special interest, since the GlnK protein shows little if any hydrophobicity, and is a highly soluble protein.

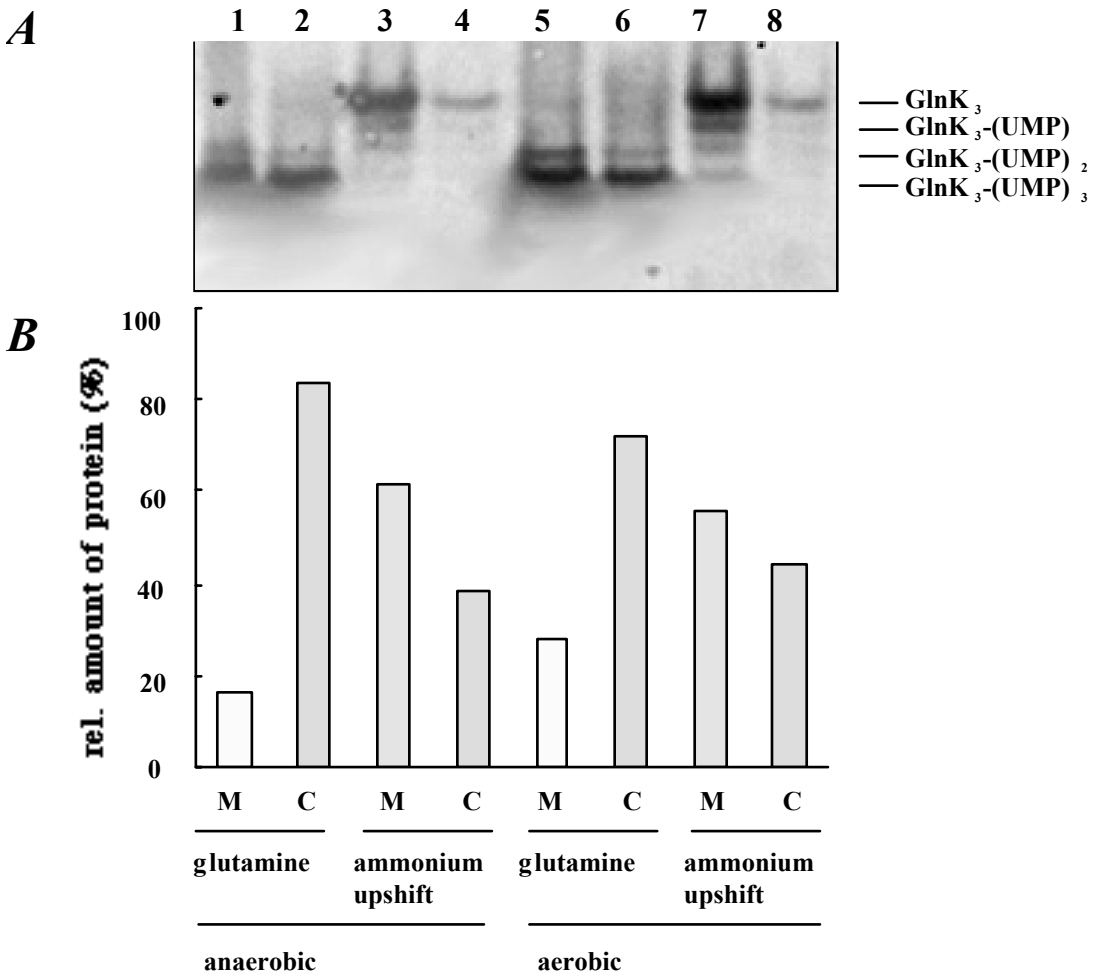


Fig. 14: Localization of GlnK in *K. pneumoniae* UN4495 grown under different conditions. *K. pneumoniae* UN4495 cells were grown, harvested and fractionated as described in Fig. 2. Equal volumes of the observed membrane and cytoplasmic fractions (20 µl) were subjected to native PAGE and subsequently analyzed by western-blotting. Polyclonal GlnK antibodies were used to detect GlnK. The western blot is shown in **A**. Lanes 1 and 2, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation; lanes 3 and 4, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation but shifted to nitrogen sufficiency and incubated for an additional 2 hours; lanes 5 and 6, membrane and cytoplasmic fraction of cells grown aerobically under nitrogen limitation; lanes 7 and 8, membrane and cytoplasmic fraction of cells grown aerobically under nitrogen limitation but shifted to nitrogen sufficiency and incubated for additional 2 hours. The amounts of GlnK found in the membrane and cytoplasmic fractions were quantified using a fluoroimager (Molecular Dynamics storm, ImageQuant software), concentrations of GlnK were corrected for fraction volume (total volume of the membrane fraction was 2 ml; total volume of the cytoplasmic fraction was 10 ml), and plotted as relative amounts of total GlnK in the respective fraction, in **B**.

When nitrogen limited *K. pneumoniae* cells were shifted from nitrogen limitation to nitrogen excess, GlnK in both the membrane and the cytoplasmic fractions was deuridylylated (Fig.

14A, lanes 3 and 4, lanes 7 and 8). However, it appeared that after a shift to nitrogen-excess independent of oxygen availability the cytoplasmic GlnK fraction decreased significantly. In contrast, the amount of membrane-associated GlnK apparently did not change (Fig. 14A, compare lanes 2 and 4, and lanes 6 and 8), thus shifting the ratio of membrane-associated GlnK to cytoplasmic GlnK from 15 % / 85 % under nitrogen limitation, to approximately 60 % / 40 % in the presence of excess nitrogen (Fig. 14B). As no new GlnK synthesis occurred during the ammonium upshift the ratio between membrane-bound and cytoplasmic GlnK should not change unless increased degradation of one fraction occurs. However, one cannot rule out that unspecific proteolysis of the cytoplasmic GlnK fraction occurred during the time of separating the two fractions, as the buffers used for cell breakage and membrane preparation were not supplemented with protease inhibitors. Thus, further analysis of the apparent faster degradation of cytoplasmic GlnK after a shift to nitrogen sufficiency is required.

Discussion

Regulatory proteins that are membrane bound and transmit an environmental signal via a cytoplasmic transmitter domain are a common principle in bacterial signal transduction. In a variety of such regulatory proteins or transducers of both prokaryotic and eukaryotic origin, conserved sequence motifs, so called PAS domains, have been identified (for review see Taylor and Zhulin, 1999). Most bacterial sensory proteins containing a PAS domain are histidine kinase sensor proteins of two-component regulatory systems, and usually contain one or more transmembrane domains (Zhulin et al., 1997; Taylor and Zhulin, 1999). The regulatory protein NifL contains a C-terminal histidine-kinase-like transmitter domain (Drummond and Wootton, 1987; Parkinson and Kofoid, 1992; Woodley and Drummond, 1994) and its N-terminal domain contains the conserved motifs of the PAS domain (Zhulin et al., 1997). NifL differs, however, in that no membrane-spanning domain can be predicted from amino acid sequence data of the protein (Drummond and Wootton, 1987). Thus, NifL is considered to be a solely cytoplasmic protein that receives and transduces the oxygen and nitrogen signal to the transcriptional activator NifA in the cytoplasm (Dixon, 1998). We have recently shown that Fnr of *K. pneumoniae* is the primary oxygen sensor for nitrogen fixation, which apparently transduces the oxygen signal to NifL by activating transcription of genes, whose products reduce the NifL-bound FAD under anaerobic conditions (Grabbe et al., 2001b). In addition, preliminary studies indicated that *K. pneumoniae* NifL is membrane-

associated during anaerobic growth. Thus we proposed that the physiological electron donor for the reduction of NifL during anaerobic growth is a component of the anaerobic electron transport chain. In order to characterize a potential membrane association of NifL as a part of the regulatory process further, we localized NifL in cells grown anaerobically and aerobically, both in the absence and presence of combined nitrogen.

Spatial separation as the potential regulatory principle in *nif* regulation by NifA and NifL. We present three lines of evidence that both conditions, nitrogen limitation and the absence of oxygen, are required for significant membrane association of *K. pneumoniae* NifL. Either signal alone is not sufficient for NifL association with the membrane. (i) Electron microscopy analysis of NifL overproduced in *K. pneumoniae* indicated that under oxygen- and nitrogen-limiting conditions, NifL is significantly membrane associated, whereas under nitrogen sufficiency NifL is located in the cell lumen (Fig. 9). (ii) Immunological quantifications of NifL synthesized from the chromosomal *nifL* gene confirmed that under oxygen and nitrogen limitation, approximately 55 % of the total NifL protein is located in the membrane fraction. A shift to nitrogen sufficiency or the presence of molecular oxygen, however, resulted in a significant decrease in membrane association of NifL, to approximately 10 % (Fig. 10). (iii) In the absence of eiher the primary oxygen sensor Fnr or the primary nitrogen sensor GlnK plus the ammonium transporter AmtB, NifL is located in the cytoplasm (Table 6). Thus, in addition to nitrogen limitation, the reduced conformation of NifL appears to be critically important for the membrane affinity of the protein. With oxidation, the membrane affinity of NifL significantly decreases, and NifL is again located in the cytoplasm. Determination of malate dehydrogenase activity and detection of quinoproteins in the different membrane and cytoplasmic fractions ruled out that the analyzed membrane fractions were contaminated with cytoplasmic proteins (Table 5). Thus, the basal amount of a maximum of 10 % membrane-bound NifL, detected under all conditions except under oxygen- and nitrogen-limitation, appears to be based on non-specific binding of the hydrophobic regions of the NifL protein to the cell membrane. This is consistent with the amounts we observed for the NifA protein in the same membrane and cytoplasmic fractions; approximately 10 % of total NifA were membrane associated under all conditions tested (Fig. 12), although NifA is a transcriptional activator and is therefore expected to be a soluble protein located in the cytoplasm (Austin et al., 1990; Lee et al., 1993). This suggests that the observed fractions of NifA and NifL, which appear to be membrane associated under all conditions, are fractions of both regulatory proteins, which bind, independent from each other, non-specifically to the membrane and are not functionally involved in the regulatory process.

The observed decrease in cytoplasmic NifL under anaerobic and nitrogen-limited conditions, conditions under which no change of NifA location is detectable, suggests that membrane association of NifL plays a critical role in the regulation of NifA activity. The spatial separation of membrane-bound NifL and cytoplasmic NifA under nitrogen and oxygen limitation may be responsible for the release of the NifL inhibition of NifA resulting in *nif* gene induction. We therefore propose a NifL conformation that integrates the oxygen and nitrogen signal in such a way that the overall conformation of the protein under anaerobic and nitrogen-limited conditions is able to bind to the cytoplasmic membrane, creating a spatial gap between NifL and its target NifA. A comparable regulatory mechanism is discussed for the transcriptional regulator PutA, which is involved in proline catabolism in *Salmonella typhimurium* and *Escherichia coli* (Maloy, 1987). PutA associates with the membrane and catalyzes the two-step oxidation of proline to glutamate when the intracellular proline concentration is high (Muro-Pastor et al., 1997; Wood, 1987); when the intracellular proline concentration decreases, PutA dissociates from the membrane and represses transcription of the proline utilization (*put*) operon by binding to an operator (Ostrovsky et al., 1991; Brown and Wood, 1993). In contrast to the observed membrane affinity of NifL in *K. pneumoniae* under oxygen and nitrogen limitation, no membrane association for *A. vinelandii* NifL has been reported to date (Dixon, 1998).

Hypothetical function for GlnK in *nif* regulation

Concerning the *nif* regulation by combined nitrogen, we observed evidence that a shift from nitrogen limitation to nitrogen sufficiency results in a decrease in membrane association of NifL (Figs. 9 and 10). Thus the question arises, how does the presence of sufficient nitrogen change the membrane affinity of NifL. We therefore localized GlnK, a highly soluble protein, which is responsible for the detection of the internal nitrogen status and for the transduction of the nitrogen signal to the *nif* regulatory system (Xu et al., 1998, He, et al., 1998; Jack et al., 1999; Arcondéguy et al., 1999 and 2001). Unexpected, we observed a significant membrane association of GlnK under nitrogen-limiting conditions (approximately 15 - 20 %, Fig. 14), which may result from its interaction with the ammonium transporter AmtB and is not dependent on a defined uridylylation state of GlnK. At the actual experimental status however, one cannot decide if the observed membrane-association of GlnK is directly linked to the NifL location or whether GlnK is regulating the NifL location indirectly as a consequence of its role in controlling the interaction of NifL with NifA. Under nitrogen excess in the absence of GlnK, cytoplasmic NifL inhibits NifA by complex formation. If

GlnK transduces the signal of nitrogen limitation either by interacting with NifL or NifA resulting in the dissociation of the NifL/NifA complex, NifL would be able to interact with the putative membrane bound electron donor and stays membrane-associated. In the presence of oxygen however, the oxidized form of NifL would be preferentially located in the cytoplasm and this conformation may interact with NifA even when GlnK is present.

