# Regulation of nitrogen fixation in *Klebsiella pneumoniae:* NifL, one protein transducing two environmental signals to the *nif* transcriptional activator NifA

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# Abbreviations

| | |
|---|---|
| ADP | adenosine-5´-diphosphate |
| AMP | adenosine-5´-monosphate |
| ATP | adenosine-5´-triphosphate |
| bp | base pairs |
| CTP | cytosine-5´-triphosphate |
| DTT | *DL*-dithiothreitol |
| FAD | flavine-adenine-dinucleotide |
| GTP | guanosine-5´-triphosphate |
| HPLC | high pressure liquid chromatography |
| MBP | maltose binding protein |
| NTP | nucleosine-5´-triphosphate |
| OD | optical density |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| SDS | sodiumdodecylsulfate |
| UTP | uracile-5´-triphosphate |
| uv | ultraviolet |
| vis | visible |
| wt | wild type |

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# Summary

• The enzymatic reduction of molecular nitrogen to ammonia requires high amounts of energy and the catalyzing nitrogenase complex is irreversible inactivated by oxygen. Thus nitrogen-fixing microorganisms tightly control synthesis and activity of nitrogenase to avoid unnecessary consumption of energy. In the free-living diazotrophs *Klebsiella pneumoniae* and *Azotobacter vinelandii*, products of the nitrogen fixation *nifLA* operon regulate transcription of the other *nif* operons. NifA activates the transcription by the alternative form of RNA-polymerase, $\sigma^{54}$-holoenzyme; NifL modulates the activity of the transcriptional activator NifA in response to combined nitrogen and external molecular oxygen. The translationally-coupled synthesis of the two regulatory proteins, immunological studies, and complex analysis imply that the inhibition of NifA activity by NifL apparently occurs via direct protein protein interaction *in vivo*.

• The inhibitory function of the negative regulator NifL appears to be located in the C-terminal domain, whereas the N-terminal domain binds FAD as a redox sensitive cofactor and is involved in signal transduction of the internal oxygen status. Changes of the internal oxygen status apparently result in a redox-sensitive conformational change of NifL, which modulates NifA activity in response to the redox state of its FAD cofactor.

• Characterization of purified NifL showed that NifL contains FAD as cofactor, when synthesized aerobically or anaerobically, under nitrogen excess or limitation. The redox potential of NifL was determined to be –277 ± 10 mV (at pH = 8.0) and was independent of the nitrogen availability during NifL synthesis.

• The two NifL fractions synthesized under nitrogen limitation and nitrogen sufficiency differed however, in that a non-reducible absorbance in the uv-vis spectrum at 420 nm was observed, only when NifL was synthesized under nitrogen excess conditions. The absorbance was stable at temperatures up to 60 °C and in the presence of 4 M guanidine hydrochloride. Further studies excluded that it is due to heme, an iron-containing cofactor, or a quinone-like cofactor. This indicates that the absorbance might be based on a covalent modification of NifL in response to ammonium presence.

• We studied the iron dependency of *nif* induction *in vivo* using a *nifH-lacZ* fusion to monitor NifA activity in *K. pneumoniae*. We observed strong evidence, that the primary oxygen sensor for *nif* gene regulation in *K. pneumoniae* appears to be Fnr, which is required for *nif* gene induction under anaerobic conditions. Thus the Fnr requirement for *nif* gene induction accounts for the iron dependency of nitrogen fixation in *K. pneumoniae*. The transcriptional activator Fnr presumably transduces the signal of anaerobiosis towards NifL by activating the transcription of gene(s) whose product(s) function to relieve NifL inhibition by reducing the FAD cofactor. Hypothetical candidates for the physiological electron donor for NifL reduction are components of the anaerobic electron transport system, which are Fnr dependent synthesized. This hypothesis is supported by the finding that NifL is membrane associated under anaerobic and nitrogen limited growth conditions.

• Studying NifL activity *in vitro* indicated that adenosine nucleotides stimulate the inhibitory function of NifL synthesized under nitrogen excess. NifL synthesized under nitrogen limitation, however, was not affected by adenosine nucleotides.

• Further characterization of purified NifL concerning the stimulatory effect of adenine nucleotides on NifL inhibitory function, revealed that NifL synthesized under nitrogen excess binds and hydrolyzes adenine nucleotides. The apparent $K_M$ and apparent $V_{max}$ were determined to be of 41 mM and 2500 mU / mg, respectively. In contrast, NifL synthesized under nitrogen limitation neither bound nor hydrolyzed adenine nucleotides.

• Intracellular localization of *K. pneumoniae* NifL and NifA revealed strong evidence that *nif* induction is dependent on a spatial separation between NifL and its target protein NifA. NifL is membrane associated under anaerobic and nitrogen limited conditions, whereas NifA is located in the cytoplasm. The localization experiments also revealed, that GlnK is partially membrane associated under anaerobic and nitrogen limited conditions. Interestingly, cytoplasmic GlnK is degraded rapidly upon ammonium upshifts, whereas membrane associated GlnK exhibits a significantly higher stability.

*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). 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). Upon the high energy requirement, in nitrogen fixing cells up to 40 % of the ATP is utilized by the nitrogenase 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 irreversible 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 expression 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 involved in this control can vary in different organisms (Fischer, 1996; Dixon, 1998; Halbleib and Ludden, 2000). 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 *Klebsiella pneumoniae* and *Azotobacter vinelandii*, two free-living diazothrophs, which belong to the γ-Proteobacteria, however, 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 (Reinhold-Hurek, pers. comm.).

**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 ($\sigma^{54}$-RNA polymerase) is generally activated by NifA, which binds to an upstream activation sequence (UAS) (Morrett and Buck, 1988) and contacts promoter-bound $\sigma^{54}$-RNA polymerase by means of a DNA loop (Buck *et al.*, 1987). Subsequently NifA catalyzes the isomerization of closed complexes between $\sigma^{54}$-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). The inhibitory protein NifL is composed of two domains separated by a hydrophilic interdomain linker (Q-linker) (Söderbäck *et al.*, 1995; Drummond and Wootton, 1987). The C-terminal domain of NifL shows homology to a histidine protein kinase (Blanco *et al.,* 1993), however no autophosphorylation or a 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*., 1999; Lei *et al.*, 1999). Thus signal transduction apparently occurs via protein interaction. 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 interaction

**Nitrogen signal transduction.** Analyses of internal pool sizes of glutamine and glutamate - the two central nitrogen intermediates - showed that under nitrogen limiting growth conditions the glutamine pool in *K. pneumoniae* is 6 to 9 fold lower than under nitrogen sufficiency, whereas the glutamate pool remains stable (Schmitz, 2000). This indicates that *K. pneumoniae* apparently senses external nitrogen limitation as a decrease in the intracellular glutamine pool size as it has been shown for *Salmonella typhimurium* (Ikeda *et al.*, 1995). Changes in the internal glutamine pool reflecting the cellular nitrogen status are mediated to the NtrB/NtrC two-component regulatory system via uridylylation and deuridylylation of the regulatory protein PII (encoded by *glnB*) by GlnD (uridylyl-transferase and uridylyl-removing-enzyme) (Merrick and Edwards, 1995; Ninfa *et al.*, 1995).

A shift from nitrogen limitation to nitrogen sufficiency results in repression of *nif* gene induction in *K. pneumoniae*, 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.* The expression of *glnK* is NtrC dependent and it is cotranscribed with *amtB* (encoding for an ammonium transporter). Upon the high similarity to the PII-protein, the GlnK-protein is a potential 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 (Xu *et al.,* 1998; van Heeswijk *et al.,* 1995). 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 is not sensed by NifL directly, but is apparently mediated by GlnK to the NifA/NifL regulatory system. Whereas NifL is a negative regulator, GlnK apparently acts positively to antagonize inhibitory effects of NifL under nitrogen limiting conditions. The uridylylation of GlnK is apparently not required for relief of NifL inhibition (He *et al*. 1998, Arcondeguy *et al*., 1999). Interestingly, the T-loops of GlnK and PII, which are supposed to be necessary for the signal transduction function, differ in *K. pneumoniae* only in three amino acid residues. 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). It is further discussed that NifL inhibitory function is rapidly restored, when ammonium is added to the growth medium upon the formation of non-functional heterotrimers between GlnK and PII (Arcondeguy *et al*., 1999). 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 interaction but it has to be elucidated, whether GlnK is interacting directly with NifL or is affecting the NifL/NifA complex formation. For diazothrophs not belonging to the γ-proteobacteria and missing NifL (e.g. *Herbaspirillum seropedicae* and *Azospirillum brasilense*) experimental data indicate that the PII protein participates 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*, apparently only has 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. 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). These findings for GlnK function in *K. pneumoniae* and *A. vinelandii* indicate that the interaction between GlnK and the NifL/NifA complex are likely to be specific to each organism.

**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 with purified proteins showed that NifL in *A. vinelandii* and *K. pneumoniae*, is a flavoprotein with a non-covalently N-terminally bound FAD as cofactor (Hill *et al.*, 1996; Söderbäck *et al.*, 1998; Schmitz, 1997). 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 from *Escherichia coli* with NADH as electron donor does not antagonize open complex formation by NifA *in vitro* (Machereoux *et al*., 1998). This indicates that reduction of the flavin moiety of NifL results in a non-inhibitory form of NifL. 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.

By reducing the cofactor of NifL, the physiological electron donor is possibly transducing the signal for anaerobiosis to NifL, therefore the physiological electron donor for NifL reduction

itself might be a potential component of the oxygen signal transduction. Thus, the key question concerning the oxygen signal transduction is, if NifL senses the oxygen status in 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 indicating the presence of a potential iron sulphur cluster in NifL (Schmitz *et al.,* 1996).

For *A. vinelandii,* an obligate aerobic microorganism, which apparently protects the nitrogenase system against oxygen damage by high consumption of oxygen (so called 'respiratory protection') (Dixon, 1998; Oelze, 2000), one has to expect a different oxygen signal transduction to NifL. *In vitro A. vinelandii* NifL is reduced by NADH catalized by the *E. coli* flavoheme protein (HMP), which is proposed to be a global oxygen sensor (Pool, 1994; Macheroux *et al*., 1998). However, the functional and physiological relevance for the reduction of NifL by HMP has not been demonstrated to date. It is currently discussed, that the reduction of *A. vinelandii* NifL occurs non-specifically depending on the availability of the reducing equivalents in the cell, as the relatively high redox potential of NifL may allow a number of different electron donors and NAD(P)H-dependent enzymes to reduce NifL (Dixon, 1998; Macheroux *et al.*, 1998).

**Effects of adenine nucleotides on NifL.** The C-terminal domain of *A. vinelandii* NifL shows sequence similarity to members of the histidine kinase family and binds adenine nucleotides indicating that the C-terminal domain might have evolved from a classical histidine protein kinase to a domain responsive to nucleotides (Söderbäck *et al.,* 1998). Interestingly, ADP-binding to the C-terminal domain specifically increases the inhibitory activity of *A. vinelandii* NifL on open complex formation by NifA *in vitro* independent of the redox response (Eydmann *et al.,* 1995; Söderbäck *et al.,* 1998). Proteolysis protection analyses further indicated that ADP-binding to the C-terminal domain induces a change in NifL conformation

(Söderbäck *et al.,* 1998; Dixon, 1998), which apparently promotes complex formation between purified NifL and NifA *in vitro* as shown by co-chromatography experiments (Money *et al.,* 1999). These findings and the higher affinity of NifL to bind ADP versus ATP suggest that the formation of the inhibitory complex between NifL and NifA might be regulated by the ATP/ADP ratio in response to the energy charge *in vivo* (Söderbäck *et al.,* 1998). The C-terminal domain of *K. pneumoniae* NifL, however, is distinct from that of *A vinelandii* NifL and lacks three out of five sequence motifs found in the histidine protein kinase family (Woodley and Drummond, 1994). This raises the question, whether adenine nucleotides also affect *K. pneumoniae* NifL.

The focus of this thesis was to study the signal transduction of molecular oxygen and combined nitrogen and the influence of adenine nucleotides on the NifL/NifA regulatory system in *K. pneumoniae*. We investigated the redox properties of purified *K. pneumoniae* NifL synthesized under different nitrogen availabilities (chapter 2), the iron dependency of *nif* gene induction (chapter 3), the influence of adenine nucleotides on *nif* gene transcription *in vitro* (chapter 4) and the cellular localization of NifL, NifA and GlnK as key regulatory proteins in nitrogen fixation (chapter 5).

*Chapter 2:*

# NifL of *Klebsiella pneumoniae:*

# Redox characterization in relation to the nitrogen source


## Abstract

In *Klebsiella pneumoniae,* NifL modulates the activity of the transcriptional activator NifA in response to combined nitrogen or external molecular oxygen. We recently showed that *K. pneumoniae* NifL is a flavoprotein which apparently senses oxygen through a redox-sensitive, conformational change. In order to study if the nitrogen signal might be transmitted to NifA through a stable modification of NifL we characterized the redox properties of NifL synthesized in *Escherichia coli* in the presence of different nitrogen sources. FAD analyses showed that purified NifL carried FAD as cofactor independent of nitrogen and oxygen availability. The redox potential of NifL synthesized in the presence of ammonium was –277 ±5 mV at pH 8.0 and 25° C, as determined by reduction with dithionite or with enzymatic reduction by xanthine oxidase in the presence of methyl viologen as redox mediator. When synthesized under nitrogen-limiting conditions, NifL showed a redox potential of - 274 ±6 mV at pH 8.0 and 25° C. Fully reduced NifL fractions, synthesized under either condition listed above, reoxidized rapidly in the presence of molecular oxygen. These results indicate that for NifL synthesized in *E. coli,* the redox potential of the NifL-bound FAD is not influenced by the nitrogen source. The two NifL fractions differed, however, in that a non-flavin specific absorbance at 420 nm was found only in NifL synthesized in the presence of ammonium.

## Introduction

In the free-living diazotroph *Klebsiella pneumoniae* expression of nitrogen fixation (*nif*) genes is regulated by the products of the *nifLA* operon (Filser *et al*., 1983; Dixon, 1998 and therein cited publications). NifA activates transcription of all *nif* genes (except *nifLA*) by an alternative holoenzyme form of RNA polymerase, $\sigma^{54}$- holoenzyme. NifL, which is the negative regulator of the *nif* genes, inhibits the transcriptional activation by NifA in response to combined nitrogen or external molecular oxygen. NifL is composed of an N-terminal and a C-terminal domain. Its inhibitory function appears to lie in the *C*-terminal domain, and the N-terminal domain is thought to mediate the response to environmental changes in the status of oxygen and combined nitrogen (Narberhaus *et al*., 1995; Sidoti *et al*., 1993).

We have recently shown that *K. pneumoniae* NifL is a flavoprotein, which contains an N-terminally bound FAD as cofactor (Schmitz, 1997). We therefore hypothesized that under nitrogen and oxygen-limiting conditions, the inhibitory form of NifL is transformed to the non-inhibitory form upon reduction of the N-terminally bound FAD. NifL apoprotein completely lacking the cofactor is still able to inhibit NifA activity *in vitro* (Schmitz, 1997), showing that the FAD-cofactor is not directly required for the inhibitory function of NifL. However this leaves open the possibility that FAD mediates the conversion of NifL to the non-inhibitory form upon reduction of this N-terminal bound moiety. In *Azotobacter vinelandii* NifA activity is also modulated by an FAD-containing NifL regulatory protein acting as a redox switch in response to environmental oxygen status (Hill *et al.*, 1996). Hill *et al*. 1996) and Machereaux *et al*. (1998) showed that the *in vitro* reduction of the flavin moiety of NifL by sodium dithionite (Hill *et al.*, 1996) or by the flavoheme protein HMP from *E. coli* with NADH (Macheroux *et al*. 1998) resulted in a non-inhibitory form of NifL.

