Oxygen control of *nif* gene expression in *Klebsiella pneumoniae* depends on NifL reduction at the cytoplasmic membrane by electrons derived from the reduced quinone pool

Roman Grabbe and Ruth A. Schmitz

Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen, Germany

In *Klebsiella pneumoniae*, the flavoprotein, NifL regulates NifA mediated transcriptional activation of the N_2-fixation (*nif*) genes in response to molecular O_2 and ammonium. We investigated the influence of membrane-bound oxidoreductases on *nif*-regulation by biochemical analysis of purified NifL and by monitoring NifA-mediated expression of *nifH*ʹ-ʹ*lacZ* reporter fusions in different mutant backgrounds. NifL-bound FAD-cofactor was reduced by NADH only in the presence of a redox-mediator or inside-out vesicles derived from anaerobically grown *K. pneumoniae* cells, indicating that *in vivo* NifL is reduced by electrons derived from membrane-bound oxidoreductases of the anaerobic respiratory chain. This mechanism is further supported by three lines of evidence: First, *K. pneumoniae* strains carrying null mutations of *fdnG* or *nuoCD* showed significantly reduced *nif*-induction under derepressing conditions, indicating that NifL inhibition of NifA was not relieved in the absence of formate dehydrogenase-N or NADH:ubiquinone oxidoreductase. The same effect was observed in a heterologous *Escherichia coli* system carrying a *ndh* null allele (coding for NADH dehydrogenaseII). Second, studying *nif*-induction in *K. pneumoniae* revealed that during anaerobic growth in glycerol, under nitrogen-limitation, the presence of the terminal electron acceptor nitrate resulted in a significant decrease of *nif*-induction. The final line of evidence is that reduced quinone derivatives, dimethylnaphthoquinol and menadiol, are able to transfer electrons to the FAD-moiety of purified NifL. On the basis of these data, we postulate that under anaerobic and nitrogen-limited conditions, NifL inhibition of NifA activity is relieved by reduction of the FAD-cofactor by electrons derived from the reduced quinone pool, generated by anaerobic respiration, that favours membrane association of NifL. We further hypothesize that the quinol/quinone ratio is important for providing the signal to NifL.

Keywords: *Klebsiella pneumoniae*; nitrogen fixation; NifL; FNR; quinol/quinone ratio.

In the free-living diazotrophs, *Klebsiella pneumoniae* and *Azotobacter vinelandii*, members of the γ-subgroup of proteobacteria, N_2-fixation is controlled tightly to avoid unnecessary consumption of energy. The transcriptional activator, NifA and the inhibitor, NifL regulate the transcription of the N_2-fixation (*nif*) operons according to the environmental signals, ammonium and O_2 (reviewed in [1,2]). Under O_2 and nitrogen-limitation, the inhibitor, NifL stays in a noninhibitory conformation and *nif*-gene expression is activated by NifA. In the presence of O_2 or ammonium, NifL antagonizes the activity of NifA resulting in a decrease of *nif*-gene expression. In *K. pneumoniae*, the translationally coupled synthesis of *nifL* and *nifA*, in addition to evidence from immunological studies of complex formation, imply that the inhibition of NifA activity by NifL occurs via a direct protein–protein interaction [3,4]. For the diazotroph *A. vinelandii*, the formation of NifL–NifA complexes has been demonstrated recently by *in vitro* cochromatography and by using the yeast two-hybrid system [5–7]. Recent studies revealed that the nitrogen signal in *K. pneumoniae* and *A. vinelandii* act on the downstream regulatory proteins, NifL and NifA, via the GlnK protein (a paralogue PII-protein). However, the mechanism appears to be opposite in *K. pneumoniae* and *A. vinelandii*. In *K. pneumoniae*, relief of NifL inhibition under nitrogen-limiting conditions depends on the presence of GlnK, the uridylylation state of which appears not to be essential for its nitrogen signaling function [8–11]. However, it is currently not known whether GlnK interacts with NifL or NifA alone, or affects the NifL/NifA-complex. In contrast to *K. pneumoniae*, nonuridylylated GlnK protein appears to activate the inhibitory function of *A. vinelandii* NifL under nitrogen excess, whereas under nitrogen-limitation the inhibitory activity of NifL is apparently relieved by elevated levels of 2-oxoglutarate [12,13]. Interactions between *A. vinelandii* GlnK and NifL were demonstrated recently using the yeast two-hybrid system and *in vitro* studies further indicated that

Correspondence to R. A. Schmitz, Institut für Mikrobiologie und Genetik, Georg-August Universität Göttingen, Göttingen, Germany.
Fax: + 49 551 393808, Tel.: + 49 551 393796,
E-mail: rschmit@gwdg.de

the nonuridylylated form of *A. vinelandii* GlnK interacts directly with NifL and prevents *nif*-gene expression [14,15].