Chapter 5:

Oxygen Control of *nif* Gene Expression in *Klebsiella pneumoniae* is dependent on NifL reduction at the cytoplasmic membrane by electrons derived from the reduced quinone pool

ROMAN GRABBE AND RUTH A. SCHMITZ*

Abstract

In *Klebsiella pneumoniae,* NifA mediated transcriptional activation of the nitrogen fixation (*nif*) genes is inhibited in the presence of molecular oxygen by the negative regulator NifL. The primary oxygen sensor Fnr transduces the signal of anaerobiosis to the negative regulator resulting in the non-inhibitory, reduced conformation of the flavoprotein NifL. We have recently demonstrated that membrane sequestration of NifL under anaerobic and nitrogen-limited conditions impairs inhibition of cytoplasmic NifA by NifL and thus seems to be involved in the regulatory mechanism for oxygen dependent *nif*-regulation in *K. pneumoniae*. We have now investigated the influence of different membrane-bound oxidoreductases of the anaerobic electron transport chain on *nif*-regulation in *K. pneumoniae* by biochemical analysis of purified NifL and by monitoring NifA-mediated expression of *nifH'-'lacZ* reporter fusions in different genetic backgrounds. *In vitro* analysis showed that NifL-bound FAD-cofactor was reduced by $NADH/H^+$ only in the presence of either a redox mediator or anaerobic inside-out vesicles derived from anaerobically grown *K. pneumoniae* cells, indicating that *in vivo* NifL is reduced by a membrane-bound component of the anaerobic electron transport chain. This mechanism is further supported by three lines of evidence: First, *Klebsiella* strains carrying null mutations of *fdnG* or *nuoCD* showed significantly reduced *nif* induction under derepressing conditions, indicating that NifL inhibition of NifA was not relieved in the absence of formate dehydrogenaseN or NADH:ubiquinone oxidoreductase. The same effect was observed in a heterologous *E. coli* system carrying a *ndh* null allele (coding for NADH dehydrogenase II). Second, studying *nif* induction in *K. pneumoniae* under different growth conditions revealed that the presence of nitrate during anaerobic growth on glycerol under nitrogen limitation resulted in a significant decrease of *nif* induction. However, when growing

on sucrose or glucose, nitrate did not effect *nif* regulation. The final line of evidence is that a reduced quinone derivative, dimethylnaphthoquinone$_{red}$ (DMN$_{red}$) is able to transfer electrons to the FAD-moiety of purified NifL resulting in the reduced conformation of NifL. On the basis of these data, we postulate that under anaerobic and nitrogen-limiting conditions NifL inhibition on NifA activity is relieved by reduction of the FAD-cofactor at the cytoplasmic membrane through the reduced quinone pool of the anaerobic electron transport chain.

INTRODUCTION

In the free-living diazotroph *Klebsiella pneumoniae*, a member of the γ-subgroup of proteobacteria, nitrogen (N_2) fixation is tightly controlled to avoid unnecessary consumption of energy. The transcriptional activator NifA and the inhibitor NifL, both under the control of the NtrB/C-system, regulate the transcription of the nitrogen fixation (*nif*) operons according to the environmental signals molecular oxygen and combined nitrogen (for review see Dixon 1998, Schmitz et al. 2002). Under oxygen and nitrogen limitation the inhibitor NifL stays in the non-inhibitory conformation and *nif*-gene expression is activated by NifA. In the presence of oxygen or combined nitrogen, NifL antagonizes the activity of NifA resulting in a decrease of *nif*-gene expression. The translationally coupled synthesis of *nifL* and *nifA* in addition to evidence from immunological studies of complex formation, imply that the inhibition of NifA activity by NifL occurs via a direct protein-protein interaction (Govantes et al. 1998; Henderson et al. 1989). Recently, in the diazotroph *Azotobacter vinelandii* formation of NifL/NifA complexes has been demonstrated by *in vitro* co-chromatography in the presence of adenine nucleotides and using the yeast-two-hybrid system (Money et al. 1999 and 2001, Lei et al. 1999).

Recent studies revealed that the nitrogen signal in *K. pneumoniae* and *A. vinelandii* is transduced towards the regulatory proteins NifL and NifA by the GlnK protein, a paralogue PII-protein. However, the mechanism appears to be opposite in *K. pneumoniae* and *A. vinelandii*. In *K. pneumoniae,* relief of NifL inhibition under nitrogen limiting conditions depends on the presence of GlnK, the uridylyation state of which appears not to be essential for its nitrogen signaling function (He et al. 1998, Jack et al. 1999, Arcondeguy et al. 1999 and 2000). It is currently not known, whether GlnK interacts with NifL or NifA alone or affects the NifL/NifA-complex in *K. pneumoniae*. In contrast to *K. pneumoniae*, non-uridylylated GlnK protein appears to activate the inhibitory function of *A. vinelandii* NifL under nitrogen excess, whereas under nitrogen limitation the inhibitory activity of NifL is

apparently relieved by elevated levels of 2-oxoglutarate (Little et al. 2000, Reyes-Ramirez et al. 2001). Very recently interactions between *A. vinelandii* GlnK and NifL was demonstrated using the yeast-two-hybrid system and *in vitro* studies further indicated that the non-uridylylated form of *A. vinelandii* GlnK directly interacts with NifL preventing *nif*-gene expression (Little et al. 2002, Rudnick et al. 2002).

For the oxygen-signaling pathway it was shown that *A. vinelandii* NifL and *K. pneumoniae* NifL act as redox-sensitive regulatory proteins. NifL modulates NifA activity in response to the redox-state of its N-terminal bound FAD-cofactor and allows NifA activity only in the absence of molecular oxygen, when the flavin cofactor is reduced (Hill et al. 1996, Schmitz 1997, Dixon 1998, Macheroux et al. 1998, Klopprogge and Schmitz 1999). Thus, under anaerobic conditions in the absence of combined nitrogen, reduction of the flavin moiety of NifL results in a non-inhibitory conformation of the NifL protein. Recently, we have demonstrated that in *K. pneumoniae* the global regulator Fnr is required to mediate the signal of anaerobiosis to NifL (Grabbe et al. 2001b). Thus, we proposed that in the absence of oxygen the primary oxygen sensor Fnr activates transcription of gene(s) the product(s) of which reduce the NifL-bound FAD-cofactor resulting in a non-inhibitory conformation of NifL, which allows NifA activity. Further localization analyses of NifL under various growth conditions showed that only under derepressing conditions NifL is highly membrane-associated impairing the inhibition of cytoplasmic NifA. This indicates that sequestration of NifL to the membrane under anaerobic and nitrogen-limited conditions is involved in the regulation of NifA activity by NifL (Klopprogge et al. 2002). Based on these findings the question arises, whether NifL reduction occurs at the cytoplasmic membrane by a component of the anaerobic electron transport chain during membrane association of NifL. In order to verify this hypothesis and to identify the electron donor - potentially localized in the cytoplasmic membrane - we analyzed the effects of different membrane-bound oxidoreductases of the anaerobic electron transport chain on *nif*-regulation in *K. pneumoniae* and in a heterologous *E. coli* system. In addition in vitro reduction of purified NifL was studied using artificial electron donors or $NADH/H^+$ in the presence of inverted vesicles derived from *K. pneumoniae* cells.

MATERIAL AND METHODS

Bacterial strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 7. Plasmid DNA was transformed into *E. coli* cells according to the method of Inoue et al. (1990) and into *K. pneumoniae* cells by electroporation. Transduction by phage P1 was performed as described previously (Silhavy et al. 1984).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	**Relevant genotype**	**Source, reference**
Strains: **Klebisella pneumoniae:**		
M5a1	Wild type	MacNeil et al. 1981
UN4495	ϕ (*nifK-lacZ*)5935 Δ*lac-4001 his D4226 Gal*^{r}	MacNeil et al. 1981
RAS 18	UN4495, but *fnr*::Ω	Grabbe et al. 2001
RAS46	UN4495, but spontaneous streptomycine resistance	This study
RAS47	UN4495, but *nuoCD*::*tet*	This study
RAS48	UN4495, but *fdnG*::*tet*	This study
RAS49	UN4495, but *frdA*::*tet*	This study
E. coli:		
NCM1529	*araD139Δ(argF-lacU)169 fthD5301 gyrA219 non-9 rspL150 ptsF25 relA1 deoC1 trpDC700putPA1303::*[Kan^r-(*nifH-lacZ*)] (Wild type)	He et al. 1998
NCM1528	NCM1529/pNH3	He et al. 1998
NCM1527	NCM1529/pJES851	He et al. 1998
RAS50	NCM1529, but *ndh::tet*	This study
RAS51	RAS50 + pNH3	This study
RAS52	RAS50 + pJES851	This study
RAS53	NCM1529, but *frd::tet*	This study
RAS54	RAS53/pNH3	This study
RAS55	RAS53/pJES851	This study
Plasmids:		
pBSK$^+$	cloning vector	Stratagene
pCR 2.1	Topo-TA cloning vector	Invitrogen
pKAS46	allelic exchange vector, *ori*R6K; *rpsL**(Strep^s), Amp^r, Kan^r	Skorupsky K. & R.K. Taylor, 1996
pNH3	*K. pneumoniae nifLA* under the control of the *tac* promoter	Henderson et al. 1989
pJES851	*K. pneumoniae nifA* under the control of *tac* promoter	Schmitz et al. 1996
pJES794	*K. pneumoniae malE-nifL* under the control of the *tac* promoter	Narberhaus et al. 1995

pRS167	*Eco*RI/*Hin*dIII *fdnG* fragment (*K. pneumoniae* M5a1) in pBSK$^+$	This study
pRS177	pRS167, but *fdnG::tet*	This study
pRS187	*frdA* fragment (*K. pneumoniae* M5a1) in pCR2.1	This study
pRS191	*Eco*RI/*Hin*dIII *nuoCD* fragment (*K. pneumoniae* M5a1) in pBSK$^+$	This study
pRS193	*fdnG::tet* fragment from pRS177 in pKAS46	This study
pRS194	pRS191, but *nuoCD::tet*	This study
pRS197	*nuoCD::tet* fragment from pRS194 in pKAS46	This study
pRS214	pRS187, but *frdA::tet*	This study
pRS215	*frdA::tet* fragment from pRS214 in pKAS46	This study

(i) *E. coli* strains:

E. coli NCM1529, containing a chromosomal *nifH´-lacZ´* fusion (He et al. 1997) was chosen to study NifA and NifL regulation in *E. coli*. The *ndh*II::*tet* allele was transferred from ANN001 (T. Friedrich, unpublished) into NCM1529 by P1 mediated transduction with selection for tetracycline resistance, resulting in RAS50. Strains RAS51 and RAS52 contain plasmid pNH3 and plasmid pJES851, respectively.

(ii) *K. pneumoniae* strains:

K. pneumoniae strain M5al (wild type, N_2-fixing) and strain UN4495 [ϕ(*nifK-lacZ*) 5935 Δ*lac-4001 his D4226 Gal*^{r}] (McNeill et al. 1981) were provided by Gary Roberts. The spontaneous streptomycin resistant UN4495 strain, RAS46, carrying a *rpsL* mutation was isolated by plating UN4495 on a Luria-Bertani (LB) agar plate containing 100 µg streptomycin per ml. *K. pneumoniae* subsp. *pneumoniae* (DSM No. 4799, not N_2-fixing) and *K. oxytoca* (DSM No. 4798, not N_2-fixing) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

Mutant strains of UN4495 were in general constructed by cloning the respective genes by PCR-techniques, inserting a tetracycline resistance cassette derived from the MiniTn*5* (DeLorenzo et al. 1990), cloning the respective interrupted genes into the suicide vector pKAS46 (Skorukpski and Taylor 1996) followed by transformation into the streptomycin resistant *K. pneumoniae* UN4495 strain (RAS46). Recombinant strains (generated by means of a double cross over) were identified by the ability to grow on LB supplemented with 400 µg streptomycin per ml and resistance to tetracycline (Skorukpski and Taylor 1996); the respective chromosomal mutations were confirmed by PCR and Southern blot analysis (Sambrock et al. 1989). For generating homologous primer for PCR amplification sequence information for genes of *K. pneumoniae* MG478578 (subsp. *pneumoniae*, not N_2-fixing) were

obtained from the database of the Genome Sequencing Center, Washington University, St. Louis (Genome Sequencing Center, personal communication) and using the database ERGO (Integrated Genomics, Inc.) (http://www.integratedgenomics.com).

nuoCD mutant: RAS47 was constructed as follows, (i) a 1.6 kb fragment carrying the *nuoCD* genes of *K. pneumoniae* M5a1 was amplified by PCR using primers with additional synthetic restriction recognition sites (underlined) nuoC/D ERI (5'CAGCGCGAATTCTCGCCG-GCA3') and primer nuoC/D HindIII (5'CTGCTGAAGCTTGCGCAGACTCTG') and cloned into pBluescript SK^+ producing pRS191, (ii) a 2.2 kb fragment containing the tetracycline resistance cassette (DeLorenzo et al. 1990) was inserted into the *Eco*RV site of *nuoCD* gene region in pRS191 yielding pRS194, (iii) the 3.8 kb *Eco*RI/*Kpn*I fragment of pRS191 carrying the interrupted *nuoCD* region was transferred into the allelic exchange vector pKAS46 (Skorukpski and Taylor 1996) creating plasmid pRS197; the correct insertion of the tetracycline cassette was checked by sequencing, (iv) pRS197 was transformed into RAS46 and recombinant strains carrying the chromosomally inserted plasmid by means of single homologous recombination were identified by their inability to grow on streptomycin agar plates as a consequence of the plasmid encoded *rpsL* mutation. Overnight selection of single colonies in liquid LB medium containing 400 µg streptomycin per ml resulted in the loss of the integrated plasmid with an integration frequency of the interrupted *nuoCD* region in 50 % of the integrands.

fdnG mutant: Primer fdnG 5‘ EcoRI (5'CCGACTGATGAATTCCGACCGCGA3') and primer fdnG 3‘ HindIII (5'GCCGAGCAGAAGCTTGATCATCGC3') were used to clone a 1 kb *fdnG* fragment from *K. pneumoniae* M5a1 into $pBSK^+$ vector creating pRS167, followed by insertion of the tetracycline resistance cassette into the *Eco*RV site of *fdnG* fragment resulting in pRS177. The 3.2 kb *Eco*RI/*Kpn*I fragment of pRS177 including the *fdnG::tet* region was cloned into pKAS46. The construction of the *K. pneumoniae* chromosomal mutant was performed using the same strategy as described in detail above, yielding RAS48.