The mechanism by which nitrogen is sensed in *K. pneumoniae* and *A. vinelandii* is currently not understood. It was shown that *K. pneumoniae* NifL He *et al.*, 1997) and *A. vinelandii* NifL (Söderbäck *et al.*, 1998) both modulate NifA activity in *E. coli* in response to the level of combined nitrogen, suggesting that NifL might respond to a generalized or globally sensing nitrogen signal transduction pathway. In *K. pneumoniae*, Holtel and Merrick (1989) showed

that PII (*glnB* gene product) is not required for signaling the nitrogen status. Recently, He *et al.* (1998) provided evidence that the second PII protein, GlnK, might be involved in signaling the nitrogen status. One possible mechanism for the nitrogen sensing pathway in *K. pneumoniae* - hypothesizing NifL as transducer for the nitrogen signal to NifA - is that in the presence of combined nitrogen the redox potential of NifL-bound FAD is lowered by a covalent post translational modification such that it can no longer be reduced by its physiological electron donor. To test whether the redox properties of NifL change with the nitrogen status of cells we determined the redox potential of NifL synthesized in *E. coli* under nitrogen-limiting conditions and in the presence of ammonium, a preferred nitrogen source.

## Materials and methods

**Plasmids**. pJES794 encoding MBP-NifL under the control of the *tac* promoter is described by Narberhaus et al. (Narberhaus *et al.*, 1995). pJES283 encoding NifL under the control of the T7 promoter is described by Lee et al. (He *et al.*, 1997).

**Cell extracts and purification of Maltose Binding Protein (MBP)-NifL.** MBP-NifL was synthesized in NMC1529 (He *et al.*, 1998) carrying pJES794. Expression of fusion protein was induced from the *tac* promoter with 50 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) when cultures reached an $O.D._{600}$ = 0.6. The cultures were grown at 30° C, aerobically or anaerobically under $N_2$ gas, and in modified K-medium containing 4 mM glutamine or 10 mM ammonium as the sole nitrogen source (Schmitz, 1997; He *et al.*, 1997]. After disruption of cells in breakage (B) buffer Schmitz *et al.*, 1996) and centrifugation at 20,000 x g, fusion proteins were purified from the supernatant by amylose affinity chromatography. Anaerobic purifications were performed in the presence of 2.0 mM dithiothreitol and under a nitrogen atmosphere in an anaerobic chamber.

**FAD analyses.** Purified MBP-NifL (0.2-2 mg) was heat denatured and extracted with 5% (wt/vol) trichloroacetic acid. The extracted compounds were analyzed by reversed-phase HPLC at room temperature as described by Schmitz (1997).

**Determination of the redox potential of NifL bound FAD.** Purified MBP-NifL fractions were concentrated to 20-50 µM using polyethylenglycol. The redox potential of these MBP-NifL fractions in B-buffer (pH 8.0) were determined according to Massey (1991) by reducing the flavoprotein in either of two ways: Either with dithionite or with the electron-generating reaction with xanthine oxidase / xanthine. 200 µl of a 20 to 50 µM MBP-NifL solution was incubated anaerobically in a cuvette at pH 8.0 and 25° C with 2 µM methyl viologen as redox mediator and a redox indicator (2 µM phenosaphranine). Anaerobiosis was achieved by flushing the sealed cuvette with molecular nitrogen several times. Dithionite reduction was started by adding dithionite stepwise to final concentrations of 10, 20 and 60 µM. Spectral changes for each reduction step were recorded (Kontron Uvikon 930). When using the electron-generating reaction with xanthine oxidase, 200 µM xanthine was included in the cuvette and the reduction started by adding xanthine oxidase to a final concentration of approximately 20 mU / ml. The redox potential was determined by plotting the data as described by Minneart (1965): For each time point the concentration of oxidized and reduced MBP-NifL was calculated from the spectra and $\log(FAD_{ox}/FAD_{red})$ was plotted versus $\log(\text{phenosaphranine}_{ox}/\text{ phenosaphranine}_{red})$.

In a separate method, the redox potential of MBP-NifL was also determined by reducing the protein with dithionite and measuring the redox potential with an electrode (Ingold Pt 4805-M8/120). For this method, a 3 ml sample of enzyme was incubated in an anaerobic cuvette in the presence of 5 µM methyl viologen and titrated in an anaerobic chamber by adding aliquots of 5 mM dithionite. After addition of dithionite, the mixture was incubated at room temperature until the redox electrode showed no further change. The redox potentials of MBP-NifL samples were calculated using the Nernst equation.

**Purification of NifL apoprotein from inclusion bodies by denaturation with urea.** NifL was synthesized in *E. coli* BL21 carrying pJES283 as described by Lee et al. [1993]. Expression of the protein was induced from the T7 promoter with 50 µM IPTG when cultures reached an $O.D._{600}$ = 0.6. The cultures were grown aerobically at 30° C in maximal induction medium (Schmitz *et al.*, 1996) or in minimal medium with glutamine as limiting nitrogen source. After disruption of cells in breakage buffer and centrifugation at 20,000 x g NifL was purified from inclusion bodies under aerobic conditions by denaturation with 8 M urea and refolding by dialysis into breakage buffer overnight at 4° C as described by Lee et al. (1993).

**Determination of non-heme iron and protein.** Non-heme iron was determined colorimetrically as described by Fish (1988). Protein was determined via the method of Bradford (1976) with the BioRad protein assay using bovine serum albumin as standard.

**SDS-PAGE Analyses and heme peroxidase activity stain.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli using 12.8% acrylamide (Laemmli, 1970). Gels were stained either for protein with Coomassie Brilliant Blue or with 3,3'5,5'-tetramethyl benzidine (TMBZ, 2 mM) and 30 mM $H_2O_2$ to reveal heme peroxidase activity as described by Thomas *et al*. (1976). Protein samples were not denatured by heating or treated with ß-mercapthoethanol when heme peroxidase activity was analyzed.

## Results

We chose to examine the possible influence of the nitrogen availability in cultures on the redox properties of *K. pneumoniae* NifL. For this study, NifL was synthesized and purified from *E. coli* grown in minimal medium with ammonium or with a limiting nitrogen source. In order to keep NifL in solution a protein fusion with the maltose binding protein (MBP) was used (Lee *et al.*, 1993). Synthesis of MBP-NifL was induced to a level at which NifL function is regulated normally in response to oxygen and combined nitrogen in *K. pneumoniae* and in a heterologous *E. coli* system (Schmitz *et al.*, 1996; He *et al.*, 1997).

**FAD analyses of *K. pneumoniae* NifL synthesized in *E. coli* under various conditions.** To test whether NifL from *K. pneumoniae* carries FAD as cofactor independent of the nitrogen source, MBP-NifL was expressed in *E. coli* grown under various conditions, purified to approximately 97 % homogeneity (data not shown) and analyzed for cofactors. MBP-NifL purified from all culturing conditions examined - aerobically or anaerobically and with a limiting nitrogen source (glutamine) or ammonium - contained FAD as cofactor. The proportions of FAD detected in proteins varied between 0.25 mol to 0.45 mol FAD per MBP-NifL, which is due to different losses of cofactor during purification procedures. These results indicate that *K. pneumoniae* NifL carries FAD as cofactor independent of the availability of nitrogen and oxygen in the culture medium.

**Determination of redox potentials.** In order to study if the nitrogen availability in cultures is affecting the redox properties of NifL-bound FAD, we determined the redox potentials of MBP-NifL, synthesized in minimal medium with ammonium or with glutamine as limiting nitrogen source. MBP-NifL preparations for the following redox titrations were in general synthezised under aerobic growth conditions followed by cell breakage and purification in the presence of oxygen. Reducing flavoproteins in the presence of a redox-sensitive dye provides a convenient method to determine the redox potential of protein-bound FAD-cofactors. This method is based on the equilibration of reducing equivalents between the flavin moiety of the flavoprotein and a dye of a known redox potential (Massey, 1991).

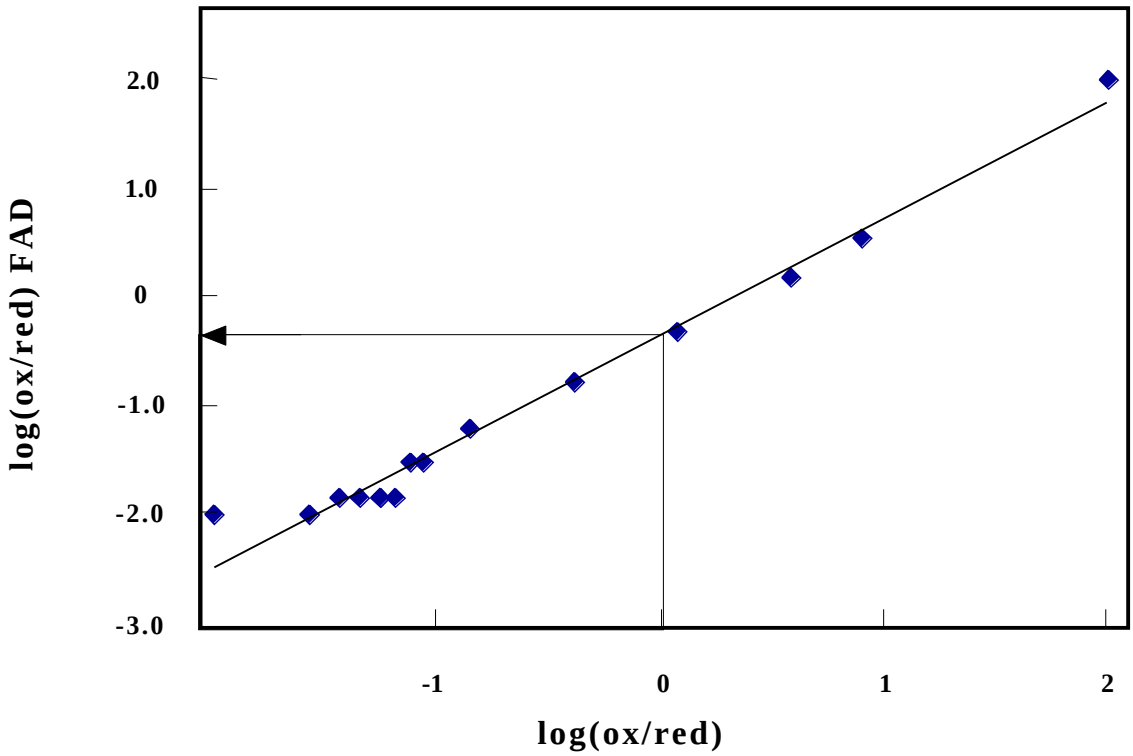


**Figure 1: Nernst plot of the reduction of MBP-NifL with dithionite.** 20 µM MBP-NifL in B-buffer pH 8.0 synthesized aerobically in minimal medium in the presence of ammonium was stepwise reduced at 25° C with dithionite to a final concentration of 60 µM in the presence of 2 µM methyl viologen and 20 µM phenosaphranine as redoxindicator ($E_0$ = -282 mV at pH 8 assuming that the pH dependence of the phenosaphranine$_{ox}$/phenosaphranine$_{red}$-couple has a slope of -30 mV). Concentrations of reduced MBP-NifL were calculated for each reducing step from the respective absorbance at 450 nm and log($FAD_{ox}/FAD_{red}$) plotted versus log(phenosaphranine$_{ox}$/phenosaphranine$_{red}$) according to Minneart (1965).

We used two methods to reduce MBP-NifL in the presence of 2 µM phenosaphranine as redox indicator ($E_0$' = -252 mV at pH 7.0): Chemically, by adding dithionite to the solutions, or enzymatically, by incubating MBP-NifL with xanthine/xanthine oxidase. For reduction by dithionite, 20 to 50 µM of purified MBP-NifL in B-buffer pH 8.0 was treated with stepwise additions of dithionite (final concentrations of 10, 20, 40 and 60 µM). Spectra were recorded after each step of addition. The flavin-specific absorbance at 450 nm revealed that the NifL-bound FAD was completely reduced after adding 60 µM dithionite, further addition of dithionite did not result in any further absorbance decline at 450 nm. In samples that were enzymatically reduced, the reducing reaction was started by adding a catalytic amount of

xanthine oxidase. The spectra of these samples were recorded every 15 minutes thereafter. To calculate the redox potential of MBP-NifL, the concentration of oxidized and reduced MBP-NifL was calculated for each time point from their respective absorbances at 450 nm and the $\log(FAD_{ox}/FAD_{red})$ plotted versus $\log(\text{phenosaphranine}_{ox}/\text{phenosaphranine}_{red})$ which was determined from the dye absorbance at 525 nm (Fig. 1, showing the Nernst plot of NifL synthesized aerobically in minimal induction medium in the presence of ammonium). The redox potential was calculated according to Minneart (1965) from the y-axis intercept where $\log(\text{phenosaphranine}_{ox} / \text{phenosaphranine}_{red})$ equals zero: $E_0$ (NifL) = -282 mV - (-0.34 x 30 mV) = -272 mV ($E_0$(phenosaphranin) at pH 8 = -282 mV, assuming that the pH dependence of the phenosaphranine(ox/red)-couple has a slope of -30 mV). From ten independent MBP-NifL preparations synthesized aerobically in the presence of ammonium, the redox potential was determined to be –277 ± 5 mV. The redox potential of MBP-NifL synthesized in the presence of oxygen under nitrogen-limiting conditions was –274 ± 6 mV (data from four independent preparations). In the presence of molecular oxygen reoxidation of MBP-NifL which was fully reduced during the process of redox titration occurred rapidly, regardless of whether it was synthesized under nitrogen limitation or in the presence of ammonium. The finding that the redox potentials do not differ between MBP-NifL preparations isolated from cells grown aerobically under conditions of nitrogen limitation or in the presence of ammonium suggests that the nitrogen source has no direct influence onto the redox characteristics of NifL when synthesized in *E. coli*.

The redox potentials were also determined by a second method. In this method, the flavin moiety of purified MBP-NifL was titrated with dithionite and the solution potential measured with a redox electrode after equilibration with each addition. The redox state of the flavin was followed spectroscopically. This method for determining the redox potentials of MBP-NifL preparations gave the same results as the dye-linked assays described above (data not shown).

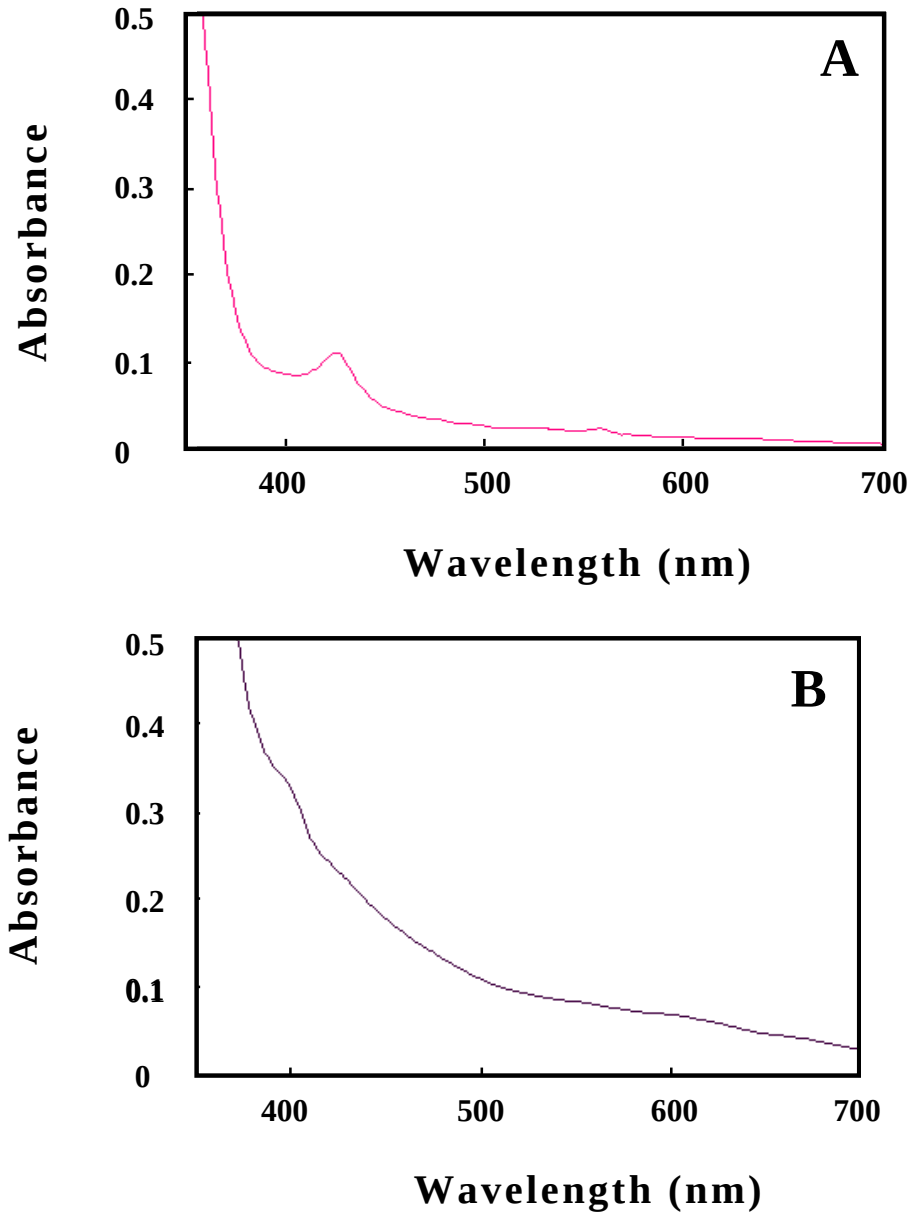


**Figure 2: Absorbance analyses after reduction of MBP-NifL with sodium dithionite**. 20 µM purified MBP-NifL in B-buffer pH 8.0 was reduced at 25° C with dithionite to a final concentration of 60 µM in the presence of 2 µM methyl viologen. MBP-NifL was synthesized in the presence of 10 mM ammonium (A) or 4 mM glutamine (B).