For the O_2-signaling pathway, it was shown that *A. vinelandii* NifL and *K. pneumoniae* NifL act as redox-sensitive regulatory proteins. NifL appears to modulate NifA activity in response to the redox-state of its N-terminal bound FAD-cofactor, and only allows NifA activity in the absence of O_2, when the flavin cofactor is reduced [16–19]. Thus, under anaerobic conditions in the absence of ammonium, reduction of the flavin moiety of NifL is required to relieve NifL inhibition of NifA. Recently, we have demonstrated that in *K. pneumoniae* the global regulator, FNR is required to mediate the signal of anaerobiosis to NifL [20]. We proposed that in the absence of O_2, the primary O_2 sensor, FNR, activates transcription of gene(s) the product(s) of which reduce the NifL-bound FAD-cofactor. Localization analyses under various growth conditions further showed that NifL is highly membrane-associated under depressing conditions, thus, impairing the inhibition of cytoplasmic NifA [21]. Upon a shift to aerobic conditions or nitrogen sufficiency, however, NifL dissociates into the cytoplasm [21]. This indicates that sequestration of NifL to the cytoplasmic membrane under anaerobic and nitrogen-limited conditions appears to be the mechanism for regulation of NifA activity by NifL. Based on these findings, the question arises whether NifL reduction occurs at the cytoplasmic membrane by an oxidoreductase of the anaerobic respiratory chain and favours membrane association of NifL.

In order to verify this hypothesis and to identify the electron donor – potentially localized in the cytoplasmic membrane – we (a) studied *in vitro* reduction of purified NifL using artificial electron donors or NADH and (b) analyzed the effect on *nif*-regulation of different membrane-bound oxidoreductases of the anaerobic respiratory chain and of terminal electron acceptors under fermentative growth conditions. Unexpectedly, during these studies we revealed that the anaerobic metabolism in *E. coli* and *K. pneumoniae* differ significantly in various aspects.

Materials and methods

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid DNA was transformed into *E. coli* cells according to the method of Inoue *et al.* [22] and into *K. pneumoniae* cells by electroporation. Transduction by phage P1 was performed as described previously [23].

E. coli strains

E. coli NCM1529, containing a chromosomal *nifH'-lacZ'* fusion [24], was chosen to study NifA and NifL regulation in *E. coli*. The *ndh*II::*tet* allele and *frdABCD::tet* allele were transferred from ANN001 (T. Friedrich, unpublished observation) and from JI222 [25], respectively, into NCM1529 by P1-mediated transduction with selection for tetracycline resistance, resulting in RAS50.

K. pneumoniae strains

K. pneumoniae strain, M5al (wild- type, N_2-fixing) and strain, UN4495 [φ(*nifK-lacZ*)5935 Δ*lac-4001 hi D4226*

Gal^r] [26] were provided by G. Roberts (Madison, Wisconsin, USA). The spontaneous streptomycin resistant UN4495 strain, RAS46, carrying a *rpsL* mutation was isolated by plating UN4495 on a Luria–Bertani (LB) agar plate containing 100 µg streptomycin per ml. *K. pneumoniae* ssp. *pneumoniae* (DSM no. 4799, not N_2-fixing) and *K. oxytoca* (DSM no. 4798, not N_2-fixing) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