Growth conditions. *E. coli* and *K pneumoniae* strains were grown anaerobically with molecular nitrogen (N_2) as gas phase at 30 °C in minimal medium supplemented with 4 mM glutamine as the sole nitrogen source (nitrogen limitation), 10 mM Na_2CO_3, 0.3 mM sulfide and 0.002% resazurin to monitor anaerobiosis (Schmitz et al. 1996). The medium was further supplemented with. 0.5 % sucrose and 0.004 % histidine for *K. pneumoniae* strains and 1% glucose and 0.002 % tryptophane for *E. coli* strains. Precultures were grown overnight in closed bottles with N_2 as gas phase in the same medium but lacking sulfide and resazurin. 25 ml main cultures were inoculated from precultures and incubated under a nitrogen atmosphere

and strictly anoxic conditions without shaking. Samples were taken anaerobically for monitoring the optical density at 600 nm and determining ß-galactosidase activity. In *E. coli* strains carrying a plasmid encoding NifL and NifA (pNH3) or NifA alone (pJES851) expression of *nifLA* or *nifA* from the *tac* promoter was induced by the addition of 10 µM IPTG (isopropyl-ß-D-thiogalactopyranoside).

ß-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 ß-galactosidase synthesis during exponential growth (units per ml per optical density at 600 nm (OD_{600}) (Schmitz et al. 1996)). Inhibitory effects of NifL on NifA activity were assessed by virtue of a decrease in *nifH* expression.

Purification of MBP-NifL. The fusion protein between maltose binding protein (MBP) and NifL was synthesized in NCM1529 carrying plasmid pJES794 (Narberhaus et al. 1995) growing aerobically at 30 °C in maximal induction medium (Mott et al. 1985) supplemented with 0.5 mM riboflavin. Expression of the fusion protein was induced with 100 µM IPTG when cultures reached an OD_{600} of 0.6. After harvesting and disruption in B buffer (20 mM Epps (N-[2-hydroxyethyl]piperazine-*N*'-3-propanesulfonic acid), 125 mM potassium glutamate, 5 % glycerol, 1.5 mM dithiothreitol, pH 8.0) using a French pressure cell, cells debris were sedimented by centrifugation at 20,000 x *g* for 30 min and fusion proteins were purified from the supernatant by amylose affinity chromatography. All purification steps were performed at 4 °C in the dark preventing degradation of the FAD moiety. The purified protein was dialyzed overnight into B buffer containing 25 mM potassium glutamate and subsequently used for biochemical analysis. The amount of FAD cofactor of the NifL fractions was calculated using a UV/Vis spectrum at 450 nm and the extinction coefficient $\in_{450}$ = 11.3 $mM^{-1}cm^{-1}$ (Whitby 1953)**.** In general an FAD content of 0.4 to 0.6 mol FAD / mol purified MBP-NifL was obtained.

Spectral analysis of purified MBP-NifL. Purified MBP-NifL was reduced under a N_2 atmosphere in the presence of $NADH/H^+$ and methyl viologen. The standard 0.2 ml assay was performed in B buffer (25 mM potassium glutamate, pH 8.0) under a nitrogen atmosphere using 40 µM MBP-NifL. Reduction of fully oxidized MBP-NifL at room temperature was followed using a spectrophotometer with an integrated diode array detector (J&M Analytische Meß- und Regeltechnik, Aalen, Germany). As reductants 1.25 mM $NADH/H^+$ (final concentration) in the presence of 0.2 µM methyl viologen or inverted vesicles (10 mg/ml) derived from *K. pneumoniae* cells and 0.12 mM (final concentration) non-physiological

electron donor, reduced dimethylnaphthoquinone (DMN_{red}) was used in the absence of a redox mediator. Stock solution of DMN was prepared in methanol. After dilution into anaerobic B-buffer containing 25 mM potassium glutamate, DMN was reduced by molecular hydrogen in the gas phase in the presence of platin oxide.

Preparation of inside-out vesicles of *K. pneumoniae*. 1 l cultures of *K. pneumoniae* cells were grown under nitrogen and oxygen-limited conditions, harvested at an optical density of OD_{600} = 1.3 and vesicles were prepared according to Krebs et al. (1999) except the addition of diisopropylfluorophosphate to the vesicle buffer. Inverted vesicles were directly used for the reduction of MBP-NifL or stored at -70 °C. All manipulations were performed under exclusion of oxygen in an anaerobic chamber at 4 °C.

Determination of NADH:ubiquinone oxidoreductase activity. The enzyme activity of the NADH:ubiquinone oxidoreductase in cell extracts prepared under anaerobic conditions was determined as described by Friedrich et al. (1989) using ferricyanide as electron acceptor. The assay contained vesicle buffer (10 mM Tris/HCl pH 7.5, 50 mM KCl, 2 mM DTT), 0.3 mM $NADH/H^+$ and 0.2 mM potassium ferricyanide. The reaction was started by adding cell extract and reduction of ferricyanide was monitored at 410 nm.

Southern blot analysis. Southern blots were performed as described by Sambrock et al. (1989) using a vacuum pump for the DNA transfer. Hybridization with DIG-labeled probes and detection using CSPD as substrate was carried out according to the detection protocol of the manufacturer (Boehringer, Germany).

Western blot analysis. 1 ml samples of exponentially growing cultures were harvested and concentrated 20-fold into sodium dodecyl sulfate (SDS) gel-loading buffer (Laemmli, 1970). Samples were separated by SDS/polyacrylamide (12%) gel electrophoresis and transferred to nitrocellulose membranes as described (Sambrock et al. 1989). Membranes were exposed to polyclonal rabbit antisera directed against the NifL or NifA proteins of *K. pneumoniae*, protein bands were detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (BioRad Laboratories). Purified NifA and NifL from *K. pneumoniae* and prestained protein markers (New England Biolabs, UK) were used as standards.

RESULTS

Under oxygen and nitrogen limitation reduction of the flavin moiety of NifL results in a non-inhibitory conformation of the NifL-protein. Localization analysis of *K. pneumoniae* NifL revealed that under those derepressed conditions NifL is membrane-associated, indicating that sequestration of NifL to the membrane is involved in the regulation of NifA activity by NifL. In order to analyze whether the association of NifL to the cytoplasmic membrane is accompanied with the reduction of NifL by a membrane-bound electron donor, we studied reduction of purified MBP-NifL *in vitro* and analyzed the influence of different oxidoreductases of the anaerobic electron transport chain on NifL reduction.

***K. pneumoniae* NifL is reduced by NADH/H$^+$ in the presence of a redox-mediator or anaerobic inside-out vesicles.** In order to demonstrate whether NADH/H$^+$ is a potential electron donor *in vivo*, reduction of purified NifL was studied *in vitro*. In general, NifL was synthesized in maximal induction medium under aerobic conditions fused to the maltose binding protein (MBP) to keep NifL in a more soluble state. Subsequently MBP-NifL was purified to apparent homogeneity by affinity chromatography. The FAD content of those purified fractions was in the range of 0.4 - 0.6 FAD per MBP-NifL. Fully oxidized MBP-NifL (40 µM) was incubated in an anaerobic cuvette under a nitrogen atmosphere in a total volume of 200 µl B-buffer containing 25 mM glutamate. The absorption spectra were recorded online using a diode array detector. In the absence of a redox mediator, the addition of 1.25 mM NADH/H$^+$ (final concentration) did not result in reduction of the NifL-bound FAD-cofactor even after long incubation periods up to 25 min (data not shown). However, in the presence of 0.2 µM methyl viologen, significant reduction of the flavin-moiety of NifL by NADH/H$^+$ was observed. After the addition of NADH/H$^+$ the flavin-specific absorbance at 450 nm decreased constantly within 50 min indicating that the flavin cofactor of NifL was reduced by electrons derived from NADH/H$^+$ (Fig. 15). This was further supported by the difference spectrum of oxidized MBP-NifL before the addition of NADH/H$^+$ corrected versus the spectrum 50 min after NADH/H$^+$ addition, which clearly showed the flavin-specific absorption maximum at 450 nm (inset of Fig. 15) and the 420 nm absorbance which is generally found in NifL preparations synthesized under nitrogen sufficiency (Klopprogge and Schmitz, 1999). These findings strongly indicate that NADH/H$^+$ is a potential electron donor for NifL reduction *in vivo*, however it appears that the reducing equivalents derived from NADH/H$^+$ have to be transferred to NifL through an additional oxidoreductase system.

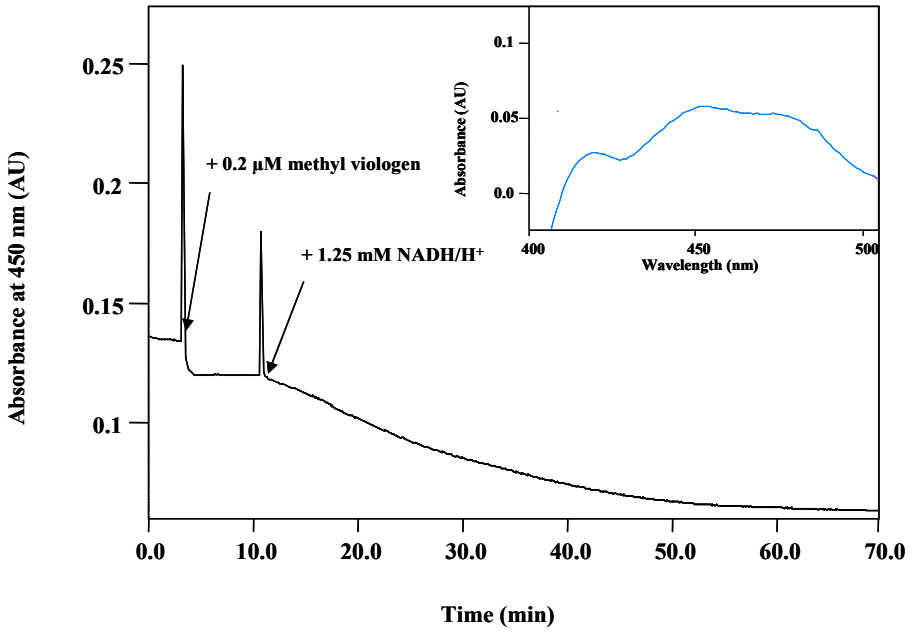


Fig. 15: Reduction of purified MBP-NifL with NADH/H$^+$ in the presence of methyl viologen. 40 µM purified fully oxidized MBP-NifL in B-buffer (pH 8.0) was incubated in an anaerobic cuvette under a nitrogen atmosphere at 25 °C. After the addition of methyl viologen to a final concentration of 0.2 µM the protein was reduced by the addition of 1.25 mM NADH/H$^+$ (indicated by arrows). The spectral changes were recorded using a spectrophotometer with an integrated diode array detector (J & M Analytische Mess- und Regeltechnik Aalen, Germany) and the reduction of the flavin moiety of the protein was monitored at 450 nm. The inset shows the difference spectrum; the fully oxidized spectrum at 10 min was corrected versus the reduced spectrum at 60 min.