**Analyses of iron and heme peroxidase activity of purified MBP-NifL.** In purified MBP-NifL fractions synthesized in the presence of ammonium an interesting absorbance centered around 420 nm was found, which partially overlaid the absorbance from the flavin at 450 nm

and persisted during reduction processes (Fig. 2A). Careful reviewing the MBP-NifL difference spectrum we published recently ((Schmitz, 1997), Fig. 1) revealed that MBP-NifL synthesized in *E. coli* under nitrogen excess in maximal induction medium also showed the presence of the 420 nm absorbance peak. Purified MBP-NifL synthesized under nitrogen limitation with glutamine as nitrogen source, however, did not show this absorbance at 420 nm (Fig. 2B). To rule out that the absorbance around 420 nm of MBP-NifL fractions synthesized in the presence of ammonium arises from an iron-containing cofactor of NifL, we measured the iron concentration of the purified protein. Using the method described by Fish (1988), no significant amounts of iron were found (less than 0.2 mol per mol which is in the same range as it was found for purified MBP). In order to analyze if the absorbance at 420 nm is based on a contamination by a heme containing protein we determined the peroxidase activity of purified MBP-NifL fractions with TMBZ-$H_2O_2$. Purified MBP-NifL fractions synthesized in the presence of ammonium (75 to 600 pmol) were analyzed by gel electrophoresis and subsequently stained for peroxidase activity with TMBZ-$H_2O_2$ (Fig. 3A), followed by staining for proteins using Coomassie Brilliant blue (Fig. 3B). As a control cytochrome C was analyzed in a range of 3 to 300 pmol. In contrast to no peroxidase activity in case of the purified MBP-NifL-fractions (maximal 600 pmol), peroxidase activity of cytochrome C was still detectable at 3 pmol. This indicates that a possible heme contamination of the MBP-NifL fractions would be significantly lower than 0.5%.

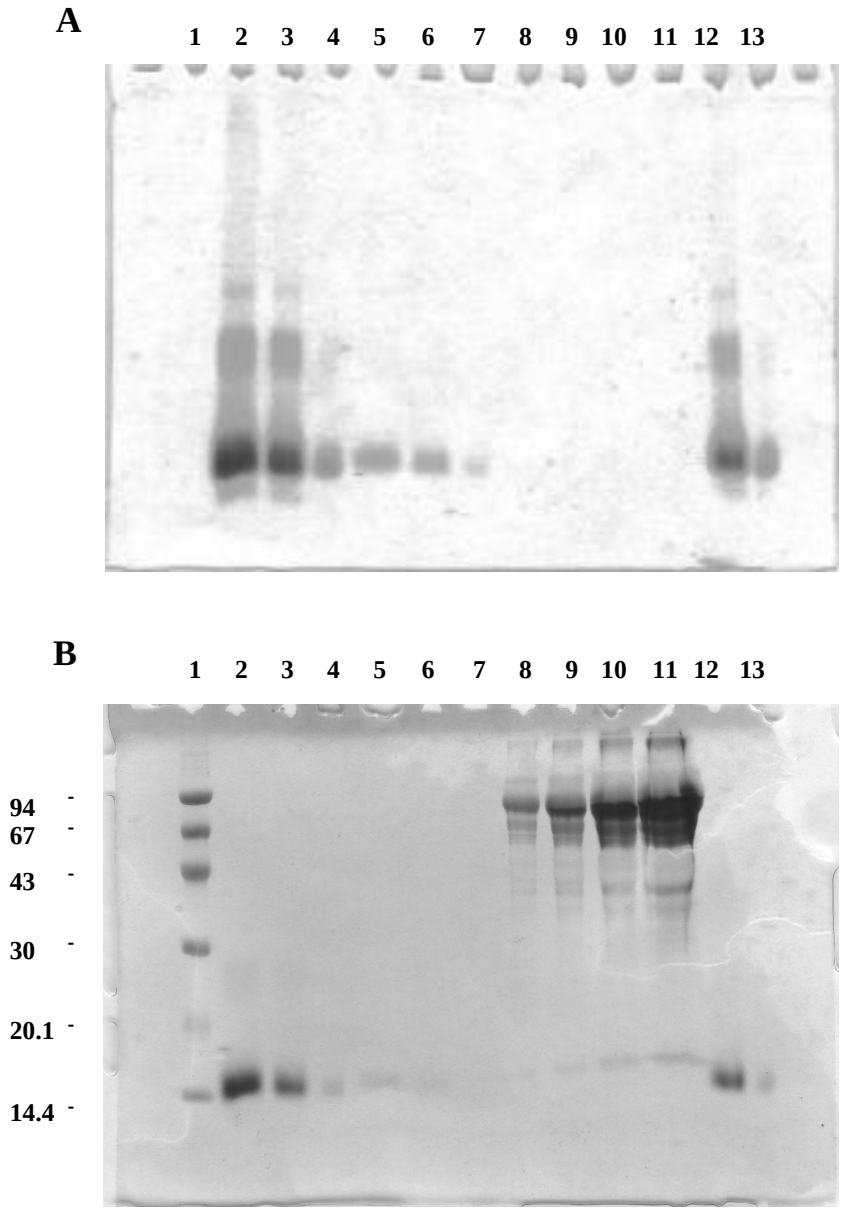


**Figure 3: Analyses of purified MBP-NifL for peroxidase activity with TMBZ and $H_2O_2$ (heme stain):** MBP-NifL synthesized in the presence of ammonium was analyzed by SDS gel electrophoresis (12.8% acrylamide) and stained with 3,3'5,5'-tetramethyl benzidine (TMBZ, 2 mM) and 30 mM $H_2O_2$ to reveal the heme peroxidase activity (A) and subsequently with Brilliant Blue for protein (B). Lane 1 contained 5 µg protein Molecular mass standard (Pharmacia); lanes 2-7 cytochrome C from horse heart (Fluka), 300, 150, 30, 30, 15, and 3 pmol, respectively; lanes 8-11 concentrated purified MBP-NifL fractions, 75, 150, 300 and 600 pmol, respectively and lanes 12 and 13, 150 and 30 pmol cytochrome C.

**Spectral properties of NifL purified from inclusion bodies by denaturation with urea.** The 420 nm absorbance feature, which is only found, when MBP-NifL is synthesized in the presence of ammonium**,** might originate from a covalent modification of the protein moiety in the presence of ammonium. To test this possibility we purified NifL apoprotein (apo-NifL) to homogeneity from inclusion bodies synthesized in *E. coli* under aerobic conditions and nitrogen excess (maximal induction medium) by denaturation with 8 M urea and subsequent renaturation in the presence of oyxgen as described in materials and methods (Lee *et al.*, 1993). Spectrofluorometric analyses of trichloroacetic acid extracts from renatured NifL preparations revealed that the renatured NifL completely lacks its FAD cofactor. However, this apoprotein fraction of NifL still retains an absorption at 420 nm which cannot be reduced by the addition of dithionite (Fig. 4A). The absorbance at 420 nm is detectable and significantly higher than the slope in absorbance between 400 and 500 nm which is also present in the MBP-NifL preparations carrying the cofactor (Fig. 2). These findings indicate that the absorbance at 420 nm is neither due to the NifL-bound FAD nor based on a contaminating protein but might originate from a different protein moiety or a covalent post translational modification upon ammonium presence.

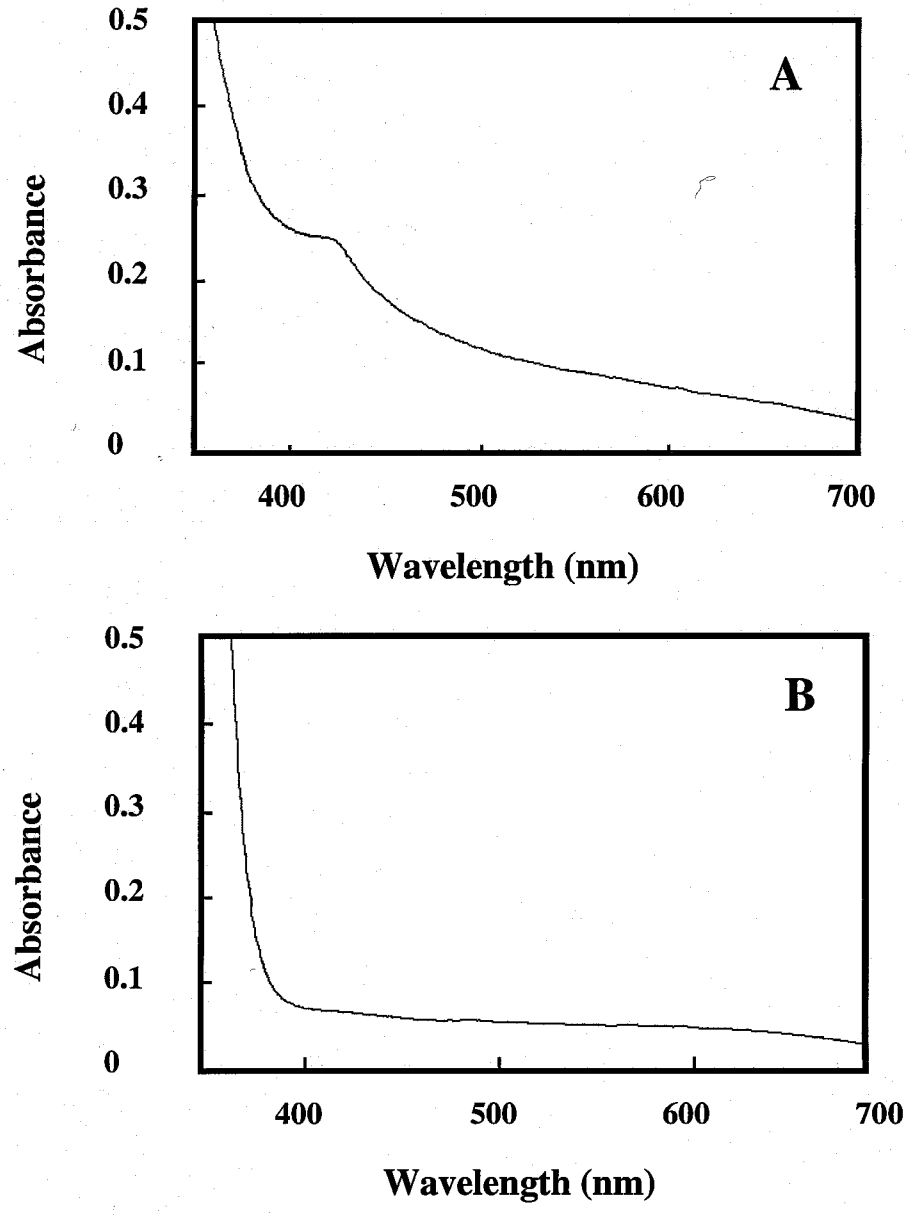


**Figure 4: Absorbance spectrum of fully reduced NifL apoprotein**. The spectra of NifL apoproteins in B-buffer (pH 8.0) were recorded at room temperature after reducing the protein fractions with sodium dithionite (final concentrations 75 µM). Apo-NifL (57 µM; and 15 µM, respectively) was purified from cells grown under nitrogen excess in maximal induction medium (A) or in minimal medium with glutamine as limiting nitrogen source (B).

To rule out that the absorbance at 420 nm results from an artefact upon the denaturation / renaturation procedure we purified apo-NifL from cells grown under aerobic conditions in minimal medium with glutamine as limiting nitrogen source. The absorbance spectrum of this apo-NifL preparation shown in Fig. 4B lacks the absorbance at 420 nm as it

was the case for the corresponding NifL preparation carrying the cofactor (Fig. 2B). This result clearly shows that the 420 nm absorbance peak in Fig. 4A is not based on an artefact during isolation and renaturation of apo-NifL.

## Discussion

Our goal is to determine how *K. pneumoniae* NifL may receive and transmit information about the levels of combined nitrogen in cells to NifA. Our starting hypothesis was that changes in the nitrogen status of the cell might affect the redox potential of the FAD-moiety bound to NifL, ultimately determining whether the flavin could be reduced by its physiological electron donor. We therefore investigated whether the NifL protein isolated from *E. coli* grown under nitrogen limitation or in the presence of ammonium had different redox potentials. To minimize unphysiological effects upon overexpression, NifL was synthesized at a level at which its function is regulated normally in vivo in response to combined nitrogen and oxygen.

We determined that the redox potential of NifL, synthesized as a fusion protein (MBP-NifL) in *E. coli* in the presence of ammonium, was -277± 5 mV at pH 8.0 and 25° C. Assuming that the pH dependence of the $FAD_{ox}/FAD_{red}$-couple has a slope of -30 mV, the redox potential of NifL at pH 7 is approximately -247± 5 mV. MBP-NifL synthesized in *E. coli* under nitrogen starvation had almost the same redox potential (-274± 6 mV at pH 8.0 and 25° C). These results indicate that the nitrogen source has no apparent influence on the redox potential of MBP-NifL when synthesized in *E. coli*. Thus the nitrogen signal is apparently not directly transmitted through NifL by stably altering the redox properties of the flavoprotein.

The two MBP-NifL fractions differed, however, in that an absorbance centered at 420 nm was found only in MBP-NifL synthesized in the presence of ammonium. Analyses of peroxidase activity showed that a possible heme content of the MBP-NifL fraction is lower than 0.5% indicating that the absorbance at 420 nm is apparently not based on the presence of a covalent bound heme cofactor. The finding that apoprotein of NifL purified from cells grown in the presence of ammonium still retained the absorbance at 420 nm further indicates that the 420

nm absorbance is also not due to the flavin cofactor. One possible explanation for the absorbance at 420 nm is a post translational covalent modification of NifL in response to ammonium**:** such a modification might yield in an inhibitory form of NifL with unchanged redox potential. At this state of experimental datas we cannot completely exclude that the absorbance at 420 nm might be based on an allosteric interaction with a small effector molecule upon ammonium presence. However, it seems to be unlikely to copurify these effector molecules with NifL when apo-NifL is purified from inclusion bodies by denaturation in the presence of 8 M urea. A covalent modification of NifL, however, should be stable during the denaturation procedure.