In general, mutant strains of the streptomycin resistant *K. pneumoniae* UN4495 strain (RAS46) were constructed using the allelic exchange system developed by Skorupsky and Taylor [27]. The respective genes were cloned by PCR-techniques, a tetracycline-resistance cassette (derived from the MiniTn5 [28]) was inserted and the resulting interrupted genes were cloned into the suicide vector, pKAS46. These constructs were then introduced into the chromosome of RAS46 by side-specific recombination. The respective chromosomal mutations were confirmed by PCR and Southern blot analysis [29]. For generation of homologous primers for PCR amplification, sequence information for genes of *K. pneumoniae* MG478578 (ssp. *pneumoniae*, not N_2-fixing) was obtained from the database of the Genome Sequencing Center, Washington University, St. Louis (Genome Sequencing Center, personal communication) and using the database, ERGO (Integrated Genomics, Inc.; http://www.integratedgenomics.com).

nuoCD mutant. RAS47 was constructed as follows (a) a 1.6-kb fragment carrying the *nuoCD* genes of *K. pneumoniae* M5a1 was amplified by PCR using primers with additional synthetic restriction recognition sites (lower case letters) nuoC/D ERI (5′-CAGCGCgaattcTCGCCGGCA-3′) and primer nuoC/D *Hin*dIII (5′-CTGCTGaagcttG CGCAGACTCTG-3′) and cloned into pBluescript SK$^+$ producing pRS191; (b) a 2.2-kb fragment containing the tetracycline resistance cassette [28] was inserted into the *Eco*RV site of *nuoCD* gene region in pRS191 yielding pRS194; (c) the 3.8-kb *Eco*RI/*Kpn*I fragment of pRS191 carrying the interrupted *nuoCD* region was transferred into the allelic exchange vector, pKAS46 [27] creating plasmid, pRS197; (d) pRS197 was transformed into RAS46 and recombinant strains carrying the chromosomally inserted plasmid (by means of single homologous recombination) were identified by their resistance to tetracycline and their inability to grow on streptomycin agar plates as a consequence of the plasmid encoded *rpsL* mutation. Overnight selection in liquid LB medium containing 400 µg streptomycin per ml and subsequent selection of single colonies onto plates resulted in loss of the integrated plasmid with an integration frequency of the interrupted *nuoCD* region in 50% of the integrants.

fdnG mutant. Primer, fdnG 5′-*Eco*RI (5′-CCGACTGAT gaattcCGACCGCGA-3′) and primer fdnG 3′-*Hin*dIII (5′-GCCGAGCAGaagcttGATCATCGC-3′) were used to clone a 1-kb *fdnG* fragment from *K. pneumoniae* M5a1 into pBSK$^+$ vector creating pRS167, followed by insertion of the tetracycline resistance cassette into the *Eco*RV site of *fdnG* fragment resulting in pRS177. The 3.2-kb *Eco*RI/*Kpn*I fragment of pRS177 including the *fdnG::tet* region was cloned into pKAS46. The construction of the

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype	Source/reference
K. pneumoniae		
M5a1	Wild-type	[26]
UN4495	φ (*nifK-lacZ*)5935 Δ*lac-4001 hi D4226 Gal*ʳ	[26]
subs. *pneumoniae*	Wild-type, not N_2-fixing	DSM number 4799
K. oxytoca	Wild-type, not N_2-fixing	DSM number 4798
RAS18	UN4495, but *fnr*::Ω	[20]
RAS46	UN4495, but spontaneous streptomycin resistance	This study
RAS47	UN4495, but *nuoCD*::tet	This study
RAS48	UN4495, but *fdnG*::tet	This study
E. coli		
NCM1529	*araD139Δ(argF-lacU)169 fthD5301 gyrA219 non9 rspL150 ptsF25 0 relA1 deoC1 trpDC700putPA1303*::[Kanʳ-(*nifH-lacZ*)] (Wild-type)	[8]
NCM1528	NCM1529/pNH3	[8]
NCM1527	NCM1529/pJES851	[8]
RAS50	NCM1529, but *ndh*::tet	This study
RAS51	RAS50/pNH3	This study
RAS52	RAS50/pJES851	This study
RAS53	NCM1529, but *frd*::tet	This study
RAS54	RAS53/pNH3	This study
RAS55	RAS53/pJES851	This study
Plasmids		
pBSK⁺	cloning vector	Stratagene
pCR 2.1	Topo-TA cloning vector	Invitrogen
pKAS46	allelic exchange vector, *ori*R6K; *rpsL**(Strepˢ), Ampʳ, Kanʳ	[27]
pNH3	*K. pneumoniae nifLA* under the control of the *tac* promoter	[4]
pJES851	*K. pneumoniae nifA* under the control of *tac* promoter	[30]
pJES794	*K. pneumoniae malE-nifL* under the control of the *tac* promoter	[31]
pRS167	*Eco*RI/*Hind*III *fdnG* fragment (*K. pneumoniae* M5a1) in pBSK⁺	This study
pRS177	pRS167, but *fdnG*::tet	This study
pRS187	*frdA* fragment (*K. pneumoniae* M5a1) in pCR2.1	This study
pRS191	*Eco*RI/*Hind*III *nuoCD* fragment (*K. pneumoniae* M5a1) in pBSK⁺	This study
pRS193	*fdnG*::tet fragment from pRS177 in pKAS46	This study
pRS194	pRS191, but *nuoCD*::tet	This study
pRS197	*nuoCD*::tet fragment from pRS194 in pKAS46	This study