To obtain further evidence for NifL reduction by NADH/H$^+$ via an oxidoreductase system in vivo we analyzed the effect of inside-out vesicles derived from *K. pneumoniae* cells on the reduction state of NifL. As NifL is membrane-associated under oxygen- and nitrogen-limitation oxidoreductases of the anaerobic electron transport chain are attractive candidates for transferring electrons derived from NADH/H$^+$ to membrane-bound NifL. To exclude the presence of contaminating redox mediators in the following experiments the cuvettes were extensively washed with chromosulfuric acid and control experiments were performed, in which no significant decrease of the NifL absorbance at 450 nm was observed after the addition of NADH/H$^+$. Inside-out vesicles were prepared under strictly anaerobic conditions from *K. pneumoniae* cells grown under nitrogen and oxygen limitation to obtain vesicles containing the anaerobic electron transport chain (see Materials and Methods). Fully oxidized MBP-NifL was incubated under a nitrogen atmosphere and the absorption spectrum was

recorded online. The addition of 100 µg inside-out vesicles resulted in a slow decrease of flavin specific absorbance at 450 nm, suggesting that NifL is partially reduced by electrons derived from reduced membrane-bound oxidoreductases (Fig. 16 A). This reduction process of NifL-bound FAD was further increased by the addition of NADH/H^+. Again, the difference spectrum of NifL before NADH/H^+ addition corrected versus the spectrum 32 min after the addition of NADH/H^+ clearly showed a significant decrease of the flavin-specific absorbance at 450 nm (Fig. 16 B). The finding, that NifL was reduced by a membrane-bound oxidoreductase system receiving reducing equivalents derived from NADH/H^+ strongly indicates that *in vivo* the NifL-bound FAD cofactor receives electrons from a reduced membrane-bound oxidoreductase system.

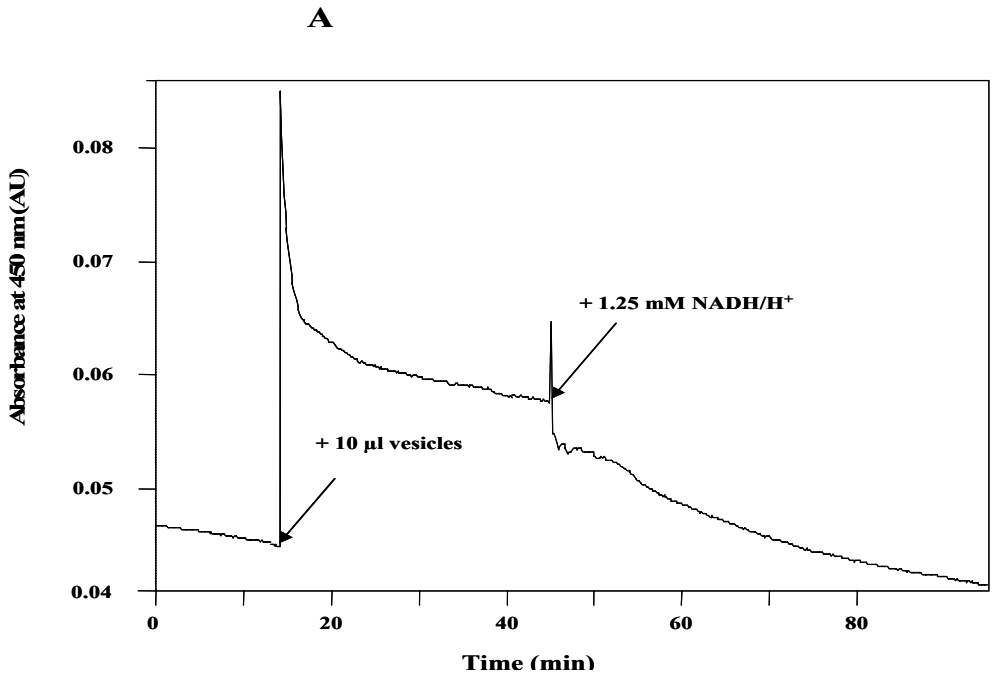
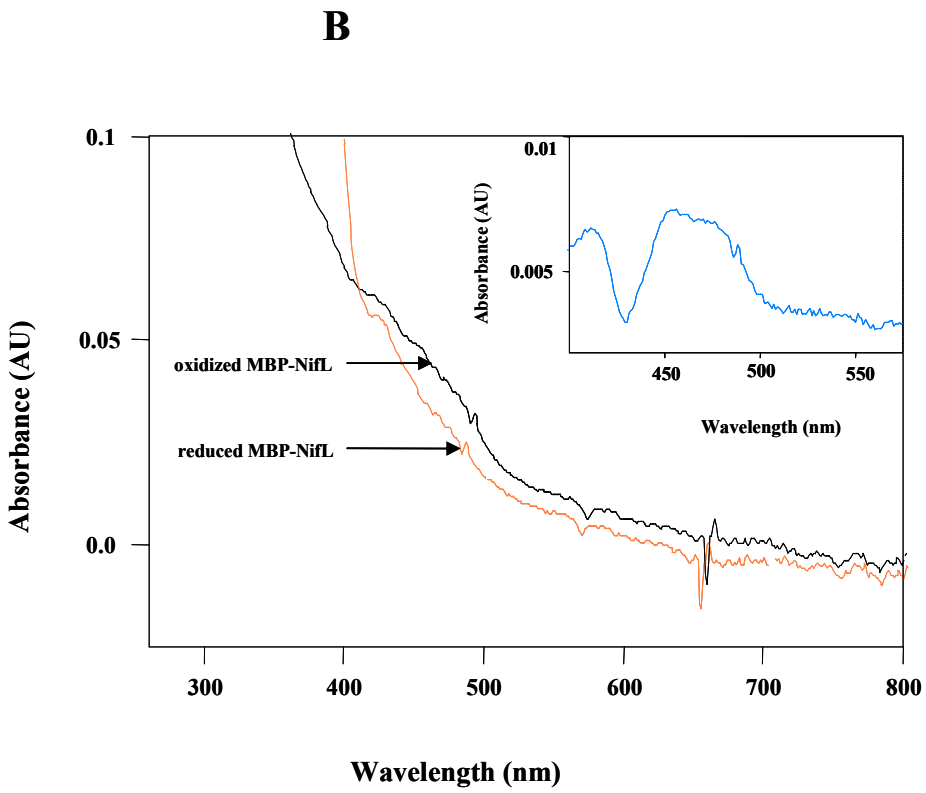


Fig. 16: Reduction of purified MBP-NifL with NADH/H^+ in the presence of inverted vesicles from *K. pneumoniae*. 40 µM purified fully oxidized MBP-NifL was incubated in an anaerobic cuvette under a nitrogen atmosphere at 25 °C in a final volume of 400 µl B buffer. 30 min after the addition of 10 µl inverted vesicles (10 mg / ml) of *K. pneumoniae* cells grown under nitrogen and oxygen limitation, the reduction was started by the addition of 1.25 mM NADH/H^+ (final concentration). Changes in absorbance upon the reduction of the flavin cofactor were recorded and monitored as described in the legend of figure 15. (**A**) Time course measurement at 450 nm of the MBP-NifL reduction with NADH/H^+ in the presence of inverted vesicles. (**B**) Absorbance spectra of MBP-NifL after vesicle injection at 40 min (oxidized MBP-NifL) and after NADH/H^+ addition at 85 min (reduced MBP-NifL). The inset shows the corresponding difference spectrum of oxidized MBP-NifL corrected versus the reduced spectrum.



Effects of chromosomal *ndh* and *frd* null mutations on *nif* induction in a heterologous *E. coli* system. The biochemical analyses of purified MBP-NifL and membrane association of NifL under derepressing conditions indicate that during the process of *nif* regulation the NifL-bound cofactor is apparently reduced by a membrane-bound oxidoreductase system. To obtain further evidence we studied the influence of two oxidoreductase systems on *nif* regulation in a heterologous *E. coli* system: (i) NADH dehydrogenaseII and (ii) fumarate reductase, which are both under transcriptional control of Fnr.

NCM1529 carrying a chromosomal *nifH'-'lacZ* fusion was used as parental strain. The *K. pneumoniae* regulatory proteins NifL and NifA were synthesized from plasmids, pNH3 (*nifLA*) or pJES851 (*nifA*) at induction levels, at which NifL function in *E. coli* is regulated normally in response to oxygen and combined nitrogen (He et al. 1997, Grabbe et al. 2001). To study the effect of the two oxidoreductases the respective null alleles, *ndh::tet* (NADH dehydrogenaseII) and *frd::tet* (fumarate reductase), were introduced by P1 transduction into the parental strain NCM1529 as described in Materials and Methods. After introducing *nifLA* and *nifA* on plasmids, the resulting strains were grown anaerobically in minimal medium with glucose as carbon source and glutamine as sole nitrogen source to study the effects of the

mutation on NifA regulation by NifL. No significant differences in growth rates or in the NifL and NifA expression levels were obtained for the mutant and the respective parental strains (Table 8). Transcription of *nifH'-'lacZ* fusion dependent on NifA activity was monitored in the two mutant backgrounds by determining synthesis rates of ß-galactosidase during exponential growth (Schmitz et al. 1996). In case of the *frd* mutation no effects on *nif* induction was detectable. Synthesis rates of ß-galactosidase determined for the *frd* mutant strain (RAS54) showed no significant difference compared to the parental strain (NCM1528) (Table 8). However, in the absence of a functional NADH dehydrogenaseII (RAS51), expression of *nifH'* significantly decreased resulting in a ß-galactosidase synthesis rate of 300 ± 20 U/ml/OD_{600}, which is equivalent to 10 % of the synthesis rate in the parental strain NCM1528 (3000 ± 100 U/ml/OD_{600}). In contrast, the *ndh* mutant strain carrying NifA alone on a plasmid (RAS52) showed no significant decrease in NifA activity compared to the parental strain (NCM1527), indicating that NifA activity is not directly affected by the *ndh* mutation. These findings suggest that in the absence of NADH dehydrogenaseII NifL apparently does not receive the signal for anaerobiosis and inhibits NifA activity. Thus, in the heterologous *E. coli* system NADH dehydrogenaseII may be responsible for reduction of membrane-associated NifL under anaerobic conditions.

Table 8: Effects of chromosomal *ndh* and *frd* null mutations on NifA activity in the heterologous *E. coli* system carrying *K. pneumoniae nifLA* on a plasmid. *E. coli* strains carrying a single copy of a ϕ(*nif'-'lacZ*) fusion (He et al. 1998) and *K. pneumoniae nifLA* (pNH3) or *nifA* (pJES851) under the control of the *tac* promoter were grown at 30 °C in minimal medium under anaerobic conditions with 4 mM glutamine as limiting nitrogen source. Expression of NifL and NifA was induced with 10 µM IPTG. Synthesis rates of ß-galactosidase were determined reflecting NifA activity in the respective strain background.

Strain	Relevant genotype	Expression of *nifH'-'lacZ* (U/min/OD_{600})[a]	Doubling time (h)
NCM1528	Wild type/*Ptac-nifLA*	3000 ± 100	5.0
NCM1527	Wild type/*Ptac-nifA*	5000 ± 200	4.8
RAS51[b]	*ndh* / *Ptac-nifLA*	300 ± 20	5.5
RAS52[b]	*ndh* / *Ptac-nifA*	4500 ± 150	5.2
RAS54[c]	*frd* / *Ptac-nifLA*	3500 ± 100	5.0
RAS55[c]	*frd* / *Ptac-nifA*	5400 ± 150	4.9

[a] Data presented as mean values (± standard aberration) of at least three independent experiments.
[b] Strain contains the *ndh::tet* allele from ANN001 (T. Friedrich, unpublished results).
[c] Strain contains the *frdABCD::tet* allele from JI222 (Imlay, 1995)

NADH:ubiquinone oxidoreductase and formate dehydrogenaseN are effecting *nif* regulation in *K. pneumoniae*. As the findings using the heterologous *E. coli* system indicated that NADH dehydrogenaseII might be the physiological electron donor for NifL reduction, we intended to construct a chromosomal null allele of the respective homologous gene in *K. pneumoniae.* In order to clone the *ndh* gene by PCR techniques we generated primers based on the 5' and 3' end sequences of the *ndh* gene of *K. pneumoniae* MGH78578 (subsp. *pneumoniae*); sequence information was obtained from the database from the Genome Sequencing Center, Washington University St. Louis (Genome Sequencing Center, personal communication). Using chromosomal DNA from *K. pneumoniae* subsp. *pneumoniae* (DSM No. 4799) as control, the *ndh* gene was amplified. However, no corresponding PCR product was obtained using chromosomal DNA from *K. pneumoniae* M5a1, although different pairs of homologous and degenerated primers were tested under various PCR-assay conditions. To confirm this finding we performed Southern blot analysis of *Sma*I digested chromosomal DNA derived from *K. pneumoniae* M5a1, *K. oxytoca* (DSM No. 4798) and *K. pneumoniae* subsp. *pneumoniae* using a DIG-labeled *ndh* probe derived from *K. pneumoniae* subsp. *pneumoniae*. Hybridization resulted in a single hybridization signal (4 kbp) for *K. pneumoniae* subsp. *pneumoniae* DNA (Fig. 17), which is in accordance with the expected fragment size based on the knowledge of the genomic sequence. However, no signal was obtained for DNA derived from *K. pneumoniae* M5a1 and *K. oxytoca* even under conditions of low stringency. These findings strongly indicate that the nitrogen fixing strain *K. pneumoniae* M5a1 and *K. oxytoca* do not exhibit an NADH-dehydrogenaseII. Thus, we decided to examine the influence of two other membrane-bound oxidoreductases of the anaerobic respiration on *nif* regulation in *K. pneumoniae*.