It still remains to be explained how the nitrogen signal is transmitted to NifL/NifA. One possible candidate for the nitrogen sensing component is the second PII protein, GlnK, which was shown to be required for the non-inhibitory form of *K. pneumoniae* NifL under nitrogen and oxygen limiting conditions in a heterologous *E. coli* system (He *et al.,* 1998). If *K. pneumoniae* GlnK is indeed the sensor or intermediate messenger responsible for carrying the nitrogen signal to the nitrogen fixation regulatory system, it remains to be found how GlnK interacts with the NifL/NifA system.

The redox potential determined for *K. pneumoniae* NifL produced under nitrogen limitation or abundance is significantly lower than that which was determined for *A. vinelandii* NifL (- 260 mV at pH 8.0 (Macheroux *et al.*, 1998)). In *K. pneumoniae* as in *A. vinelandii*, the redox state of the flavoprotein is thought to influence its ability to modulate the NifA activity in response to the oxygen levels. The physiological electron donors for NifL in *A. vinelandii* and *K. pneumoniae* have not yet been identified. Because *nif* induction in *K. pneumoniae* under nitrogen and oxygen limiting conditions requires iron, we postulated that the oxygen signal is passed to NifL via an iron-containing protein (Schmitz *et al.*, 1996). One possible candidate for this role is a flavoheme protein from *E. coli* studied by Poole and his colleagues, which is believed to function as an oxygen sensor in *E. coli* (Pool, 1994). In vitro, this flavoheme protein can reduce NifL of *A. vinelandii* with NADH, as it is the case for the spinach ferredoxin:NADP oxidoreductase in the absence of an electron mediator (Macheroux *et al.*, 1998). However, NifL reduction by these electron donors in vitro is very slow, indicating that

they might not be the physiological electron donors. In addition to the differences in their redox potentials, the NifL of *K. pneumoniae* and *A. vinelandii* differ significantly in that the apoprotein of NifL *K. pneumoniae* completely lacking its cofactor can inhibit NifA activity in vitro, indicating that FAD is not essential for the inhibition process (Schmitz, 1997). However, Hill *et al.* (1996) showed for *A. vinelandii* that the refolded apoprotein of NifL - lacking the flavin moiety - failed to inactivate NifA under oxidizing conditions. These differences between the NifL proteins might indicate that the mechanisms for sensing and or transmission of the environmental signals to NifA differ in some regards in the two species.

*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 $\sigma^{54}$ (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 1. 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 (9), and derivatives of NCM1529 were chosen to study NifA/NifL regulation in *E. coli*. The *fnr*::Tn*10* allele was transferred from the *fnr*::Tn*10* derivative of M182 (Jayaraman *et al.*, 1988) into NCM1529 by P1-mediated transduction with selection for tetracycline resistance, resulting in RAS1

(Grabbe *et al.*, 2000). 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 1: 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) | 10 |
| NCM1528 | NCM1529/pNH3 | 10 |
| NCM1527 | NCM1529/pJES851 | 10 |
| RAS1 | NCM1529 but *fnr*::Tn*10* | 6 |
| RAS2 | NCM1529/pRS107 | This study |
| RAS6 | RAS1/pRS107 | This study |
| RAS7 | RAS1/pNH3 | This study |
| RAS8 | RAS1/pJES851 | This study |
| RAS9 | RAS1/pNH3 and pRS79 | This study |
| RAS10 | RAS1/pNH3 and pRS120 | This study |

| | | |
|---|---|---|
| RAS11 | RAS1/pNH3 and pMCL210 | This study |
| RAS12 | RAS1/pNH3 and pACYC184 | This study |
| RM101 | MC4100 but Δ *fnr* | 31 |
| 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$^r$ | 24 |
| 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 | 9 |
| RAS29 | RAS18/pJES839 | This study |
| RAS30 | UN4495Δ(*nifLA*)*6293::*Km / pJES839 | 33 and this study |
| Plasmids | | |
| pNH3 | *K. pneumoniae nifLA* controlled by the *tac* promoter | 12 |
| pJES839 | pNH3 but additional tetracycline resistance cassette | 33 |
| pJES851 | *K. pneumoniae nifA* controlled by the *tac* promoter | 33 |
| 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 | 6 |

| | | |
|---|---|---|
| pRS127 | 2.1 kbp fragment in pBluescript $SK^+$ containing *K. pneumoniae fnr* | 6 |
| pRS137 | *K. pneumoniae fnr* controlled by the*tet* promoter on pACYC184 | 6 |
| pACYC184 | Low copy vector | New England Biolabs, UK |
| pMCL210 | Low copy vector | 28 |
| 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.*, 2000).

**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 2). 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 2).

**Table 2**: **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 2). 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 2). 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 2). 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).

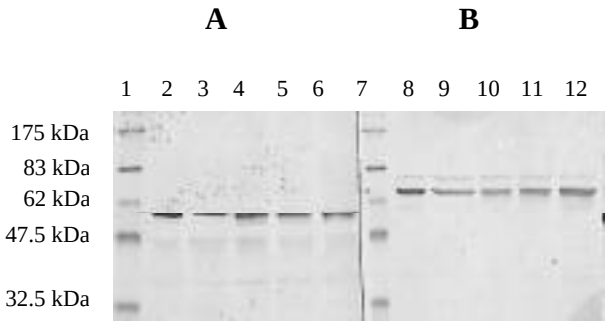


**Figure 5: Amounts of NifA and NifL in wild type and *fnr*⁻ strains of *E. coli*.** Cultures were grown at 30° C in minimal medium under anaerobic conditions with 4 mM glutamine as limiting nitrogen source. The strains carried *K. pneumoniae* NifL and NifA under the control of the *tac* promoter on 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 7) 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 5 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 6. The *nif* induction under anaerobic conditions in both mutant strains was restored to the induction level of the parental strains (NCM1528 and RAS22, respectively) by expressing *E. coli fnr* from promoter P*tet* on pACYC184 or promoter P*lac* on pMCL210, whereas the vectors pACYC184 and pMCL210 alone did not restore *nif*-induction (Table 2). These results and the finding that Fnr affects NifA only in the presence of NifL (see above) strongly indicate that in the heterologous *E. coli* system, Fnr is required for release of NifL inhibition of NifA activity under anaerobic conditions.

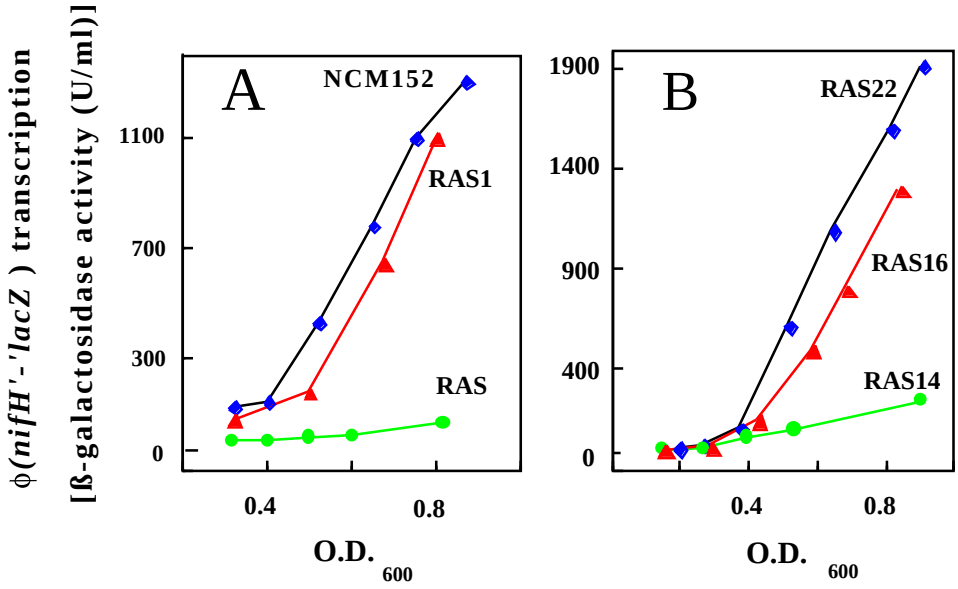


**Figure 6: Effects of *fnr* null alleles on expression of a ø(*nifH'-'lacZ*) fusion in heterologous *E. coli* strains carrying *K. pneumonia nifLA* on a plasmid.** The activity of ß-galactosidase was plotted as a function of $OD_{600}$ for cultures grown at 30° C in minimal medium under anaerobic conditions 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 (9) and plasmid pNH3 encoding NifL and NifA under the control of the *tac* promoter. **A** *fnr* null allele transduced from M182 (*fnr*::Tn*10*): Wild type NCM1528 (diamonds), the respective *fnr* null allele in NCM1528 (RAS7) (circles), 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.

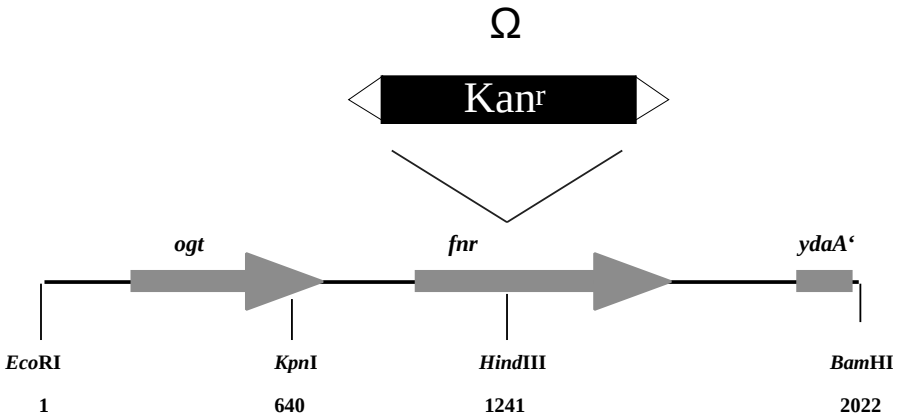


**Figure 7:** Map of the cloned *Eco*RI-*Bam*HI fragment (pRS127) showing the side of insertion of the Ω interposon fragment with a kanamycin resistance gene derived from plasmid pHP45Ω (29) in *K. pneumoniae fnr*. The Ω interposon fragment is flanked by short inverted repeats including strong transcription 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 7), 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 3). 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 8). *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 3**: **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 3). These results indicate that the Fnr effect on *nif* regulation observed in the native background is based on the Fnr requirement for relief of NifL inhibition under oxygen-limiting growth conditions. Based on our findings, we hypothesize that in *K. pneumoniae,* Fnr is the primary oxygen sensor for the *nif* regulation, which transduces the signal directly or indirectly to NifL.

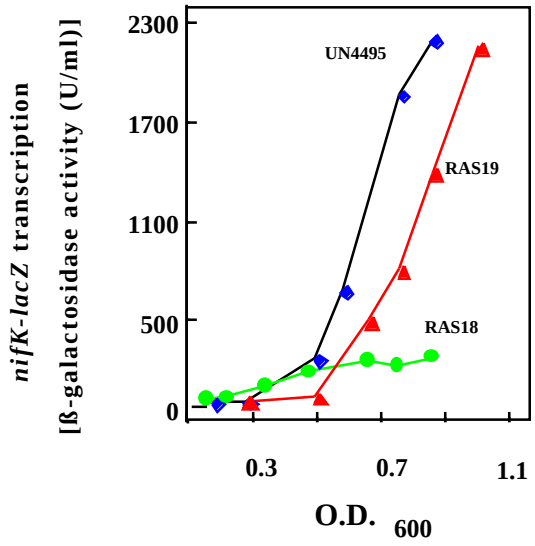


**Figure 8: Effects of an *fnr* null allele on expression of a *nifK-LacZ* fusion in *K. pneumoniae* strain UN4495.**

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.

**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. 9), 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.

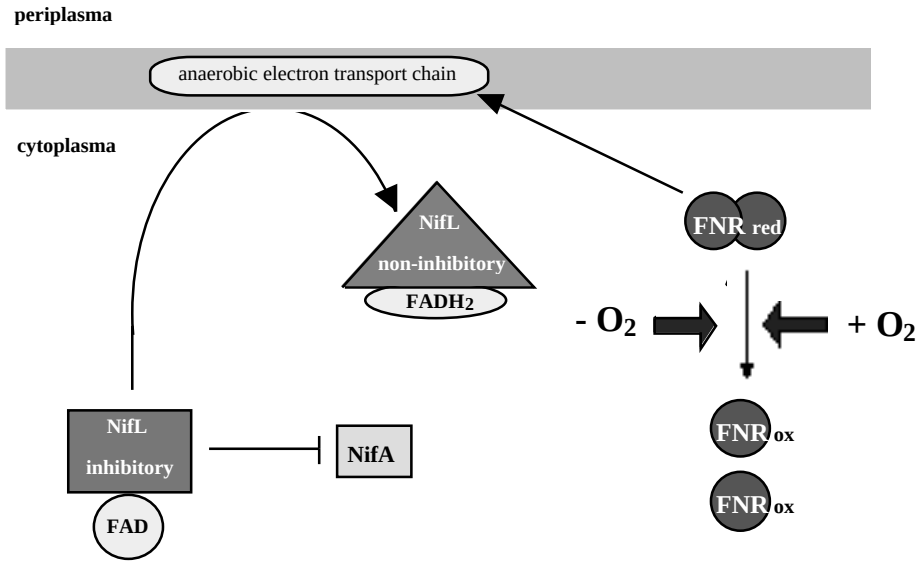


**Figure 9: Hypothetical model of oxygen signal transduction in *K. pneumoniae*.**

*Chapter 4:*

# The inhibitory form of NifL from *Klebsiella pneumoniae* exhibits ATP hydrolyzing activity only when synthesized under nitrogen sufficiency


## Abstract

The inhibitory function of *Klebsiella pneumoniae* NifL on NifA transcriptional activity *in vitro* increases upon addition of ATP and ADP, when NifL was synthesized under nitrogen sufficiency ($NifL_{NH4}$). Interestingly, $NifL_{NH4}$ also hydrolyzes ATP (2500 mU/mg). In contrary, NifL synthesized under nitrogen limitation is not affected by adenine nucleotides and exhibits no ATP hydrolyzing activity. These major differences indicate that stimulation of the inhibitory function and ATP-hydrolysis depend on a specific NifL conformation induced by ammonium. We hypothesize that the presence of ammonium alters the conformation of NifL, enabling NifL to use the energy of ATP-hydrolysis to form NifL-NifA complexes more efficiently.

## Introduction

*Klebsiella pneumoniae* is a free-living diazotroph whose *nifLA* operon regulates the transcription of the *nif* operons in response to environmental oxygen and nitrogen availabilities (Hill *et al.*, 1981; Merrick *et al.*, 1982; Filser *et al.*, 1983; Dixon, 1998). NifA activates the transcription of the *nif* genes and NifL antagonizes the transcriptional activation in the presence of molecular oxygen or combined nitrogen (Merrick *et al.*, 1982; Morett and Buck, 1988). The translationally coupled synthesis, immunological studies and complex analysis imply that the inhibition of NifA activity by NifL occurs via a direct protein protein interaction (Govantes *et al.*, 1998; Money *et al.*, 1998; Lei *et al.*, 1999; Henderson *et al.*, 1989). Concerning the oxygen signal transduction it was recently shown that the flavoprotein NifL senses oxygen apparently via a redox sensitive conformational change, which is mediated by the reduction state of the FAD cofactor (Klopprogge and Schmitz, 1999; Hill *et al.*, 1996; Macheroux *et al.*, 1998). Under oxygen-limiting conditions, NifA transcriptional activity is inhibited by NifL only in the presence of combined nitrogen indicating that in the absence of oxygen NifL exists at least in two different conformational forms, one of which is capable to inhibit NifA. We recently showed that only when synthesized in the presence of nitrogen sufficiency, NifL shows a distinct non-flavin specific absorbance at 420 nm, which might be based on a covalent modification upon the presence of ammonium (Klopprogge and Schmitz, 1999). A similar phenomenon was observed for *Azotobacter vinelandii* NifL synthesized under different nitrogen availabilities (Klopprogge and Schmitz, unpublished results). These findings support the hypothesis that in response to excess nitrogen a conformational change of NifL occurs resulting in an inhibitory conformation.