chromosomal mutant was performed as described above, yielding RAS48.

Growth conditions

In *E. coli* and *K. pneumoniae* strains carrying chromosomal *nifH′-lacZ′* fusions, *nif* gene expression (synthesis of nitrogenase) can be monitored by NifA activity. Cultures were grown anaerobically with N_2 as the gas phase at 30 °C in minimal medium, supplemented with 4 mM glutamine as the sole (limiting) nitrogen source, that allows full induction of *nif* gene expression as shown recently [17,20,30]. The medium was further supplemented with 10 mM Na_2CO_3, 0.3 mM sulfide and 0.002% resazurin to monitor anaerobiosis, and 0.4% sucrose plus 0.004% histidine for *K. pneumoniae* strains and 1% glucose plus 0.002% tryptophane for *E. coli* strains. Precultures were grown overnight in closed bottles in the same medium but lacking sulfide and resazurin and with N_2 as the gas phase. Main cultures (25 mL) were inoculated from precultures and incubated under a N_2 atmosphere and strictly anaerobic conditions without shaking. Anaerobic samples were taken for monitoring of growth at 600 nm and β-galactosidase activity determined.

In *E. coli* strains carrying a plasmid encoding NifL and NifA (pNH3) or NifA alone (pJES851) expression of *nifLA* or *nifA* from the *tac* promoter was induced by the addition of 10 μM isopropyl-β-D-thiogalactopyranoside (IPTG).

β-Galactosidase assay

NifA-mediated activation of transcription from the *nifHDK* promoter in *K. pneumoniae* UN4495 and *E. coli* strains was monitored by measuring the differential rate of β-galactosidase synthesis during exponential growth (units per ml per cell turbidity at 600 nm [D_{600}] [30]). Inhibitory effects of NifL on NifA activity in response to ammonium and O_2 were assessed by virtue of a decrease in *nifH* expression.

Purification of MBP-NifL

The fusion protein between maltose binding protein (MBP) and NifL was synthesized in NCM1529, carrying the plasmid pJES794 [31], grown aerobically at 30 °C in maximal induction medium [32] supplemented with 0.5 mM riboflavin. Expression of the fusion protein was induced with 100 μM IPTG when cultures reached a

turbidity of 0.6 at 600 nm. After harvesting and disruption in B buffer (20 mM Epps (*N*-[2-hydroxyethyl]piperazine-*N'*-3-propanesulfonic acid), 125 mM potassium glutamate, 5% glycerol, 1.5 mM dithiothreitol, pH 8.0) using a French pressure cell, cell debris was sedimented by centrifugation at 20 000 *g* for 30 min and the fusion protein was purified from the supernatant by amylose affinity chromatography. All purification steps were performed at 4 °C in the dark, preventing degradation of the FAD moiety. The purified protein was dialyzed overnight into B buffer containing 25 mM potassium glutamate and used subsequently for biochemical analysis. The amount of FAD cofactor of the NifL fractions was calculated using a UV/Visual spectrum at 450 nm and the extinction coefficient $\in_{450} = 11.3$ mM^{-1}·cm^{-1} [33] and was in the range of 0.4–0.6 mol FAD per mol MBP-NifL.