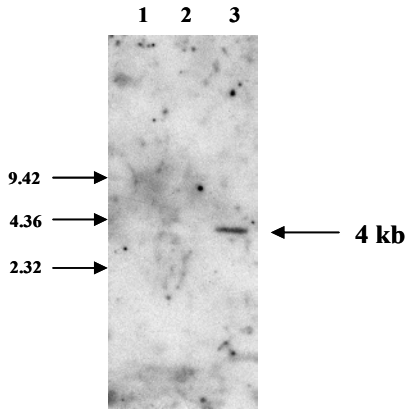
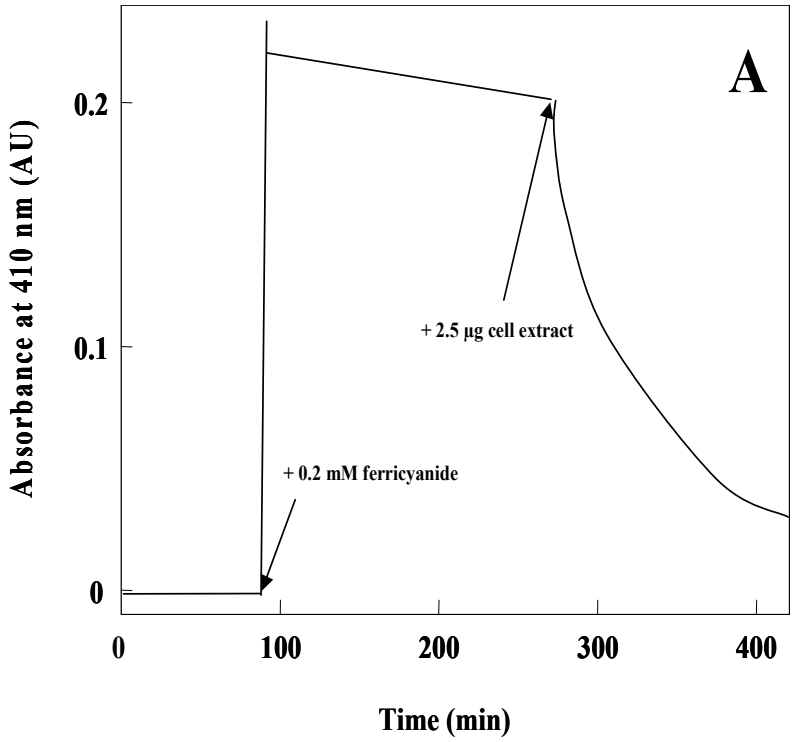


Fig. 17: Southern blot analysis using a *ndh*II probe derived from *K. pneumoniae* subs. *pneumoniae*. Genomic DNA from *K. pneumoniae* strains was completely digested with *Sma*I and equal amounts analyzed by Southern hybridization performed according to Sambrock et al. (1989) using a DIG-labeled *ndh*II probe (see Materials and methods). Lane 1, *K. pneumoniae* M5a1; lane 2, *K. oxytoca* (DSM 4798); lane 3, *K. pneumoniae* subs. *pneumoniae* (DSM 4799). Numbers on the left are molecular sizes in kilobases, the estimated size of the hybridizing fragment is indicated on the right.

K. pneumoniae strain UN4495 carrying *nifLA* and a *nifK'-'lacZ* fusion on the chromosome was used as parental strain which allows to monitor NifA mediated transcription from the *nifHDK* promoter by measuring the differential rates of ß-galactosidase synthesis during exponential growth (Schmitz et al. 1996). Two mutant strains of *K. pneumoniae* UN4495 were constructed carrying either a chromosomal *nuoCD* null allele (encoding for subunits C and D of the coupling NADH: ubiquinone oxidoreductase) or a chromosomal *fdnG* null allele (encoding for the γ subunit of formate dehydrogenaseN). The mutant strains were constructed by cloning the respective genes, inserting a tetracycline resistance cassette derived from the MiniTn*5* (DeLorenzo et al. 1990) and introducing the interrupted genes into the *K. pneumoniae* UN4495 chromosome using the allelic exchange system described by Skorupsky and Taylor (1996) (see Materials and Methods). The disruption of *nuoCD* and *fdnG* in the respective mutant strains was confirmed by PCR and Southern blot analysis (data not shown). In addition, the *nuoCD* mutant strain (RAS47) was further characterized biochemically by determining the specific activity of NADH-oxidation in anaerobic cell extracts. The specific activity of NADH-oxidation obtained for the *nuoCD* mutant strain (0.4 U/mg cell extract

protein) was significantly lower than the oxidation rate determined for the parental strain (10 U/mg cell extract protein) and might be based on unspecific NADH-oxidation (Fig. 18). In contrast, the residual NADH-oxidation rate of an *E. coli nuo* mutant strain was determined to be equivalent to 20 % of the NADH-oxidation rate obtained for the parental strain and is supposed to be dependent on NADH-dehydrogenaseII activity (Falk-Krzesinski and Wolfe, 1998). Thus, the significantly lower residual NADH-oxidation rate of the *K. pneumoniae nuo* mutant strain (4 %) in comparison to the respective *E. coli* mutant further supports our finding that *K. pneumoniae* M5a1 does not exhibit a second NADH-dehydrogenase.



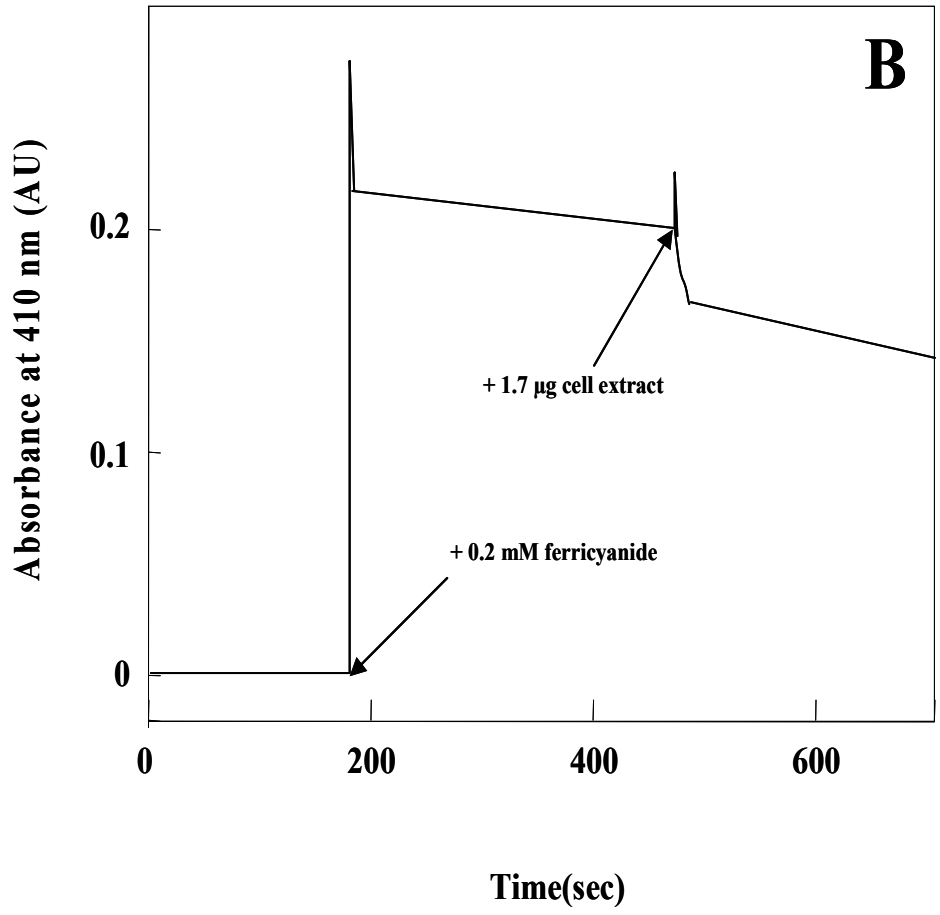
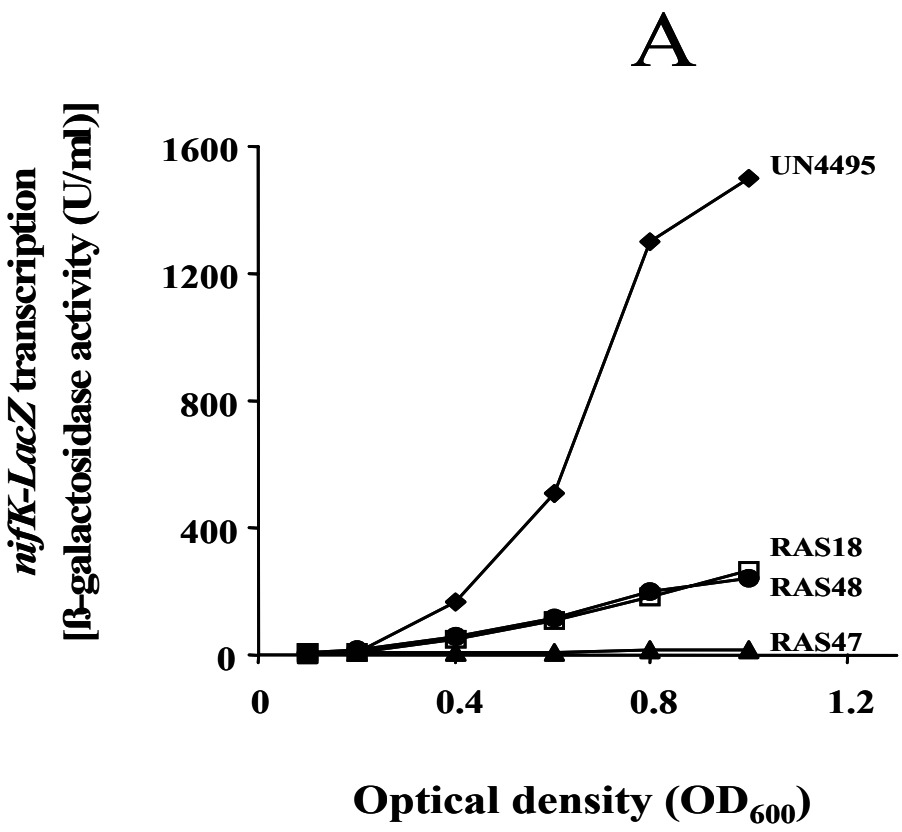


Fig. 18: Determination of NADH:ubiquinone oxidoreductase activity in *K. pneumoniae*. Activity of NADH oxidation by NADH:ubiquinone oxidoreductase was determined in cell extracts of anaerobically grown cultures (see Materials and methods). The decrease of absorption reflecting reduction of ferricyanide by $NADH/H^+$ was monitored at 410 nm. (A) wild type (UN4495) and (B) *nuoCD* mutant strain (RAS47). The addition of ferricyanide and crude cell extract is indicated by arrows.

K. pneumoniae wild type and the respective mutant strains were grown in minimal medium under oxygen limitation with glutamine as sole nitrogen source to exclude NifA inhibition by NifL in response to ammonium. Both mutant strains showed increased doubling times (td = 5 h) compared to the parental strain UN4495 (td = 3.5 h). This decrease in growth rates under anoxic conditions indicates that in the absence of either NADH:ubiquinone oxidoreductase or formate dehydrogenaseN the energy yield per mol glucose decreased based on reduced anaerobic respiration and increased fermentative recycling of NAD^+ from $NADH/H^+$. Unexpected both, the *nuoCD* and the *fdnG* mutant strain, showed significantly reduced levels of ß-galactosidase synthesis rates under derepressing conditions (Fig. 19). The ß-galactosidase synthesis rates determined for the *fdnG* mutant strain RAS48 (400 ± 30 U/ml/OD_{600}) were

similar to synthesis rates found in the *fnr* mutant RAS18 (Fig. 19 B, Grabbe et al. 2001b). The observed *nif* induction level determined for the *K. pneumoniae nuo* mutant strain decreased even more dramatically to levels of approximately 60 U/ml/OD_{600} (Figure 19 B), which is in the range of *nif* induction in the presence of oxygen and indicates that all NifL protein is in the oxidized inhibitory conformation (Grabbe et al. 2001b). Determination of NifL and NifA proteins in the mutant strain revealed no differences in the amount of the regulatory proteins compared to those of the parental strain (data not shown). These findings clearly show that in *K. pneumoniae* apparently more than one membrane-bound oxidoreductase system can provide electrons for NifL reduction under anaerobic conditions. They further indicate that NADH:ubiquinone oxidoreductase appears to play a major role in providing reducing equivalents for NifL *in vivo*.