The C-terminal domain of *A. vinelandii*-NifL contains a nucleotide-binding motif (Söderbäck *et al.*, 1998). Dixon and coworkers showed that the inhibitory activity of *A. vinelandii*-NifL on open complex formation by NifA is specifically increased in the presence of adenine nucleotides (Söderbäck *et al.*, 1998; Eydmann *et al.*, 1995). Limited proteolysis experiments further indicated that ADP-binding to the C-terminal domain induces a change in NifL conformation (Söderbäck *et al.*, 1998) and promotes complex formation between purified

NifL and NifA *in vitro* (Money *et al.*, 1998). These findings suggest that the formation of the inhibitory complex between NifL and NifA might be regulated by the ATP/ADP ratio in order to sense the energy charge *in vivo* (Eydmann *et al.*, 1995). For *K. pneumoniae* NifL, however, no adenine nucleotide specific requirement for effective inhibition of either maltose binding protein (MBP)-NifA fusion or the central domain of NifA was observed (Berger *et al.*, 1994; Lee *et al.*, 1993). In order to determine a possible regulatory function by adenine nucleotides we started to study ATP-binding by *K. pneumoniae*-NifL and the effect of adenine nucleotides on NifL inhibitory function on NifA. Unexpectedly, we observed that purified *K. pneumoniae*-NifL synthesized under nitrogen sufficiency hydrolyzes ATP with significant rates. This is the first report on ATPase activity of NifL in diazotrophic bacteria, the physiological role of which has to be elucidated.

## Materials and Methods

**Plasmids**. pJES794, pJES597 and pJES738 encoding *K. pneumoniae* MBP-NifL, MBP-NifA and MBP-NifA-central domain under the control of the *tac* promoter, respectively, are described by Narberhaus *et al.* (1995), by Lee *et al.* (1993) and Berger *et al.* (1994). pRS20 encoding *A. vinelandii* NifL under the control of the *tac* promoter was constructed by PCR-cloning. PCR-generated restriction sites, *Eco*RI upstream of the start codon and *Hind*III downstream of the stop codon, were used to clone the gene into the expression vector pKK223-3 (Pharmacia).

**Cell extracts and protein purification.** As *K. pneumoniae* NifL and NifA are highly insoluble we worked with maltose binding (MBP)-fusion proteins. MBP-NifL, MBP-NifA-central domain (MBP-cdNifA) and MBP-NifA were synthesized in NMC1529 (He *et al.*, 1997) carrying pJES794, pJES738 and pJES597, respectively. 1-l cultures were grown at 30 °C aerobically either in modified K-medium containing 4 mM glutamine as the sole nitrogen source (nitrogen limiting conditions) (Schmitz, 1997) or in maximal induction medium (Ow and Ausubel, 1983) containing additional 10 mM ammonium. When cultures reached an

$O.D._{600}$ = 0.6 expression of fusion proteins was induced from the *tac* promoter with 100 µM isopropyl-ß-D-thiogalactopyranoside (IPTG). After disruption of the cells in B-buffer (20 mM potassium EPPS (*N*-[2-hydroxyethyl]piperazine-*N*'-3-propanesulfonic acid, pH 8.0), 25 mM potassium glutamate, 10 % (vol/vol) glycerol, 1.5 mM dithiothreitol) fusion proteins were purified by amylose affinity chromatography as described previously (Klopprogge and Schmitz, 1999; Berger *et al.*, 1994). *A. vinelandii*-NifL was synthesized in NMC1529 carrying pRS20 under nitrogen sufficiency and nitrogen limitation as described for the *K. pneumoniae* proteins. After cell breakage in B-buffer NifL was purified from inclusion bodies. Washing the inclusion bodies with water and subsequent centrifugation at 20.000 x g yielded in high amounts of purified NifL protein in the supernatant, which was concentrated using polyethylene glycol. Protein was determined via the method of Bradford (1976) with the BioRad protein assay using bovine serum albumin as standard.

**Nucleotide binding assays.** Up to 10 µg purified protein fractions were spotted onto a nitrocellulose membrane (BA82, Schleicher and Schüll). The membrane was incubated with 20 ml B-buffer containing 700 µM MgATP and 2 MBq [$\alpha^{32}$P]ATP at room temperature for 60 min. After extensive washing with B-buffer, ATP-binding was analyzed by quantifying the radioactivity using a PhosphoImager and the ImageQuant 1.2 software (Molecular Dynamics).

**Nucleoside triphosphatase assays.** Nucleoside triphosphatase (NTPase) hydrolysis reactions were generally performed with 10 µM protein at 30 °C in the reaction buffer (50 mM Tris / acetate pH 8.0, 100 mM potassium acetate, 8 mM magnesium acetate, 27 mM ammonium acetate, 3.5 % polyethylene glycol 6000 and 1 mM dithiothreithol) in a total volume of 50 µl. After 5 minutes preincubation at 30 °C the reaction was started by adding 5 mM MgATP and 0.1 MBq [$\alpha^{32}$P]ATP with a specific activity of 110 TBq/mmol. The reaction was stopped at a given time point by the addition of 10 µl ethanol to a 4 µl aliquot of the reaction mixture. After 10 min on ice the precipitated protein was removed by centrifugation. Reaction products

in 0.5 µl of the supernatant were separated by thin layer chromatography (TCL) using poly(ethyleneimine)-cellulose FTLC plates (Merck) with 2 M formic acid containing 0.5 M LiCl as running buffer (Mangonlg, 1969). The plate was analyzed by using a PhosphoImager and the ImageQuant 1.2 software (Molecular Dynamics). ATP-hydrolysis activity was calculated using the initial ATP-hydrolysis rate expressed as nmol ATP hydrolysis per min (mU).

**In vitro transcription assays.** Single cycle transcription assays were performed at 30 °C with purified $\sigma^{54}$RNA-polymerase ($\sigma^{54}$RNAP) as described by Narberhaus et al. (Narberhaus *et al.*, 1995) using 0.5 µM cdNifA and pJES128 as template, which contains the *K. pneumoniae* *nifH*-promoter regulatory region (Berger *et al.*, 1994). [α-$^{32}$P]-CTP labeled transcripts were analyzed by electrophoresis in denaturing 6% polyacrylamide gels and quantified using the ImageQuant 1.2 software (Molecular Dynamics). When analyzing the effect of ATP or ADP on NifL inhibitory activity, MBP-NifL, cdNifA, template and $\sigma^{54}$RNAP were preincubated in the presence of ATP or ADP (25 µM) for 10 min prior to initiation of open complex formation by adding 1.6 mM GTP.

## Results

In order to analyze whether the energy charge of *K. pneumoniae* cells influences NifL activity via the ATP/ADP ratio, we studied the effect of adenine nucleotides on the NifL inhibitory function and ATP-binding by NifL. As NifL is highly insoluble we worked with a MalE-fusion protein (MBP-NifL).

**Influence of ATP and ADP on the inhibitory function of NifL *in vitro*.** To determine whether ATP or ADP affect the inhibitory function of NifL we studied NifL inhibition of transcriptional NifA activity in *in vitro* transcription assays in the presence or absence of adenine nucleotides. Open complex formation at the *nifH*-promoter was assessed at 30 °C in a single-cycle transcription assay as described by Berger et al. (1994) using 0.5 µM NifA

central domain (cd-NifA) and 1.6 mM GTP to initiate open complex formation. Transcription activated by cd-NifA from the *nifH*-promoter was equally well inhibited by purified MBP-NifL synthesized aerobically under nitrogen excess or nitrogen limitation. To analyze the effect of additional ATP or ADP on the inhibitory activity we preincubated different amounts of MBP-NifL (0.1 - 0.4 µM), cd-NifA (0.5 µM), template and $\sigma^{54}$RNAP in the presence of ATP or ADP for 10 min prior to initiation of open complex formation. For MBP-NifL synthesized in the presence of ammonium (MBP-$NifL_{NH4}$) the inhibitory activity significantly increased when either ATP or ADP was present (Fig. 10). However, the presence of additional ATP or ADP in the absence of MBP-NifL had no effect on NifA transcriptional activity. These findings indicate that adenine nucleotides increase the inhibitory activity of MBP-$NifL_{NH4}$, possibly by stabilizing the inhibitory $NifL_{NH4}$-form or the inhibitory $NifL_{NH4}$-NifA complex. In contrast, when synthesized under nitrogen limitation with glutamine as nitrogen source the inhibitory activity of MBP-NifL (MBP-$NifL_{Gln}$) was not affected by either ATP or ADP. Inhibition of open complex formation by MBP-$NifL_{Gln}$ in the presence of adenine nucleotides occurred to approximately the same amount as it was the case in the absence of additional nucleotides (Fig. 10). This indicates that the stimulatory effect of adenine nucleotides onto NifL inhibitory function is highly specific for $NifL_{NH4}$.

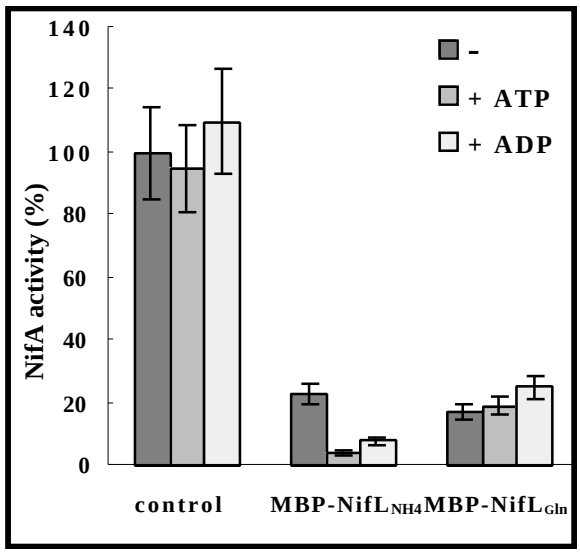


**Figure 10**: **Influence of ADP and ATP on the inhibitory function of MBP-NifL onto NifA transcriptional activity**. Single cycle transcription assays were performed at 30 °C with purified $\sigma^{54}$RNAP as described by

Narberhaus *et al.* (1994) using 0.5 µM NifA central domain and 0.3 µM MBP-NifL. [α-$^{32}$P]-CTP labeled transcripts were analyzed by electrophoresis in denaturing 6 % polyacrylamide gels and quantified using the ImageQuant 1.2 software (Molecular Dynamics). Inhibition by MBP-NifL was performed with MBP-NifL, synthesized in the presence of ammonium (MBPNifL$_{NH4}$), or under nitrogen starvation (MBP-NifL$_{Gln}$). Preincubation in the presence of 25 µM ATP (+ ATP) or 25 µM ADP (+ ADP) occurred for 10 min prior to initiation of open complex formation by adding 1.6 mM GTP. Residual NifA activity is plotted as a percentage of the maximum value and presents mean value of three independent measurements and MBP-NifL purifications. 100 % NifA activity corresponded to ca. 0.6 fmol of transcript.

***K. pneumoniae* NifL binds ATP when synthesized under nitrogen sufficiency**. ATP-binding assays were performed with purified MBP-NifL fractions spotted on a nitrocellulose membrane using [α$^{32}$P]-ATP as described in Materials and Methods. Quantification of radioactivity bound to the protein spots after extensive washing indicated that *K. pneumoniae* MBP-NifL bound significant amounts of ATP when synthesized under nitrogen excess (Fig. 11). Purified MBP-fractions used as a control in the same concentration range as the MBP-fusion proteins did not

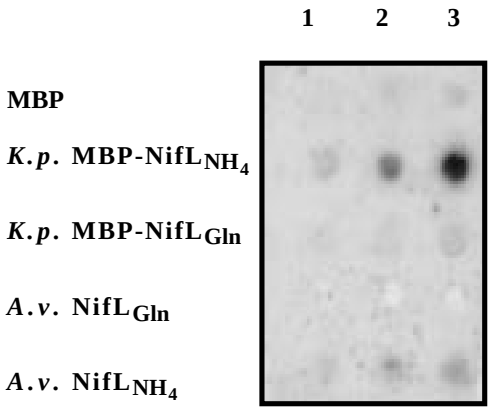


**Figure 11: ATP binding by purified MBP-NifL**. 2.5 µg, 5 µg and 10 µg fractions of purified proteins were spotted onto nitrocellulose membranes (lanes 1, 2 and 3 respectively) and binding of [α$^{32}$P]ATP was analyzed as described in Materials and Methods using a PhosphoImager. *K. pneumoniae*-MBP-NifL and *Azotobacter vinelandii*-NifL were synthesized in the presence of ammonium (*K.p.* MBP-NifL$_{NH4}$ and *A.v.* NifL$_{NH4}$, respectively) or under nitrogen starvation (*K.p.* MBP-NifL$_{Gln}$ and *A.v.* NifL$_{Gln}$, respectively) as described in Materials and Methods.

show unspecific binding of ATP. Therefore, ATP bound specifically to the NifL portion of MBP-NifL$_{NH4}$. However, when MBP-NifL was synthesized under nitrogen limiting conditions (MBP-NifL$_{Gln}$) no significant binding of ATP was observed (Fig. 11). To obtain additional evidence we investigated ATP-binding by purified *Azotobacter vinelandii*-NifL. Again, only when synthesized under nitrogen excess *A. vinelandii*-NifL was able to bind significant amounts of ATP (Fig. 11). The finding that *K. pneumoniae* MBP-NifL$_{Gln}$, NifL$_{Gln}$ from *A. vinelandii* and purified MBP did not show ATP binding strongly suggests that ATP specifically binds to *K. pneumoniae* MBP-NifL$_{NH4}$ and *A. vinelandii* NifL$_{NH4}$.

***K. pneumoniae* NifL shows NTP-hydrolyzing activity when synthesized under nitrogen sufficiency**. Purified MBP-NifL synthesized in the presence of ammonium is able to bind ATP resulting in an increase of its inhibitory function on NifA activity. We therefore investigated, whether MBP-NifL$_{NH4}$ contains a general NTP-hydrolyzing activity. Hydrolysis of radioactively labeled NTPs was followed by analyzing the reaction products of nucleoside triphosphatase assays after separation by thin layer chromatography using a PhosphoImager (see Materials and Methods). Unexpectedly, MBP-NifL$_{NH4}$ purified to apparent homogeneity showed significant adenosine triphosphatase activity resulting in hydrolysis of ATP to ADP and AMP (data not shown). As expected, purified MBP used as a control showed no activity indicating that the observed ATP- and ADP-hydrolysis is catalyzed by the NifL protein moiety of MBP-NifL$_{NH4}$.

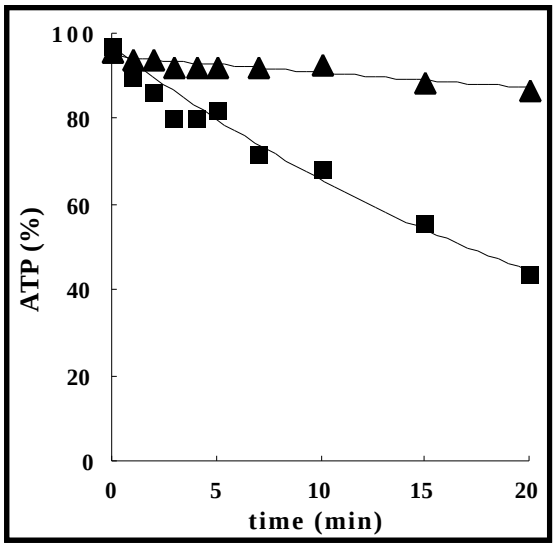


**Figure 12: ATP-hydrolysis catalyzed by purified MBP-NifL**. ATPase assays contained 5 mM ATP, 0.1 MBq [$\alpha^{32}$P]ATP and 10 µM MBP-NifL in a total volume of 50 µl. At different times of incubation at 30 °C the hydrolysis products were separated on thin layer chromatography (TLC) plates and analyzed using the ImageQuant 1.2 software as described in Materials and Methods. Residual [$\alpha^{32}$P]ATP is plotted in respect to the reaction time. MBP-NifL used was synthesized in the presence of ammonium (MBP-$NifL_{NH4}$) (squares), or under nitrogen starvation (MBP-$NifL_{Gln}$) (triangles).