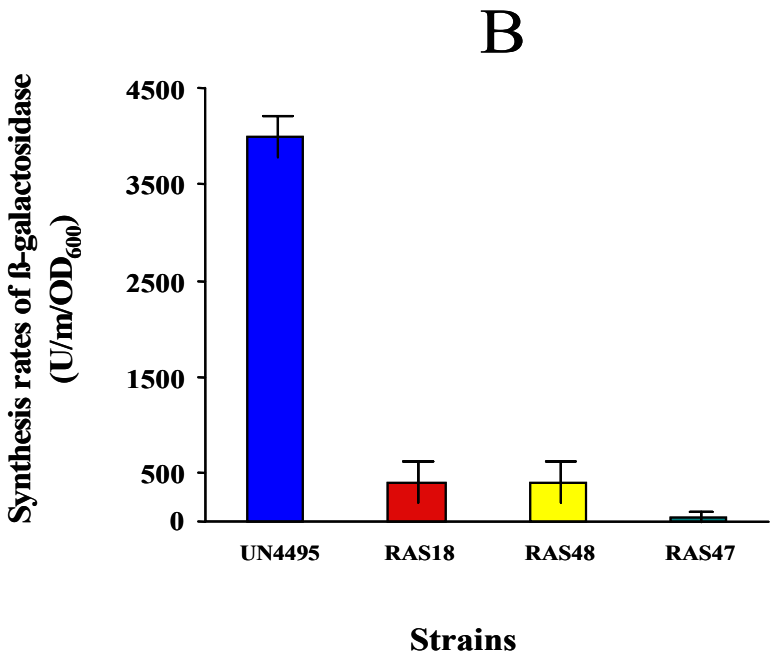


Fig. 19: Effects of chromosomal deletions in gene clusters encoding NADH:ubiquinone oxidoreductase (*nuo*) and formate dehydrogenaseN (*fdn*) on NifA activity in *K. pneumoniae* UN4495. NifA-mediated activation of transcription from the *nifHDK*-promoter in *K. pneumoniae* UN4495 and mutant derivatives was monitored by measuring the ß-galactosidase activity during anaerobic growth at 30 °C in minimal medium with glutamine (4 mM) as limiting nitrogen source. Activities of ß-galactosidase were plotted as a function of OD_{600} for *K. pneumoniae* UN4495 (wild type), the *fnr* mutant strain of UN4495 (RAS18), the *fdnG* mutant strain of UN4495 (RAS48) and the *nuoCD* mutant strain of UN4495 (RAS47) carrying a chromosomal *nifK'-'lacZ* fusion (**A**). Synthesis rates of ß-galactosidase from the *nifHDK* promoter were determined from the slope of these plots and are presented as bars reflecting *nif*-induction in the respective *K. pneumoniae* strains (**B**).

Effects of additional electron acceptors on *nif* regulation in *K. pneumoniae*. The finding that more than one oxidoreductase system can provide electrons for NifL reduction *in vivo* indicates that NifL apparently receives electrons at the cytoplasmic membrane provided by the quinone pool. To obtain additional evidence we studied *nif* induction in *K. pneumoniae* strain UN4495 in the presence of additional electron acceptors. The cultures were grown under nitrogen- and oxygen-limitation with glutamine as sole nitrogen source and sucrose, glucose or glycerol as carbon and energy source. In general, *nif* induction was significantly reduced when growing with glycerol as carbon and energy source and was equivalent to 25 % of the induction level obtained with sucrose (Table 9). As we assayed *nif* induction by determining the differential rate of ß-galactosidase synthesis, the calculated induction levels

are normalized for differences in growth rates. Thus, the observed reduction of *nif* induction when growing on glycerol appears to be based on the lower energy charge of glycerol grown cells compared to cells grown with glucose. Supplementing the medium with the additional electron acceptors nitrate or fumarate did not effect *nif* induction when cells were growing on sucrose or glucose (Table 9). This indicates that the presence of nitrate, which is also an alternative nitrogen source, does not repress *nif* induction. However, when growing on glycerol, the presence of nitrate resulted in a significant decrease of *nif* induction (200 ± 20 U/ml/OD_{600}) compared to cells grown on glycerol in the absence of nitrate (1000 ± 20 U/ml/OD_{600}). No effect was observed when fumarate was added (Table 9). The growth rate did not change significantly upon the addition of fumarate or nitrate as it is also reported for *E. coli* (Tran and Unden, 1998). Taking together, these findings indicate that at conditions of low cellular energy charge, e.g. anaerobic growth on glycerol, electrons from the quinone pool are preferentially transferred onto nitrate via respiratory nitrate reductase to obtain higher energy yields. Thus, fewer electrons from the quinone pool are available to reduce NifL, resulting in the inhibition of NifA activity. Fumarate apparently is not competing for electrons as no effect on *nif* induction is observed in the presence of fumarate.

Table 9: Effects of additional electron acceptors on the *nif* induction in *K. pneumoniae* using different carbon and energy sources. *K. pneumoniae* UN4495 cultures were grown anaerobically at 30 °C in minimal medium with 4 mM glutamine as limiting nitrogen source. The medium contained sucrose (0.5%), glucose (0.8%) or glycerol (1 %) as carbon and energy source; fumarate or nitrate were added to a final concentration of 20 mM. Differential rates of transcription from the *nifHDK*-promoter were determined, reflecting the *nif*-induction under the respective growth conditions.

Carbon and energy source	Additional electron acceptor	ß-galactosidase activity (U/ml/OD_{600})[a]	Doubling time (h)
sucrose	-	4000 ± 100	3.5
sucrose	fumarate	4100 ± 150	3.5
sucrose	nitrate	3900 ± 150	3.5
glucose	-	3000 ± 90	3.5
glucose	fumarate	2850 ± 85	3.5
glucose	nitrate	3100 ± 90	3.5
glycerol	-	1000 ± 40	5.5
glycerol	fumarate	1100 ± 60	5.7
glycerol	nitrate	200 ± 20	5.5

[a] Data are presented as mean values (± standard aberration) of 3 independent experiments.

Reduced dimethylnaphthoquinone is able to reduce the flavin cofactor of MBP-NifL. In order to verify our finding that under derepressing conditions NifL receives electrons from the quinone pool of the anaerobic electron transport chain we examined whether reduced quinone derivatives can transfer electrons onto NifL. Dimethylnaphthoquinone (DMN) was reduced with molecular hydrogen in the presence of platin oxide and the reduction was confirmed by monitoring the changes in absorbance at 270 and 290 nm. Fully oxidized MBP-NifL was incubated in an anaerobic cuvette under a nitrogen atmosphere in the absence of a redox mediator in a total volume of 200 µl B-buffer and the absorption spectrum was recorded online using a diode array detector. After the addition of 120 µM DMN_{red} the flavin-specific absorbance at 450 nm decreased significantly indicating that electrons are transferred from DMN_{red} to the FAD-cofactor of NifL (Fig. 20). The reduction of NifL-bound FAD by DMN_{red} was confirmed by analyzing the difference spectrum of oxidized MBP-NifL (before DMN_{red} addition) corrected versus the spectrum 110 min after the addition of DMN_{red}, which showed the flavin-specific absorption maxima at 450 and 350 nm (inset Fig. 20). The finding that DMN_{red} ($E°'$=-240 mV) transfers electrons onto NifL-bound FAD strongly supports the model that *in vivo* NifL is reduced at the cytoplasmic membrane and receives electrons from the anaerobic quinone pool.

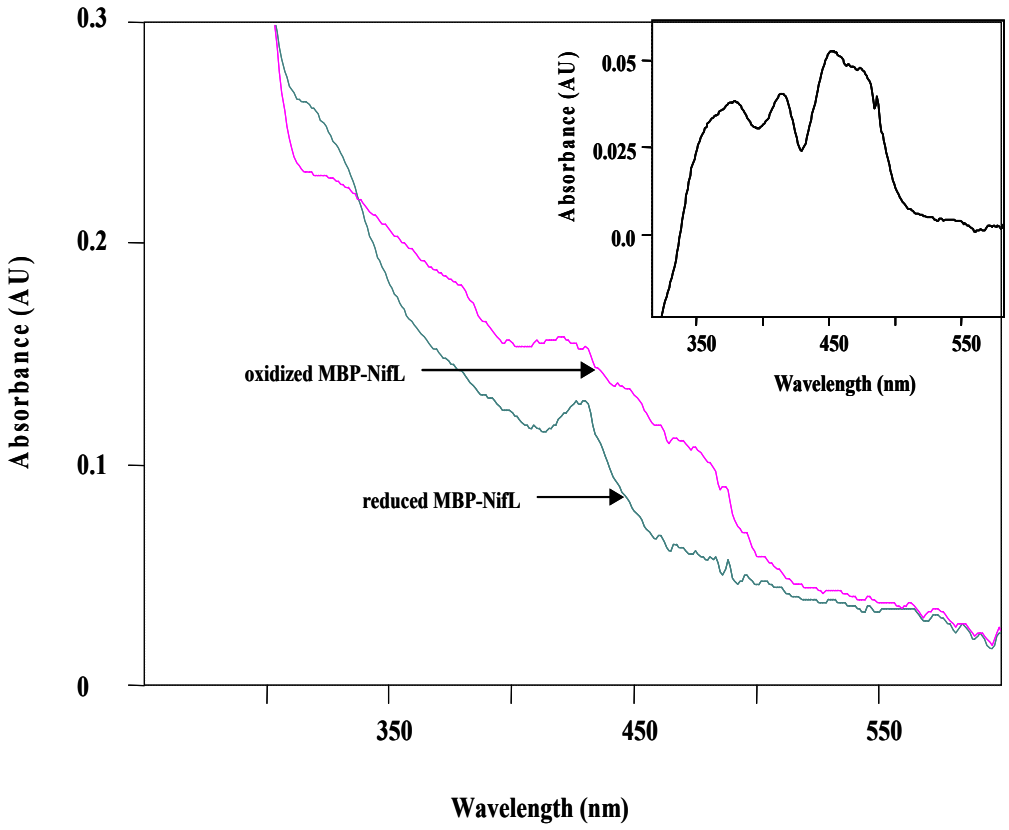


Fig. 20: Reduction of MBP-NifL using reduced dimethylnaphthoquinone as artificial electron donor. 40 µM fully oxidized MBP-NifL was incubated in B buffer under a nitrogen atmosphere at room temperature. Reduced dimethylnaphthoquinone (DMN_{red}) was added to a final concentration of 0.2 mM and the changes in absorbance were recorded using a spectrophotometer with an integrated diode array detector (J & M Analytische Mess- und Regeltechnik Aalen, Germany). Absorbance spectra of MBP-NifL before (oxidized MBP-NifL) and 40 min after the addition of 0.2 mM DMN_{red} (reduced MBP-NifL) are shown. The corresponding difference spectrum of oxidized MBP-NifL corrected versus the reduced spectrum after addition of DMN_{red} is visualized in the inset.

DISCUSSION

The NifL-bound FAD receives electrons from the reduced quinone pool at the cytoplasmic membrane. We recently showed that in *K. pneumoniae* membrane-sequestration of NifL under nitrogen- and oxygen-limited conditions seems to be the mechanism for *nif* regulation in response to molecular oxygen and combined nitrogen (Klopprogge et al. 2002). In order to verify our model that membrane-association of NifL is accompanied by the reduction of the NifL-bound FAD cofactor, we studied the process of NifL reduction. Based on our findings in this study we propose that the FAD cofactor of NifL is reduced by a membrane-bound component of the anaerobic electron transport chain resulting in a reduced non-inhibitory conformation of NifL, which is membrane-associated. A first line of evidence was provided by biochemical analyses of the purified MBP-NifL protein. Spectral analysis clearly showed that in the absence of a redox mediator no change in absorbance of the flavoprotein was detectable upon the addition of $NADH/H^+$. However, the presence of methyl viologen or inside-out vesicles derived from *K. pneumoniae* cells grown under anaerobic conditions allowed NifL reduction by $NADH/H^+$ (Figs. 15 and 16). This strongly indicates that *in vivo* NifL-bound FAD receives electrons from a membrane-bound oxidoreductase system. Two other lines of evidence derived from *in vivo* studies of *nif* regulation support this view. First, using *K. pneumoniae* or *E. coli* strains carrying null mutations of different membrane-bound oxidoreductases we showed that the absence of formate dehydrogenaseN or NADH:ubiquinone oxidoreductase in *K. pneumoniae* and the absence of NADH dehydrogenaseII in the heterologous *E. coli* system affects *nif* regulation dramatically. In the respective mutant strains *nif* induction was low, similar to induction levels under aerobic conditions, even though cells were grown under oxygen- and nitrogen-limitation (Table 8 and Fig. 19). These findings indicate that in the absence of the respective membrane-bound oxidoreductases the FAD-cofactor of NifL was not reduced at the cytoplasmic membrane

resulting in cytoplasmic NifL, which inhibits NifA activity. Second, additional studies of *nif* induction in *K. pneumoniae* growing anaerobically on glycerol under nitrogen limitation revealed that the presence of nitrate as additional electron acceptor resulted in a significant decrease in *nif* induction (Table 9). It appears that under those energy-limited growth conditions, electrons of the reduced quinone pool are preferentially transferred onto nitrate allowing anaerobic respiration and energy conservation by the respiratory nitrate reductase (Tran et al. 1997, Unden and Bongaerts 1997). Thus, a high percentage of NifL protein does not receive electrons at the membrane and stays in its oxidized conformation in the cytoplasm inhibiting NifA activity resulting in decreased *nif* induction. Taking together, these data strongly indicate that more than one membrane-bound oxidoreductase system can provide electrons for NifL reduction under anaerobic conditions and we propose that under anaerobic conditions NifL receives electrons from the reduced quinone pool generated by different membrane-bound oxidoreductase systems. Reduction of the NifL-bound cofactor finally results in higher membrane-association of NifL, allowing cytoplasmic NifA to activate *nif* induction. The demonstration that the reduced soluble quinone derivative dimethylnaphthoquinone ($E^{\circ\prime}$=-240 mV, Krafft et al. 1995) is able to reduce the FAD cofactor of purified NifL in the absence of a redox mediator fully supports this model (Fig. 6). The reduction of NifL under anaerobic conditions at the cytoplasmic membrane by electrons derived from the reduced quinone pool rather than by a single specific membrane-bound enzyme is a particularly attractive model for several reasons. It explains (i) that the absence of different membrane-bound oxidoreductases in *K. pneumoniae*, which transfer electrons onto the quinone pool, results in the oxidized inhibitory conformation of NifL; and (ii) that NADH dehydrogenaseII in the heterologous *E. coli* system significantly affects *nif* regulation, although a homologous oxidoreductase appears not to be present in *K. pneumoniae.* Finally, reduction of NifL by the reduced quinone pool potentially allows the simultaneous signal integration of the cell's energy status for *nif* regulation.

In contrast to *K. pneumoniae* NifL no membrane-association for *A. vinelandii* NifL has been reported to date (Austin et al. 1994, Hill et al. 1996, Dixon, 1998). Thus, a different mechanism for the transduction of the oxygen signal to NifL is expected. In *in vitro* experiments *A. vinelandii-NifL* is reduced by $NADH/H^+$ when catalyzed by the *E. coli* cytoplasmic flavoheme protein (HMP), which is proposed to be a global oxygen sensor or an oxidoreductase preventing cells from endogenous oxygen stress (Pool 1994, Macheroux et al. 1998, Stevanin et al. 2000;). However, the functional and physiological relevance of NifL reduction by HMP has not been demonstrated *in vivo*. It is currently hypothesized, that the

reduction of *A. vinelandii*-NifL occurs non-specifically and dependant on the availability of reducing equivalents in the cell. Because of the relatively high redox potential of NifL, there are a number of electron donors and cytoplasmic NAD(P)H-dependent enzymes that could potentially be involved in reduction of NifL (Dixon 1998, Machereux et al. 1998). Interestingly, it was recently found that nitrogen fixation in the endophytic diazotroph *Azoarcus sp.* BH72 - belonging to the β-Proteobacteria - is also regulated by the coordinated activities of the homologous *nifL* and *nifA* gene products in response to environmental signals (Reinhold-Hurek et al. 1993, Egener and Reinhold-Hurek unpublished). However, it is currently not known whether NifL is membrane-associated under derepressing conditions or how reduction of the NifL-bound flavin cofactor occurs in the oxygen signal transduction.

Nitrogen fixing *K. pneumoniae* M5a1 does not contain an NADH dehydrogenaseII homologous protein. *E. coli* contains two NADH:oxidoreductase systems. One enzyme, NADH:ubiquinone oxidoreductase (NDH-I) encoded by the *nuo* operon, couples $NADH/H^+$ oxidation to proton translocation and thus conserves the redox energy in a proton gradient (Weidner et al. 1993; Calhoun et al. 1993, Friedrich 2001). In contrast, the second enzyme, NADH dehydrogenaseII (NDH-II) encoded by *ndh* does not couple the redox reaction to proton translocation (Matsushita et al. 1987, Calhoun et al. 1993). NDH-II is induced under aerobic conditions, whereas under anaerobic conditions NDH-II is apparently repressed by Fnr (Spiro 1989, Green and Guest 1994, Meng et al. 1997). When growing under anaerobic conditions in the presence of an electron acceptor for anaerobic respiration except conditions with high energy consumption, the coupling enzyme NDH-I is primarily expressed for higher ATP yields (Bongaerts et al. 1995, Tran et al. 1997, Wackwitz et al. 1999). However, expression patterns of the two enzymes can vary depending on specific requirements, e.g. conditions under which ATP yields are more important than growth rate (Unden et al. 2002). Contrary to *E. coli* we obtained strong evidence by Southern blot- and PCR-analysis that the nitrogen-fixing *K. pneumoniae* M5a1 strain does not exhibit a homologous NADH-dehydrogenaseII in addition to the coupling NADH:ubiquinone oxidoreductase encoded by the *nuo* operon (Fig. 17). The finding that $NADH/H^+$ oxidation activity in a *K. pneumoniae* M5a1 *nuo* mutant strain was neglectable compared to the oxidation rates of an *E. coli nuo* mutant strain (Falk-Krezesinski and Wolfe, 1998) further confirmed that *K. pneumoniae* M5a1 exhibits only a single NADH:oxidoreductase (Fig. 18). In contrast, the non nitrogen-fixing strain *K. pneumoniae* subsp. *pneumoniae* exhibits both NADH:oxidoreductase systems (Fig. 17 and Sequencing Center, University of Washington, St. Louis, personal

communication). These findings indicate that the presence of a single coupling NADH:oxidoreductase in *K. pneumoniae* M5a1 may be due to the high energy requirement for nitrogen fixation. We propose that the electrons transferred by the coupling enzyme NADH:ubiquinone oxidoreductase to the quinone pool are mainly transferred to fumarate reductase system for anaerobic fumarate respiration yielding in higher ATP yields.

Hypothetical model for oxygen control of *nif* regulation in *K. pneumoniae*. We have previously shown, that the global regulator Fnr is required for *nif* gene induction and proposed that Fnr transduces the signal of anaerobiosis towards NifL by activating genes the products of which reduce the FAD-cofactor of NifL (Grabbe et al. 2001b). We further demonstrated that under oxygen- and nitrogen-limited conditions NifL is membrane-associated and dissociates into the cytoplasm upon a shift to aerobic conditions (Klopprogge et al. 2002). In this study we obtained strong evidence that NifL is reduced at the cytoplasmic membrane by electrons derived from the quinone pool resulting in higher membrane affinity. In the presence of oxygen or nitrate as electron acceptors, NifL stays oxidized and is localized in the cytoplasm resulting in NifA inhibition. Thus, it is attractive to speculate that in *K. pneumoniae* M5a1 the membrane-associated oxidoreductases which transfer electrons to the quinone pool under anaerobic conditions are transcriptionally regulated by Fnr and thus are the downstream signal transductants following Fnr in the oxygen signal cascade.

As in *E. coli* the genes encoding formate dehydrogenaseN are transcribed in a Fnr dependent manner (Li and Steward 1992, Leonhartsberger et al. 2002) one can expect that expression of formate dehydrogenaseN in *K. pneumoniae* is also transcriptionally controlled by Fnr. This is supported by sequence analysis of the *fdnG* promoter upstream region, which indicates the presence of potential Fnr-boxes (data not shown). Transcription of the *nuo* operon in *E. coli* is regulated by molecular oxygen mainly through the transcriptional regulator ArcA which represses *nuo* transcription under aerobic conditions (Bongaerts et al. 1995). Only small negative effects of Fnr onto *nuo* transcription have been reported under anaerobic growth, which might be due to effects of Fnr on *arcA* expression (Compan and Touati 1994, Bongaerts et al. 1995). However, as the nitrogen-fixing *K. pneumoniae* strain contains only a single NADH oxidizing enzyme, one can expect that the regulation of the *nuo* operon in *K. pneumoniae* differ significantly from the regulation of the *nuo* operon in *E. coli*. Based on preliminary sequence analysis of the promoter upstream regions of the *nuoA* gene, we speculate that in *K. pneumoniae* transcription of the *nuo* operon is up-regulated by Fnr under anaerobic conditions. Thus, in our current working model for oxygen signal transduction in *K.*

pneumoniae we propose that under anaerobic conditions the primary oxygen sensor Fnr activates transcription of membrane-bound oxidoreductases leading to a reduced quinone pool, which provides electrons for NifL reduction. NifL reduction finally results in a higher membrane affinity of NifL and thus in a sequestration of NifL to the membrane, allowing cytoplasmic NifA to activate *nif* genes. However, as in *E. coli* under oxygen limitation the quinol oxidase (CydAB) is repressed by Fnr (Cotter et al. 1997, Govantes et al. 2000) we cannot completely rule out that the Fnr effect on *nif* induction in *K. pneumoniae* is based on derepression of a quinol oxidase in an *fnr* mutant strain, which would also result in a decreased reduction of the quinone pool.

Concerning the nitrogen signal transduction it is not yet known how the primary nitrogen sensor GlnK transduces the nitrogen signal to the *nif* regulatory system in *K. pneumoniae* (He et al. 1998, Jack et al. 1999, Arcondeguy et al. 1999, Schmitz et al. 2002, Klopprogge et al. 2002). It is currently discussed that uridylylated GlnK transduces the signal of nitrogen limitation either by interacting with NifL or NifA, resulting in the dissociation of the inhibitory NifL/NifA complex. Thus, under nitrogen-limitation in the absence of oxygen, NifL would be able to receive electrons from the quinone pool and stay membrane-associated. We have previously shown that under anaerobic conditions a shift to nitrogen sufficiency results in dissociation of NifL from the cytoplasmic membrane, whereas the percentage of membrane-bound GlnK significantly increased (Klopprogge et al. 2002). Very recently, Merrick and coworkers provided evidence that upon a shift to nitrogen sufficiency deuridylylated GlnK in *E. coli* binds to the membrane in an AmtB-dependent manner (Coutts et al. 2002). If this is also the case in *K. pneumoniae,* it is attractive to speculate, that based on a shift to nitrogen sufficiency sequestration of non-uridylylated GlnK by AmtB would rapidly lower the cytoplasmic GlnK pool and thereby lowering the NifL fraction released from the inhibitory NifL/NifA complex by GlnK, which can be reduced at the membrane.

Conclusions

In *Klebsiella pneumoniae* and *Azotobacter vinelandii* the nitrogen regulatory proteins NifL and NifA tightly control synthesis of nitrogen fixation genes in response to the environmental signals molecular oxygen and combined nitrogen. In this regulation the negative regulator NifL inhibits NifA transcriptional activity. Immunological studies, co-chromatography and complex formation analyses using the yeast two-hybrid system demonstrated that NifA interacts directly with NifL by protein-protein interaction (Henderson *et al*., 1989; Money *et al*. 1999 and 2001; Lei *et al*., 1999). This indicates that the signals of nitrogen sufficiency and/or molecular oxygen finally result in complex formation between NifL and NifA, which inhibits NifA activity and thus prevents unnecessary consumption of energy for nitrogen fixation. The inhibitor NifL is a flavoprotein, which regulates NifA activity depending on the reduction status of its N-terminally bound FAD-cofactor, allowing NifA transcriptional activity only under anaerobic conditions. Thus, the redox-sensitive FAD-cofactor appears to be involved in oxygen signal-transduction (Hill *et al*., 1996; Schmitz, 1997; Macheroux *et al*., 1998; Klopprogge and Schmitz, 1999). Both proteins, *K. pneumoniae* NifL and *A. vinelandii* NifL, were biochemically analyzed (Macheroux *et al*., 1998; Klopprogge and Schmitz, 1999), however, the physiological electron donor for NifL reduction is still discussed to date. In *A. vinelandii,* NifL reduction under anaerobic conditions apparently occurs unspecifically, depending on reducing equivalents available in the cell (Macheroux *et al*., 1998; Dixon, 1998). In *K. pneumoniae*, the relief of NifL inhibition under anaerobic conditions requires the presence of iron (Schmitz *et al*. 1996). As the NifL-protein does not contain iron or acid-labile sulphur (Schmitz, 1997), this finding indicates the involvement of an iron-containing protein in the oxygen-signaling mechanism, which is directly or indirectly required for NifL reduction. In Fig. 21 a model for oxygen signal-transduction in *K. pneumoniae* is presented.