Interestingly, MBP-NifL synthesized under nitrogen starvation (MBP-$NifL_{Gln}$) did not show significant adenosine triphosphatase activity (Fig. 12) indicating that the observed ATP-hydrolysis activity is specific for MBP-$NifL_{NH4}$ and is not due to a copurified contamination in the MBP-$NifL_{NH4}$ fraction. Thus, these results further support the hypothesis that NifL exists in two different conformations depending on the nitrogen availability.

Characterizing the kinetic properties of ATP-hydrolysis catalyzed by MBP-NifL$_{NH4}$ we determined the apparent $K_M$ for ATP and apparent $V_{max}$ to be 41 mM and 2500 mU / mg, respectively (Fig. 13A). Additionally, we characterized as a control the kinetic properties of ATP-hydrolysis by *K. pneumoniae* MBP-NifA, which is known to contain an ATPase function (Fig. 13B) (Berger *et al.*, 1994). Using the same assay conditions as for MBP-NifL an apparent $K_M$ for ATP of 610 µM and an

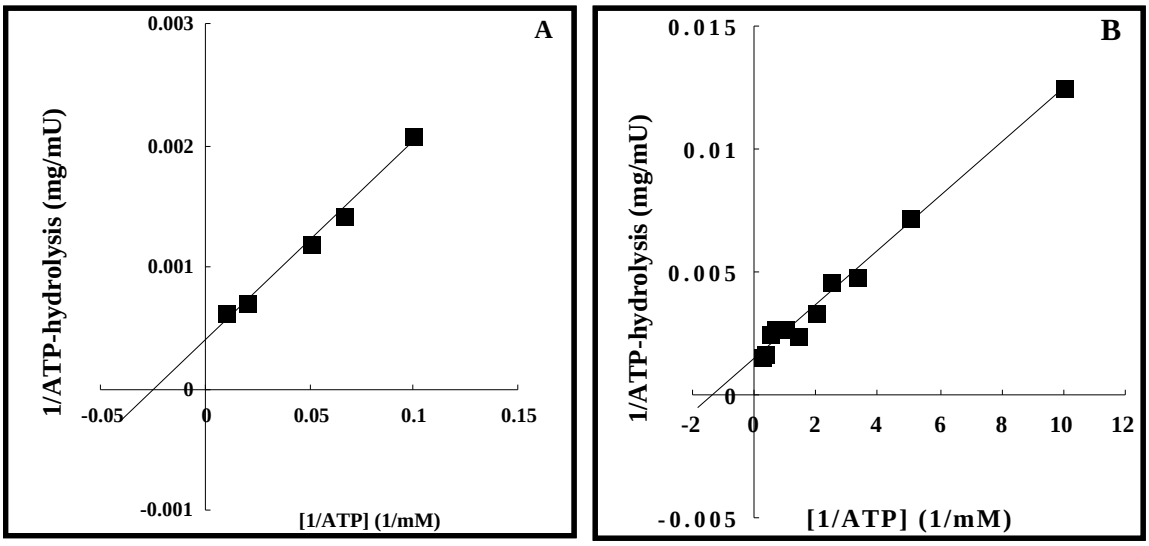


**Figure 13: ATP-hydrolysis activity in respect to the substrate concentration** ATP hydrolysis activity was determined in a total assay volume of 50 µl as described in legend of Fig. 12. The standard assay contained (A) 10 µM purified MBP-NifL synthesized in the presence of ammonium and ATP concentration between 0 and 100 mM; (B) 1 µM MBP-NifA and the ATP concentration was varied between 0 and 5 mM. The specific activities determined were plotted in a double reciprocal plot according to Lineweaver-Burk (1934).

apparent $V_{max}$ of 560 mU / mg were determined for ATP-hydrolysis by purified MBP-NifA (see Fig. 13B). Studying the capability to hydrolyze other nucleoside triphosphates as CTP, GTP, and UTP, we observed that MBP-NifL$_{NH4}$ also hydrolyzed CTP and GTP with 61 % and UTP with 91 % of the maximum ATP-hydrolysis rate. In comparison, MBP-NifA showed highest specific activities with GTP as substrate which was approximately 2.4-fold higher than the ATP-hydrolysis rate. Significant lower activities were observed with UTP or CTP as

substrate (80 % and 41 % of the ATP-hydrolysis rate, respectively) (see also (Berger *et al.*, 1994)).

Interestingly, when purified MBP-NifL$_{NH4}$ (10 µM) and MBP-NifA (5 µM) were incubated together in an ATP hydrolysis assay, MBP-NifL$_{NH4}$ did not affect NifA-ATPase activity (see also (Berger *et al.*, 1994)). This differs significantly from what is known for *A. vinelandii* NifL, which has been shown to strongly inhibit NifA-ATPase activity (Eydmann *et al.*, 1995).

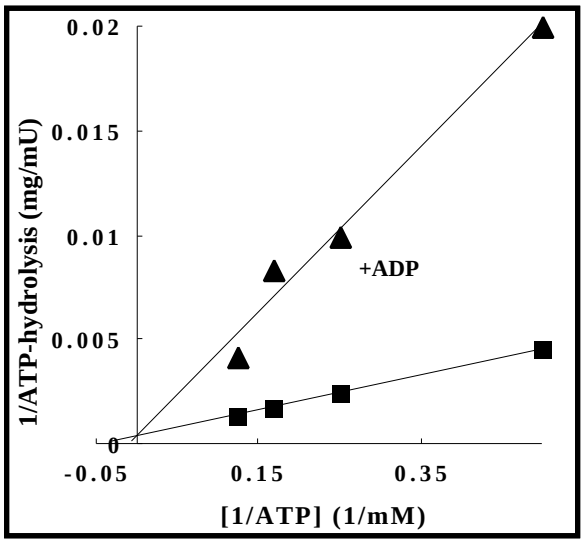


**Figure 14: Influence of additional ADP on the ATP-hydrolysis activity of MBP-NifL.** ATP hydrolysis activity was determined in a total assay volume of 50 µl as described in legend of Fig. 12. The 50 µl standard assay contained 10 µM purified MBP-NifL synthesized in the presence of ammonium and ATP concentrations between 0 and 8 mM. The calculated specific activity was plotted in a double reciprocal plot according to Lineweaver-Burk (1934). (squares), standard assay; (triangles), standard assay containing additional 5 mM ADP.

**ADP inhibits ATP-hydrolysis activity of MBP-NifL$_{NH4}$.** As the total amount of ATP during a kinetic hydrolysis experiment was not completely hydrolyzed to ADP and AMP (see Fig. 12), we studied the effect of the hydrolysis product ADP on ATP-hydrolyzing activity of MBP-NifL$_{NH4}$. The substrate dependence of ATP-hydrolysis was determined in the absence and presence of additional 5 mM ADP, which itself is hydrolyzed to AMP by MBP-NifL$_{NH4}$ (see above). The experimental data, shown in Figure 14, indicate that additional ADP inhibits ATP-hydrolysis of MBP-NifL$_{NH4}$ by competitive inhibition. The addition of 5 mM ADP

resulted in an increase of the apparent $K_M$ for ATP by a factor of six, whereas the $V_{max}$ was not affected.

## Discussion

Our intention was to examine the effect of adenine nucleotides on *K. pneumoniae* NifL in order to analyze, whether the energy charge of the cells influences NifL activity via the ATP/ADP ratio as discussed for *A. vinelandii* NifL (Söderbäck *et al.*, 1998; Eydmann *et al.*, 1995; Money *et al.*, 1998). We obtained strong evidence (i) that NifL is able to bind and hydrolyze adenine nucleotides with significant rates (Figures 11 and 12) and (ii) that the presence of ATP or ADP prior to open complex formation stimulates NifL inhibition of NifA transcriptional activity *in vitro* (Fig. 10). However, ATP-binding, ATP-hydrolysis and stimulation of the NifL inhibitory function, was only observed, when NifL was synthesized in the presence of sufficient nitrogen (MBP-$NifL_{NH4}$). In contrast, NifL, synthesized under nitrogen starvation (MBP-$NifL_{Gln}$), neither showed significant hydrolysis of ATP nor was affected by adenine nucleotides. These findings further support our model, that *in vivo* at least two conformational forms of NifL exist depending on nitrogen availability (Klopprogge and Schmitz, 1999).

**Effects of adenine nucleotides on *K. pneumoniae* NifL and *A. vinelandii* NifL.** For *A. vinelandii*, Dixon and coworkers obtained evidence that ADP stimulates the inhibitory function of NifL, which is based on a conformational change upon ADP-binding. They propose a model in which NifL modulates NifA activity in response to the energy charge of the cells via the ADP/ATP ratio (Söderbäck *et al.*, 1998; Eydmann *et al.*, 1995; Money *et al.*, 1998). In *K. pneumoniae*, adenine nucleotides are also affecting the inhibitory function of NifL, however, the two NifL proteins differ significantly in this respect. (i) In contrast to *A. vinelandii*, NifL from *K. pneumoniae* is stimulated in its inhibitory activity by adenine nucleotides only, when synthesized in the presence of ammonium. (ii) *K. pneumoniae*-NifL, when synthesized under nitrogen sufficiency, exhibits significant ATP- and ADP-hydrolyzing

activity, whereas purified *A. vinelandii*-NifL has no detectable nucleoside triphosphatase activity (Eydmann *et al.*, 1995). These major differences strongly suggest that the signal transduction of energy charge in *K. pneumniae* is not mediated by NifL to NifA by sensing the ADP/ATP ratio in the cell.

Using continuous cultures, Mortenson and colleagues showed that the ADP/ATP ratio in *K. pneumoniae* increased from 0.2 to 0.8 upon changing from nitrogen sufficiency to nitrogen limitation (Upchurch and Mortensen, 1980). Thus under nitrogen fixation conditions - when NifL is in its non-inhibitory form - the ADP concentration is high compared to growth under nitrogen sufficiency. This is consistent with our finding that ADP is not affecting the inhibitory function of NifL, when synthesized under nitrogen limitation. The obtained ratios of ADP/ATP under nitrogen excess and limitation further argue against a signal transduction of the energy charge to the *nif* regulatory system via the ADP/ATP ratio in *K. pneumoniae*.

**Functions of ATP- and ADP-hydrolysis by *K. pneumoniae* NifL**. Although both, NifL and NifA from *K. pneumoniae* comprise an ATPase activity, neither autophosphorylation nor a possible phosphor transfer between the regulatory proteins has been detected (Lee *et al.*, 1993; Austin *et al.*, 1994). Thus one has to expect that the obtained stimulation of the inhibitory function of $NifL_{NH4}$ in the presence of adenine nucleotides is based on an increase of stable complex formation between NifL and NifA. Stimulation of the inhibitory function by adenine nucleotides only occurs, when NifL was synthesized under nitrogen sufficiency, conditions under which NifL exhibits an ATPase- and an ADPase-activity. These findings suggest that hydrolysis of adenine nucleotides may play a key role in the stimulation of NifL inhibition by adenine nucleotides. We propose that *K. pneumoniae* $NifL_{NH4}$ is able to use the energy obtained by hydrolyzing ATP or ADP to form inhibitory NifL-NifA complexes more efficiently. Hydrolysis of adenine nucleotides might induce a NifL conformation, which promotes tighter or more stable complex formation between NifA and NifL. Thus the hydrolyzing activity of $NifL_{NH4}$ might comprise a chaperonine function.

*Chapter 5:*

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


## Abstract

In the diazotroph *Klebsiella pneumoniae,* NifL and NifA regulate transcription of the nitrogen fixation (*nif)* 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 was found in the membrane fraction. However, when the cells were grown under aerobic conditions or shifted to nitrogen sufficiency significantly less membrane-associated NifL was detectable (less than 10 % of total NifL). These findings suggest that a significant higher membrane affinity of NifL under derepressing conditions may create a spatial gap between NifL and its target protein NifA impairing inhibition of NifA by NifL. Localization analysis of the primary nitrogen sensor GlnK further showed that under nitrogen-limited conditions but independent of oxygen presence 15 - 20 % of the total GlnK is membrane associated. A shift to nitrogen sufficiency resulted in rapid degradation of the cytosolic GlnK fraction, whereas the membrane associated GlnK fraction displayed a significantly longer half-life. These findings suggest that proteolytic cleavage of cytosolic GlnK may be the mechanism for restoration of NifL activity upon a shift to nitrogen sufficiency.

## 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 (*nif*)*LA* operon (for review see Dixon, 1998; Schmitz *et al.,* 2001). Products of this regulatory *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 molecular oxygen and combined nitrogen 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).

External nitrogen availability is apparently perceived by *K. pneumoniae* through changes in the internal glutamine pool (Schmitz, 2000), which are subsequently mediated to the *nif* regulatory system. Recent evidence strongly suggest, 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; Arcondeguy *et al.*, 1999 and 2000; Little *et al.,* 2000). 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).

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 he 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*., 2001). 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.,* 2001; Schmitz *et al.,* 2001). 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 FAD-containing *Escherichia coli* 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 investigated the localization of NifL protein in *K. pneumoniae* under various growth 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 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 his D4226* Gal$^{r}$) (MacNeil *et al.,* 1981) were used in this study. Plasmid pHN3 carries the *K. pneumoniae nifLA* operon under the control of the *tac* promoter (Henderson *et al*., 1989).

**Growth.** *K. pneumoniae* strains were grown under anaerobic conditions 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 % resazurine. Precultures were grown overnight in closed bottles with $N_2$ as gas phase in the same medium but lacking sulfide and resazurine. The 1 liter main cultures were inoculated from precultures and grown in closed bottles with molecular nitrogen as gas phase under strictly anaerobic conditions without shaking. Samples were taken anaerobically for monitoring growth at 600 nm and determining ß-galactosidase activity. Aerobic cultures were grown in 2 liter flasks under vigorous shaking (130 rpm) using the same medium and culture supplements as described for the anaerobic growth but lacking $Na_2CO_3$, sulfide and resazurine. For ammonium shift experiments, 1 liter 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.

**ß-Galactosidase assay.** NifA-mediated activation of transcription from the *nifHDK* promoter in *K. pneumoniae* UN4495 was monitored by measuring the differential rate of ß-galactosidase synthesis during exponential growth (units per milliliter per $OD_{600}$) 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 was used carrying a plasmid born *nifLA*-operon under the control of the *tac* promoter (pNH3, Henderson *et al.*, 1989) in addition to the chromosomal *nifLA* operon. 50 ml cultures were grown 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 optical density of $OD_{600}$ = 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), 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 aerobic conditions (Roth *et al.*, 1981; Hoppert and Holzenburg, 1998).

Resin sections of 80 - 100 nm in thickness were cut with glass knives. Immunolocalization on resin sections was performed with specific polyclonal antisera directed against NifL and goat-anti-rabbit-IgG linked to colloidal gold (10 nm in diameter, BBI, Cadiff, 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 study the localization of chromosomally synthesized NifL, cytoplasmic and membrane fractions of *K. pneumoniae* UN4495 were separated by several centrifugation steps. Membrane preparations of anaerobically grown cells were performed under strictly anaerobic conditions in the presence of 2 mM dithiothreitol under a nitrogen atmosphere; aerobic membrane preparations were performed in the absence of dithiothreitol. To separate the membrane and cytoplasmic fractions, exponentially growing 1 liter 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 at 120,000 x g, for 2 h two times to sediment the membrane fraction. The resulting supernatant was designated the cytoplasmic fraction and stored at 4 °C for further

studies. 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 hydrophobic pellet was resuspended in 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 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, redox-cycle staining for the specific detection of quinoproteins was performed to detect leakage of membrane proteins into the cytoplasmic fraction. 5 µl aliquots of membrane and cytoplasmic fractions were spotted on a nitrocellulose membrane. Redox-cycle staining was performed using 0.24 mM nitroblue tetrazolium (NBT) in 2 M potassium glycinate (pH 10) as described by Flückiger *et al.* (1995). The nitrocellulose membrane was immersed in the NBT / 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.** Samples of the membrane and cytoplasmic fractions were diluted 1:1 with either sodium dodecyl sulfate (SDS) gel-loading buffer or native gel-loading buffer and subsequently separated by SDS polyacrylamide (12 %) gel electrophoresis (Laemmli, 1970)

or native (12.5 %) gel electrophoresis (Atkinson *et al.,* 1994), respectively. 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*. 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), and the protein bands were quantified for each growth condition in three independent membrane preparations using the ImageQuant v1.2 software (Molecular Dynamics). The quantifications were normalized to total NifL, NifA or GlnK content for both membrane and cytoplasmic fractions. Purified NifA and NifL from *K. pneumoniae* and prestained protein markers (New England Biolabs, UK) were used as standards. Polyclonal antibodies directed against NifL, NifA and GlnK from *K. pneumoniae* were specific for the *Klebsiella* proteins NifL, NifA and GlnK, respectively.

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). Native gel electrohoresis was performed by 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. Gels were run using a BioRad Miniprotein I electrophoresis apparatus and 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 (Grabbe *et al.,* 2001). If the reduction of NifL indeed occurs by a membrane-bound electron donor, then NifL must contact the cell membrane. We therefore investigated the localization of NifL in *K. pneumoniae* growing under various conditions.

**Localization analysis of NifL in *K. pneumoniae* cells by electron microscopical studies .**

We analyzed the localization of 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 chromosomally expressed NifL 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 *tac* promoter, with 10 µM IPTG, in *K. pneumoniae* M5a1 containing the plasmid pNH3 (Henderson *et al.,* 1989). Cells grown under anaerobic conditions were harvested anaerobically in mid exponential phase, and prepared for electron microscopy under a nitrogen atmosphere in a glove box, as described in Materials and Methods. Immunogold detection by electron microscopical analysis of the overexpressed protein indicated that NifL is membrane associated when cells were grown under nitrogen-limiting conditions; most of the gold particles were found in close proximity to the cell membrane (Fig. 15 A). 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 (Fig. 15 B). 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.

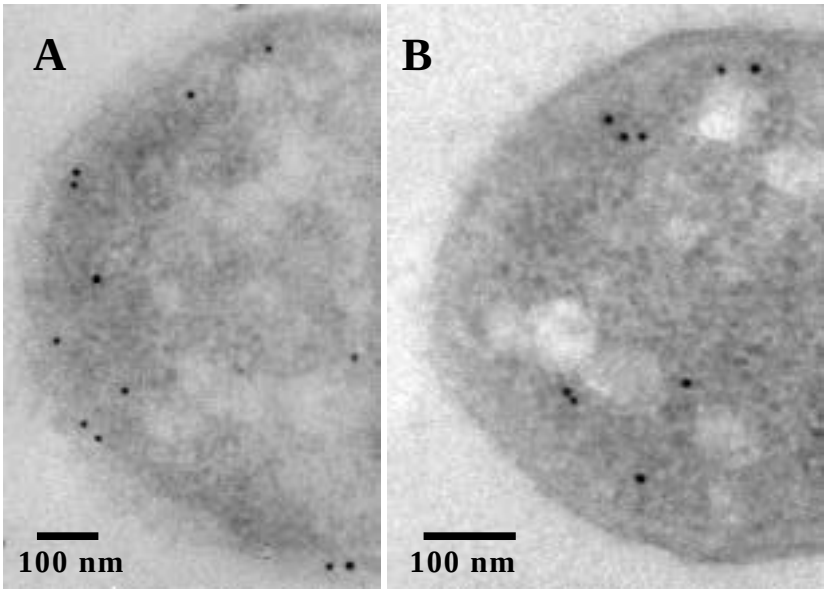


**Fig. 15: Effect of ammonium on NifL localization in *K. pneumoniae*.** NifL was overexpressed from the *tac* promoter by 10 µM IPTG in *K. pneumoniae* growing anaerobically under nitrogen-limited (A) and nitrogen excess (B) conditions. Cells were harvested in mid exponential growth and prepared for electron microscopic analysis as described in Materials and Methods. NifL identified by immunogold labelling appears as dark spots (colloidal gold particles). Horizontal bars equal 0.1 µM.

**Chromosomally synthesized NifL is highly membrane associated under derepressing growth conditions.** Localization analysis 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 chromosomally expressed NifL was too insignificant for localization by immunogold labelling, we used immunological means for the detection and quantification of NifL chromosomally synthesized in cytoplasmic and membrane fractions of *K. pneumoniae* cells grown under various conditions.

*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 in the absence or presence of molecular oxygen. In order to monitor NifL regulation of NifA activity in the respective cultures, we analyzed NifA activity by determining ß-galactosidase activity. In general, anaerobically growing cultures exhibited a β-galactosidase synthesis rate of

approximately 4000 U / ml · $OD_{600}$, whereas the synthesis rate of aerobic cultures was determined to be approximately 200 U / ml · $OD_{600}$. This indicated that *nif* gene induction was fully induced under nitrogen- and oxygen-limiting conditions, and repressed in the presence of oxygen (Schmitz *et al.*, 1996). In order to localize NifL in these cells, we performed membrane preparations under anaerobic or aerobic conditions, respectively, as described in Materials and Methods, and separated the membrane and cytoplasmic fractions. 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. Determination of the malate dehydrogenase activity, and detection of the quinones by a redox-cycle stain, were performed as described in 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 4). 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. Quantification of the NifL protein in the different fractions using the fluoroimager and the ImageQuant software (Molecular Dynamics, see Materials and Methods) was performed for each growth condition in three independent membrane preparations. The quantifications were normalized to total NifL content for both membrane and cytoplasmic fractions.

**Table 4: Quality of membrane preparations.** The quality of the membrane preparations was evaluated by determination of malate dehydrogenase activity and quinoproteins in the respective fractions, as described in Materials and Methods.

| Growth condition | Cell fraction | Malate dehydrogenase activity (U x fraction$^{-1}$) | Redox cycle stain |
|---|---|---|---|
| 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 chromosomally-expressed NifL was found in the membrane fraction (Fig. 16, lanes 1 and 2). In contrast, 6 % or less of total NifL synthesized under aerobic growth conditions was found in the aerobic membrane fraction (Fig. 16, lanes 5 and 6).

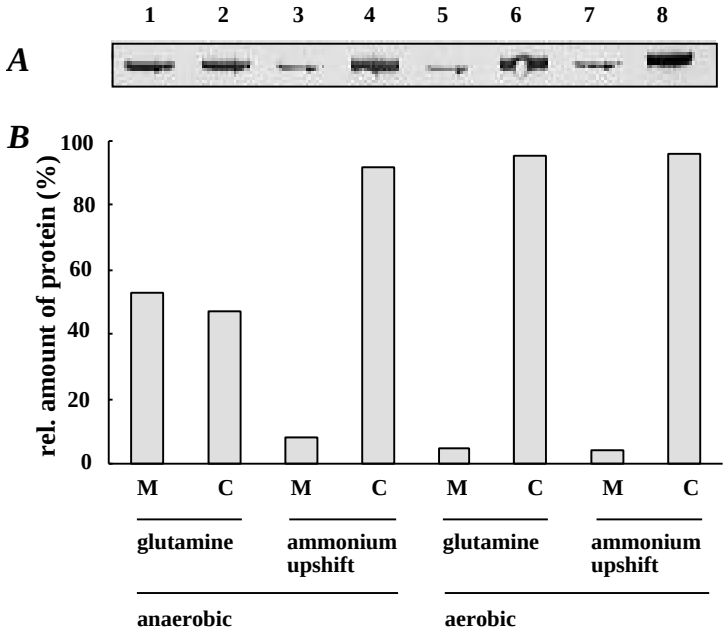


**Fig. 16: Localization of chromosomally expressed NifL in *K. pneumoniae* UN4495 under different growth conditions.** Cells of *K. pneumoniae* UN4495 were grown aerobically and anaerobically in minimal medium containing 2 mM glutamine as the sole nitrogen source. Exponentially growing cells were split 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 were subjected to SDS-PAGE, and subsequently analyzed by western-blot. Polyclonal NifL antibodies were used to detect chromosomally expressed NifL in the fractions. Quantification of NifL found in the membrane and cytoplasmic fractions was done with a fluoroimager (Molecular Dynamics storm, ImageQuant software). **A**, original western blot data. 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.

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 examined anaerobically growing cultures that were shifted from nitrogen-limited growth to nitrogen excess conditions and further grown for an additional two hours. Interestingly, although growing anaerobically these cells also showed approximately 10 % of total NifL in the membrane fraction (Fig. 16 lanes 3 and 4). The total amount of NifL, however, did not significantly decrease after the shift to nitrogen sufficiency. Within the two hour 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; Blanco *et al.*, 1993). Thus the presence of ammonium apparently resulted in a significant dissociation of NifL from the cytoplasmic membrane. This is consistent with the results obtained by electron microscopy for the localization of overproduced NifL under nitrogen-sufficient growth conditions (Fig. 15B). These findings indicate that NifL is membrane associated only when cells are growing under derepressing nitrogen-fixation conditions. However, both 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.

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 analyzed the localization of chromosomally synthesized NifA using the same membrane and cytoplasmic fractions in which we had localized NifL. We found that approximately 10 % of total chromosomally expressed NifA are found in the membrane

fraction under all growth conditions tested (data not shown). As shown for NifL, no difference in total NifA protein was detected under the various growth conditions. Taking into account (i), that NifA has to be localized in the cytoplasm to activate *nif* transcription, and (ii), that 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.

**Cytoplasmic GlnK is rapidly degraded in the presence of ammonium**

NifL is membrane associated under oxygen- and nitrogen-limited conditions and dissociates from the membrane upon a shift to nitrogen sufficiency (Fig. 16). 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 (He *et al.,* 1998; Jack *et al.,* 1999), we studied the localization of GlnK 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 (van Heeswjik *et al.* 1996; Jack *et al*., 1999; Arcondeguy *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 as is GlnB (PII-protein). 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 are up to 70 % completely uridylylated ($GlnK_3$-$(UMP)_3$) as detected by native gel electrophoresis and subsequent western blot analysis (Fig. 3, lanes 2 and 4); the uridylylation apparently changes the overall charge of the trimer resulting in a faster migration of the uridylylated forms compared to the non-modified trimers (Forchhammer and Hedler, 1997). Two hours after

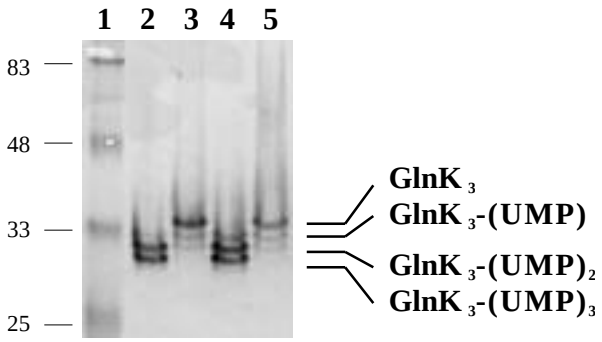


**Fig. 17: Uridylylation states of GlnK upon an ammonium upshift.** Cultures were grown aerobically and anaerobically in the presence of 2 mM glutamine as sole nitrogen source as described in Materials and Methods. During mid exponential growth, cells were split and one part was shifted to nitrogen-excess 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 chromosomally synthesized uridylylated and deuridylylated GlnK. 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.

an ammonium upshift, the same cultures showed fully deuridylylated GlnK trimers (Fig. 17, compare lanes 2 and 4 with lanes 3 and 5). These findings show that the uridylylation state of *K. pneumoniae* GlnK, like that of the paralogue PII-protein (*glnB* gene product), is dependent on the nitrogen status of the cell. In order to analyze the localization of GlnK trimers upon 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 with a further incubation of two hours. 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. During this analysis we found that under nitrogen-limiting conditions, in the cell-free extracts GlnK is approximately 80 % in its completely uridylylated form ($GlnK_3$-$(UMP)_3$). 15 to 20 % of the total GlnK protein was found in the membrane fraction under nitrogen-limiting conditions, in both the anaerobic

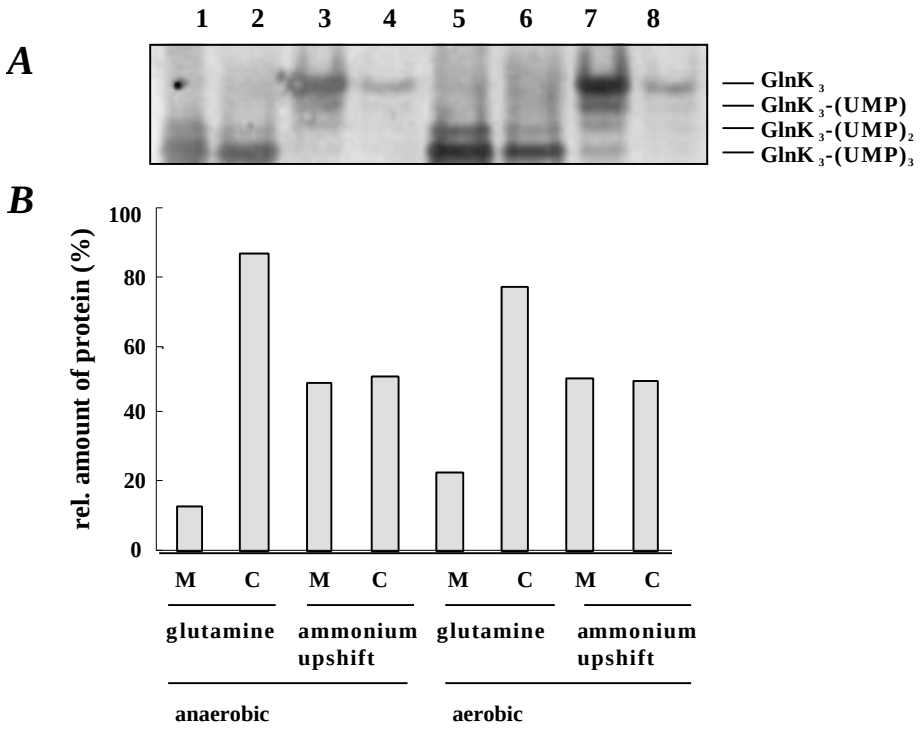


**Fig. 18: Localization of GlnK in *K. pneumoniae* UN4495 under different growth conditions.** *K. pneumoniae* UN4495 cells were grown, harvested and fractionated as described in Fig. 2. Total volumes of the membrane and cytoplasmic fraction differed considerable. Equal volumes of the observed membrane and cytoplasmic fractions, were subjected to native PAGE and subsequently analyzed by western-blotting. Polyclonal GlnK antibodies were used to detect chromosomally expressed GlnK. The original western blot data are 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 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 blotted as relative amounts of total GlnK in the respective fraction, in **B**.

and the aerobic preparation (Fig. 18B, 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. 18A, 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. When nitrogen limited *K. pneumoniae* cells were shifted from nitrogen limitation to nitrogen excess, GlnK was deuridylylated in both the membrane and the cytoplasmic fractions (Fig. 18A, lanes 3 and 4, lanes 7 and 8).