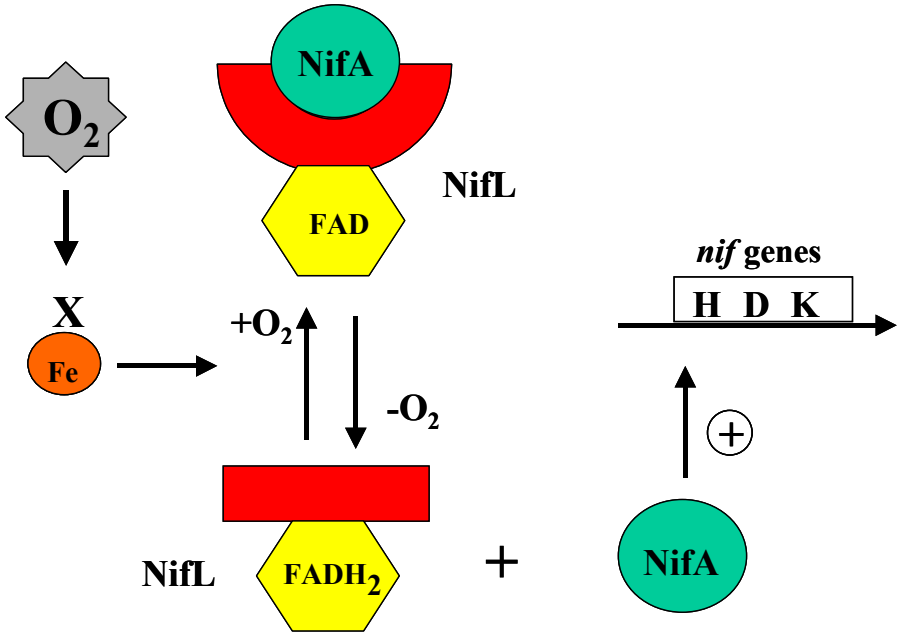


Fig. 21: Model for oxygen signal-transduction in *K. pneumoniae.*

The presence of an iron-containing protein in the oxygen signal transduction towards the flavoprotein NifL rises the question, whether a more general oxygen-sensing system might be involved in signal-transduction, which senses the redox status of the cell through an oxygen-sensitive iron-sulphur cluster. In *Escherichia coli* the Fnr-protein plays an overarching role as oxygen sensor in the cell and regulates transcription of respective genes. DNA-binding to regions upstream of the controlled genes (Fnr boxes) requires dimerization of Fnr monomers, which directly depends on the protein conformation induced by the [4Fe-4S]-cluster in each monomer (Ralph *et al*., 2001). After exposure to oxygen the [4Fe-4S]-clusters are destroyed and subsequent decay of the dimer into monomers results in an inactive form of Fnr containing an oxidized [2Fe-2S]-cluster (Green *et al*., 1996, Khoroshilova *et al*., 1997; Unden and Schirawski, 1997; Kiley and Beinert, 1998; Moore and Kiley, 2001). The gene product of *K. pneumoniae fnr* shows 98 % homology to the Fnr-protein of *E. coli*. The *K. pneumoniae* protein contains the four conserved cystein residues in the N-terminus, which build up the iron-sulphur cluster (Grabbe *et al*., 2001a). We obtained significantly decreased amounts of

iron in *K. pneumoniae* Fnr under aerobic conditions indicating that *K. pneumoniae* Fnr is sensing the redox status of the cell via a redox-sensitive [4Fe-4S]-cluster (Chapter 2, Grabbe *et al*., 2001a). The goal of this thesis was to analyze the potential role of Fnr in oxygen signal-transduction to the *nif* regulatory system.

We obtained strong evidence that in *K. pneumoniae* Fnr influences the oxygen-dependent nitrogen regulation by analyzing (i) NifA dependent *nif* induction in different *fnr* mutant strains (Chapter 3, Grabbe *et al*., 2001b), (ii) membrane-association of NifL in *K. pneumoniae* wild-type and *fnr* mutant strains (Chapter 4, Klopprogge *et al*., 2002), and finally (iii) by studying the influence of different Fnr-dependent membrane-bound oxidoreductase systems on NifL modulated NifA activity (Chapter 5, Grabbe and Schmitz, 2002). In the following, each line of evidence is discussed in more detail.

(i) In the absence of Fnr, NifA mediated transcription of *nif* genes decreased significantly under nitrogen- and oxygen-limitation (Grabbe *et al*., 2001b). This indicates that the FAD moiety of NifL is not reduced, resulting in an inhibitory conformation of NifL. As we can rule out that the transcriptional activator Fnr provides electrons to reduce NifL, we postulate that Fnr transcriptionally controls genes, the products of which function to reduce the NifL-bound FAD-cofactor, resulting in a non-inhibitory conformation of NifL. Attractive candidates for the physiological electron donor are members of the anaerobic electron transport chain.

(ii) Localization of NifL in *K. pneumoniae* revealed that under anaerobic and nitrogen-limited conditions NifL is highly membrane-associated. Based on a shift to oxygen or nitrogen sufficiency in the absence of Fnr NifL is located mainly in the cytoplasm (Klopprogge *et al*., 2002). This indicates that NifL is apparently reduced during membrane-association by a membrane-bound oxidoreductase.

(iii) Studying the influence of different oxidoreductase systems on *nif* induction in *K. pneumoniae* we observed a remarkable decrease in NifA activity under oxygen and nitrogen limitation in the absence of a functional NADH:ubiquinone oxidoreductase or formate

dehydrogenaseN. Analyzing *nif* induction in a heterologous *E. coli* system showed that in a *ndh* mutant background NifL inhibition was not relieved under nitrogen and oxygen limitation (Grabbe and Schmitz, 2002). Based on these findings we conclude that under anaerobic conditions in *K. pneumoniae* the oxidoreductase systems NADH:ubiquinone oxidoreductase and formate dehydrogenaseN generate a reduced quinone pool in the cytoplasmic membrane. In the absence of molecular oxygen, NifL contacts the membrane and receives electrons from the reduced quinone pool resulting in a non-inhibitory conformation of NifL. Subsequent analysis to verify the role of the reduced quinone pool towards the reduction of the NifL-bound FAD-cofactor revealed, that reduced dimethylnaphthoquinone, a soluble quinone derivative, is able to function as electron donor for NifL *in vitro* (Grabbe and Schmitz, 2002). This further supports the model of NifL reduction by the reduced quinone pool, which is generated by those oxidoreductase systems. We further hypothesize that transcription of formate dehydrogenaseN and NADH:ubiquinone oxidoreductase is Fnr-dependent (Grabbe and Schmitz, 2002).

Current working model for oxygen-dependent control of NifA in *K. pneumoniae*. The results presented in this thesis are summarized in Fig. 22. Under anaerobic nitrogen-limiting conditions the NifL-protein of *K. pneumoniae* contacts the cytoplasmic membrane. This membrane-association is accompanied by reducing the FAD-cofactor with electrons from the reduced quinone pool, which apparently results in a membrane-associated NifL protein. Upon a shift to aerobiosis the FAD-cofactor is oxidized, NifL switches into an inhibitory conformation and dissociates from the membrane. The increased amount of inhibitory NifL in the cytoplasm interacts with NifA, resulting in a decrease of *nif* induction. Thus, we propose that sequestration of reduced NifL to the membrane under oxygen- and nitrogen-limitation creates a spatial gap between NifL and its target NifA, which is the regulatory mechanism for oxygen dependent control of NifA activity in *K. pneumoniae*.

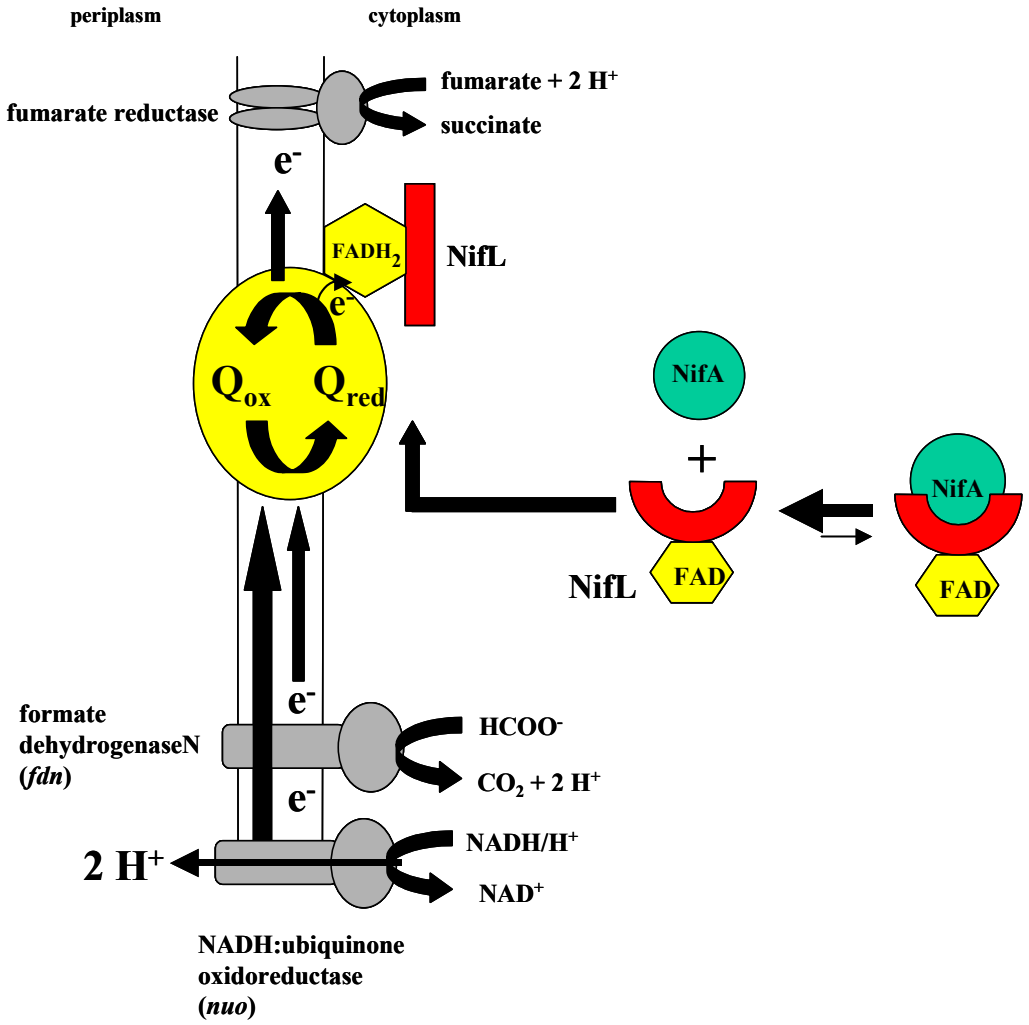


Fig. 22: Current working model for oxygen-dependent control of NifA activity in *K. pneumoniae.*

The nitrogen signal is mediated by the PII-like protein GlnK towards the nitrogen regulatory system in both organisms, *K. pneumoniae* and *A. vinelandii*. However, it is discussed that species-specific mechanisms are involved in the signaling cascade towards the nitrogen fixation regulon. In *A. vinelandii* non-uridylylated GlnK enhances NifL inhibitory functions under nitrogen sufficiency (Little *et al*., 2000), whereas in *K. pneumoniae* GlnK is required for relief of NifL inhibition (He *et al*., 1998; Jack *et al*., 1999). Recently, it was demonstrated that in *E. coli* and *A. vinelandii* after a shift to nitrogen sufficiency non-uridylylated GlnK is membrane-associated by binding to the ammonium transporter AmtB (Coutts *et al*., 2002). This is contradictory to the model Dixon and coworkers proposed, that under nitrogen

sufficiency in *A. vinelandii* unmodified GlnK interacts with NifL in the cytoplasm and activates NifL inhibitory functions (Little *et al*., 2002). In *K. pneumoniae,* GlnK antagonizes NifL inhibitory function towards NifA activity, but uridylylation of GlnK is apparently not required for relief of NifL inhibition (He *et al*. 1998; Jack *et al*., 1999; Arcondeguy *et al*., 1999). If this is also the case in *K. pneumoniae* it is attractive to speculate that under nitrogen- and oxygen-limitation GlnK in its uridylylated form remains located in the cytoplasm preventing complex formation between NifL and NifA. Upon a shift to nitrogen sufficiency non-uridylylated GlnK binds to AmtB and stays membrane-associated. As a consequence, NifL interacts with NifA inhibiting NifA activity.

Further studies

Due to the fact that the reduced quinone pool, generated by oxidoreductase systems of the anaerobic respiratory chain, is responsible for NifL reduction, the localization of NifL in the NADH:ubiquinone oxidoreductase and the formate dehydrogenaseN mutant has to be examined. Second, the proposed Fnr-dependency of those oxidoreductase systems has to be analyzed. Finally, it has to be determined, whether the dramatically reduced NifA activity in the *K. pneumoniae fdn* and *nuo* mutants is also a consequence of reduced energy charge in the cell or other metabolic signals.

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