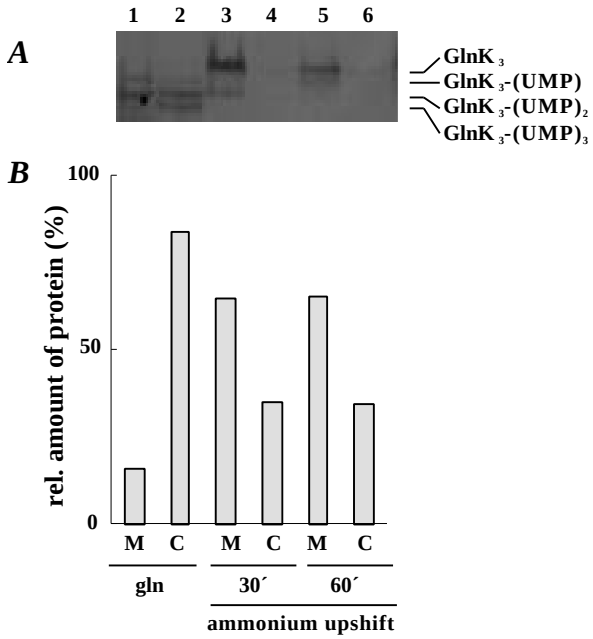


**Fig. 19: Proteolytic degradation of cytoplasmic GlnK upon the presence of ammonium.** *K. pneumoniae* UN4495 cells were grown anaerobically under nitrogen limitation. Ammonium upshift was performed in mid-exponential growth phase with part of the cells. Cells were harvested before, 30 and 60 minutes, after ammonium addition, and fractionated as described in Fig. 2. Total volumes of the membrane and cytoplasmic fraction differed considerable. Equal volumes of the observed membrane and cytoplasmic fractions were subjected to native PAGE, and were subsequently analyzed by western-blotting. Polyclonal GlnK antibodies were used to detect chromosomally-expressed GlnK. The original western blot data are shown in **A**. Lanes 1 and 2, membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation; membrane and cytoplasmic fraction of cells grown anaerobically under nitrogen limitation, which were shifted to nitrogen sufficiency and further incubated for additional 30 min, lanes 3 and 4, or for additional 60 min, lanes 5 and 6. 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 the fraction volume (total volume of the membrane fraction was 2 ml; total volume of the cytoplasmic fraction was 10 ml), and blotted as relative amounts of total NifL in the respective fraction, in **B**.

However, completely unexpectedly, a rapid degradation of the cytoplasmic GlnK fractions occurred upon the presence of ammonium, and independent of oxygen availability (Fig. 18A,

compare lanes 2 and 4, and lanes 6 and 8). Further analysis of this observed degradation of cytoplasmic GlnK after a shift to nitrogen sufficiency showed that approximately 80 % of cytoplasmic GlnK protein were degraded within 30 min after the addition of ammonium to the cultures (Fig. 19, compare lane 2 with lane 4). In contrast, membrane associated GlnK was apparently protected from degradation, thus shifting the ratio of membrane associated GlnK to cytoplasmic GlnK from 15 % / 85 % under nitrogen limitation, to 60 % / 40 % in the presence of excess nitrogen (Fig. 18 and Fig. 19B). Both the deuridylylation and the degradation of the cytoplasmic GlnK were independent of the oxygen availability during growth (Fig. 18).

## 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; Woodly 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; Schmitz *et al.*, 2001). For *K. pneumoniae* we have recently shown that Fnr 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.*, 2001). In addition, preliminary studies indicate that *K. pneumoniae*

NifL is membrane-associated under anaerobic conditions. Thus we proposed that the physiological electron donor for the reduction of NifL under anaerobic conditions is a component of the anaerobic electron transport chain. In order to further characterize a potential membrane association of NifL as a part of the regulatory process, we investigated the localization of NifL under anaerobic and aerobic growth conditions, both in the absence and presence of combined nitrogen.

**Spatial separation as the potential regulatory principle in *nif* regulation by NifA and NifL.** In this study, we present two 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 microscopical 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 localized in the cell lumen (Fig. 15). (ii) Immunological quantifications of chromosomally synthesized NifL confirmed that under oxygen and nitrogen limitation, approximately 55 % of the total NifL protein is localized 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. 16). 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 NifL significantly decreases, and it is again localized 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 4). 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, 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 localization is detectable, suggests that membrane association of NifL plays a 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 thyphimurium* 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. 15 and 16). Thus the question arises, how does the presence of sufficient nitrogen change the membrane affinity of NifL. We therefore studied the localization of 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; Arcondeguy *et al.,* 1999). Surprisingly, we observed a significant membrane association of GlnK under nitrogen limiting conditions (approximately 15 - 20 %), which is not dependent on a defined uridylylation state of GlnK (Fig. 18A). Upon an ammonium upshift, however, a fast degradation of cytoplasmic GlnK occurred ($t_{1/2}$ = approx. 10 min), whereas the amount of membrane-bound GlnK remained constant (Fig. 19). Taking into account that GlnK is required to relief NifL inhibition these findings suggest a model whereby under nitrogen-limited conditions, NifL receives the nitrogen signal by interacting with GlnK, resulting in a GlnK-mediated NifL transport to the membrane and thus in membrane association of the NifL/GlnK complex. When, in addition, oxygen is absent, the FAD cofactor of NifL is reduced by a membrane component, resulting in a significant increase of NifL-membrane association. Upon a shift to nitrogen sufficiency, the cytoplasmic GlnK is degraded rapidly, which may result in a decrease of GlnK-mediated NifL transport to the membrane and increase the release of bound GlnK/NifL through a shift in the equilibrium with cytoplasmic GlnK, thus increasing the fraction of NifL available to interact with NifA in the cytoplasm. We therefore propose a model for the signal integration of oxygen and nitrogen limitation through a conformational change in NifL, resulting in the membrane association of the protein. In addition, the observed rapid degradation of cytoplasmic GlnK upon a return to nitrogen sufficiency suggests the possibility that the mechanism for restoration of NifL activity under these conditions occurs through the proteolytic degradation of GlnK. This is in contrast to the mechanism proposed by Merrick and coworkers, that GlnK is inactivated under nitrogen excess conditions upon the formation of non-functional heterotrimers between GlnK and PII (Arcondeguy *et al.*, 1999).

# Conclusions

In *Klebsiella pneumoniae* and *Azotobacter vinelandii* NifL is inhibiting NifA transcriptional activity in response to environmental changes to tightly control nitrogen fixation and avoid unnecessary consumption of energy. The inhibition of NifA activity by NifL occurs via direct protein protein interaction as shown by immunological studies, demonstration of complex formation between NifL and NifA by co-chromatography and using the yeast two hybrid system (Henderson *et al.,* 1989; Money *et al.,* 1999; Money *et al.,* 2001; Lei *et al.,* 1999). Thus it can be expected that changes in the oxygen, nitrogen or energy level finally result in either conformational changes of the NifL protein promoting complex formation with NifA or affecting the NifL/NifA-complex formation directly. This hypothesis is supported by the findings that (i) the presence of molecular oxygen apparently results in a redox induced conformational change of NifL, which strongly interacts with NifA (Hill *et al.,* 1996; Machereux *et al.,* 1998; Schmitz, 1997), (ii) the nitrogen signal is transduced by GlnK to the *nif* system apparently by affecting NifL conformation or the NifL/NifA complex, (iii) adenine nucleotides stimulate inhibitory function of NifL apparently upon a conformational change of NifL (Eydmann *et al.*, 1995, Söderbäck *et al.*, 1998, Klopprogge and Schmitz, 2001) and (iv) *K. pneumoniae* NifL shows higher affinity for the cytoplasmic membrane under anaerobic and nitrogen limited growth conditions than in the presence of either oxygen or combined nitrogen (Klopprogge *et al.*, 2001). Although signal transduction to the *nif* regulatory system appears to result generally in conformational changes of NifL or the NifL/NifA complex, experimental data suggest that the mechanisms of the signal transduction pathways in *A. vinelandii* and *K. pneumoniae* differ significantly.

**Oxygen signal control of the NifL/NifA regulatory system.** We showed that in *K. pneumoniae* the primary oxygen sensor is the global regulator Fnr, which transduces the signal to NifL resulting in NifL reduction (Grabbe *et al.,* 2001), whereas in *A. vinelandii* the primary oxygen sensor appears to be NifL, which is reduced depending on the availability of the reducing equivalents in the cell (Macheroux *et al.,* 1998; Dixon, 1998). We hypothesize

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 in the absence of a sufficient nitrogen source. Hypothetical candidates for the physiological electron donor for NifL reduction are those components of the anaerobic electron transport system, which are Fnr dependent synthesized (Grabbe *et al.* 2001; Schmitz *et al.* 2001). This model is supported by the fact that NifL is membrane associated under anaerobic and nitrogen limited conditions (Klopprogge *et al.,* 2001). The observed decrease in cytoplasmic NifL under anaerobic and nitrogen limited conditions, whereas no change of NifA localization is detectable, further suggests, that membrane association of NifL plays a role in the regulation of NifA activity by the NifL protein. The spatial separation of membrane-bound NifL and cytoplasmic NifA under nitrogen and oxygen limitation might be responsible for active NifA resulting in *nif* gene induction. We therefore propose a NifL conformation that integrates the oxygen and nitrogen signal in 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.

**Nitrogen signal control of the NifL/NifA regulatory system.** The *nif* gene induction of *K. pneumoniae* is GlnK dependent (He *et al.,* 1997; Jack *et al.,* 1998). Although GlnK responds with uridylylation or deuridylylation in response to the nitrogen status of the cell (Klopprogge *et al.,* 2001) uridylylation of GlnK is not required for *nif* gene regulation (He *et al.,* 1997; Jack *et al.,* 1998; Arcondeguy *et al.,* 1999). In the presence of combined nitrogen the positive effect of GlnK on NifA activity disappears. Using a heterologous *Escherichia coli* system, Merrick and coworkers presented evidence that the formation of non-functional heterotrimers between GlnK and PII might be involved in the fast inactivation of NifL upon the presence of combined nitrogen (Arcondeguy *et al.,* 1999). However, the findings presented in this work indicate that proteolytic degradation of GlnK is responsible to restore NifL inhibitory activity on NifA in the presence of excess nitrogen (Klopprogge *et al.,* 2001). In *K. pneumoniae* proteolytic degradation of cytosolic GlnK occured only, when combined

nitrogen was present, however, membrane bound GlnK was not degraded (Klopprogge *et al.*, 2001). Why membrane associated GlnK remains stable under nitrogen excess conditions remains to be elucidated.

We showed that the nitrogen status of the cell does not affect the redox potential of NifL-bound FAD. The redox potential of NifL, synthesized under nitrogen limited or excess conditions, was determined to be -277 mV at pH 8.0 (Klopprogge and Schmitz, 1999). Interestingly, NifL shows a covalent modification resulting in an absorbance maximum at 420 nm only when synthesized under nitrogen excess conditions. We excluded that the absorbance is based on an iron containing cofactor, heme or quinone-like cofactor (Klopprogge and Schmitz, 1999). However, the potential modification of NifL upon nitrogen sufficiency has still to be identified and its importance for the nitrogen signal transduction still has to be elucidated.

**Influence of the energy charge on *nif* induction.** Binding of adenine nucleotides to the C-terminal domain induces a conformational change of *A. vinelandii* NifL, which apparently promotes complex formation between NifA and NifL. Interestingly and in contrast to *A. vinelandii*, the stimulatory effects of adenine nucleotides on the inhibitory function of *K. pneumoniae* NifL only occurs, when NifL is synthesized under nitrogen sufficiency and correlates with the ability of NifL to hydrolyze ATP (Klopprogge and Schmitz, 2001). No binding and hydrolysis of adenine nucleotides, nor stimulation of inhibitory activity *in vitro* was observed for NifL synthesized under nitrogen limited conditions. Hydrolysis of adenine nucleotides apparently induce a conformational change of NifL synthesized under nitrogen sufficiency resulting in increased formation of inhibitory NifL/NifA complexes. The finding that triphosphates stimulate the NifL inhibitory activity correlates with the report of Upchurch and Mortensen (1982), that the ratio between ATP and ADP in the cell considerably shifts to ADP under nitrogen fixing conditions. Why diphosphates also stimulate the NifL inhibitory activity remains unclear, since under nitrogen fixing conditions the amount of ADP increases. In contrast Dixon and coworkers proposed a model for *A. vinelandii*, in which NifL modulates NifA activity in response to the energy charge of the cell via the ATP / ADP ratio (Söderback

*et al.*, 1998). Thus one has to expect species specific mechanisms of energy charge transduction towards NifL in *K. pneumoniae* and *A. vinelandii*.

**Current working model.** The findings presented in this thesis lead to our current working model presented in Fig. 20. In *K. pneumoniae* NifL inhibits the NifA transcriptional activation of the *nif* genes through direct protein protein interaction in response to molecular oxygen or combined nitrogen. Under nitrogen limited conditions NifL receives the nitrogen signal by interacting with GlnK apparently resulting in membrane association of the NifL/GlnK complex. When in addition oxygen is absent, the FAD cofactor of NifL is reduced by a membrane component, which is under Fnr transcriptional control, resulting in a significant increase of NifL membrane association. Upon an ammonium upshift however, cytoplasmic GlnK is degraded rapidly, which might result in a decrease of GlnK mediated NifL transport to the membrane, thus an increasing fraction of NifL can interact with NifA in the cytoplasm. We therefore propose a NifL conformation, which integrates the signal of nitrogen and oxygen limitation resulting in membrane association of the NifL-protein. In addition, the observed rapid degradation of cytoplasmic GlnK upon an ammonium upshift suggests, that restoring NifL activity upon an ammonium upshift occurs by proteolytic degradation of GlnK.

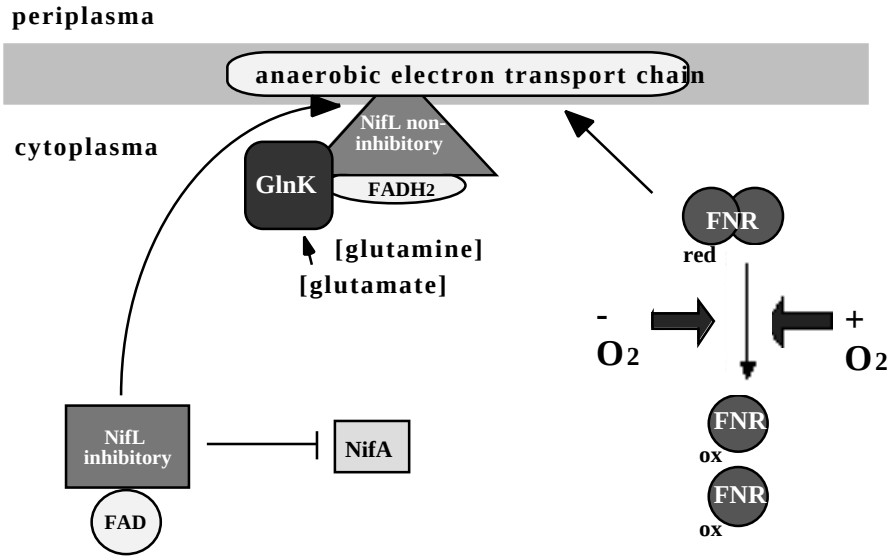


**Figure 20: Model for signal transduction to the NifL/NifA regulatory system.**

The physiological role of GlnK uridylylation and deuridylylation is still unclear since uridylylation of GlnK is not required for relief of NifL inhibition under anaerobic and nitrogen limited conditions (He *et al.*, 1998; Arcondeguy *et al.*, 1999). The finding that GlnK is uridylylated under nitrogen limited conditions, without the uridylylation state having an effect on the NifA activity, rises the question, whether GlnK plays in addition to the *nif* gene regulation a role in nitrogen metabolism, which depends on uridylylation. In contrast to the positive role of GlnK on *nif* induction in *K. pneumoniae*, *A. vinelandii* GlnK apparently promotes the formation of the inhibitory NifL/NifA complex under nitrogen excess conditions, when GlnK is deuridylylated (Little *et al.*, 2000; Reyes-Ramirez *et al.* 2000; Arcondeguy *et al.*, 1999).

Further characterization of the differences between the NifL/NifA regulatory systems in *K. pneumoniae* and *A. vinelandii* will lead to a deeper understanding of signal transduction and protein protein interaction. One example is the mentioned discrepancy that in *K. pneumoniae* GlnK is responsible for NifL relief under nitrogen limited conditions, whereas in *A. vinelandii* GlnK promotes NifA inhibition under nitrogen excess conditions. Although

the NifL and GlnK proteins of *K. pneumoniae* and *A. vinelandii* regulate in the opposite way they share high homologies on the genetic and protein level.

The function of more general factors in cell signaling like the chaperone system and proteolytic enzymes has not been investigated thoroughly in respect to nitrogen regulation to date. The degradation of GlnK under nitrogen excess conditions implies the involvement of proteolytic enzymes in regulation of signal transduction. Future experiments addressing those potential functions will deepen the insight in how the two environmental signals oxygen and nitrogen are actually perceived and transduced in an organism like *K. pneumoniae*.

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# *Curriculum vitae*

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