Spectral analysis of purified MBP-NifL

Purified MBP-NifL was reduced at room temperature under a N_2 atmosphere in the presence of NADH and methyl viologen. The standard 0.2 mL assay was performed in B buffer (25 mM potassium glutamate, pH 8.0) under a N_2 atmosphere using 10–40 μM MBP-NifL. Reduction of fully oxidized MBP-NifL was followed using a spectrophotometer with an integrated diode array detector (J & M Analytische Meβ- und Regeltechnik, Aalen, Germany). NADH (final concentration, 1.25 mM) was used as a reductant in the presence of 0.2 μM methyl viologen or inverted vesicles (10 mg·mL^{-1}) derived from *K. pneumoniae* cells grown under anaerobic conditions and nitrogen-limitation. Reduced soluble quinone derivatives, dimethyl-naphthoquinol (DMNH$_2$) and menadiol (MDH$_2$) (0.12 mM final concentration), were used in the absence of a redox mediator. Stock solutions of DMN and MD were prepared in ethanol. After dilution into anaerobic B buffer containing 25 mM potassium glutamate, DMN and MD were reduced by molecular hydrogen in the gas phase in the presence of platin oxide and the reduction was confirmed by monitoring the changes in absorbance at 270 and 290 nm (DMN) or 280 and 320 nm (MD).

Preparation of inside-out vesicles of *K. pneumoniae*

One litre cultures of *K. pneumoniae* cells were grown under nitrogen- and O_2-limited conditions, harvested at a D_{600} value of 1.3 and vesicles were prepared according to Krebs *et al.* [34] – that favours the formation of inside-out vesicles – with the exception that we added diisopropyl fluorophosphate to the vesicle buffer to inhibit proteases (J. Steuber, ETH, Zürich, Switzerland, personal communication). All manipulations were performed under exclusion of O_2 in an anaerobic cabinet at 4 °C. The inverted vesicle preparations were either used for the reduction of MBP-NifL or stored at −70 °C. Generally, inside-out vesicles were found preferentially when analyzed by electron microscopy.

Determination of NADH:ubiquinone oxidoreductase activity

The enzyme activity of the NADH:ubiquinone oxidoreductase in cell extracts prepared under anaerobic conditions was determined as described by Friedrich *et al.* [35] using ferricyanide as an electron acceptor. The assay contained vesicle buffer (10 mM Tris/HCl pH 7.5, 50 mM KCl, 2 mM dithiothreitol), 0.3 mM NADH and 0.2 mM potassium ferricyanide. The reaction was started with the addition of cell extract and the decrease of the A_{410} value reflecting reduction of ferricyanide by NADH was monitored.

Southern blot analysis

Southern blots were performed as described by Sambrook *et al.* [29], hybridization with DIG-labeled probes and chemiluminescent detection using CSPD was carried out according to the protocol of the manufacturer (Boehringer, Germany). In order to identify potential *ndh* genes in *Klebsiella* strains, Southern blot analysis was performed using a *ndh* probe derived from *K. pneumoniae* ssp. *pneumoniae* and *Sma*I digested chromosomal DNA derived from *K. pneumoniae* M5a1, *K. oxytoca* and *K. pneumoniae* ssp. *pneumoniae* as control.

Western blot analysis

Samples (1 mL) of exponentially growing cultures were harvested and concentrated 20-fold SDS gel-loading buffer [36]. Samples were separated by SDS/PAGE (12% gel) and transferred to nitrocellulose membranes as described previously [29]. Membranes were exposed to polyclonal rabbit antisera directed against the NifL or NifA proteins of *K. pneumoniae*, protein bands were detected with secondary antibodies directed against rabbit IgG and coupled to horseradish peroxidase (Bio-Rad Laboratories). Purified NifA and NifL from *K. pneumoniae* were used as standards.

Membrane preparation

Cytoplasmic and membrane fractions of *K. pneumoniae* UN4495 and mutant derivatives were separated by several centrifugation steps as recently described by Klopprogge *et al.* [21]. The NifL bands of cytoplasmic and membrane fractions were visualized in Western blot analyses using the ECLplus system (Amersham Pharmacia) with a fluoroimager (Storm, Molecular Dynamics). The protein bands were quantified for each growth condition in three independent membrane preparations using the IMAGEQUANT v1.2 software (Molecular Dynamics) and known amounts of the respective purified proteins.

Results

Our goal was to identify the physiological electron donor of *K. pneumoniae* NifL and its localization in the cell. Thus, we studied the reduction of purified MBP–NifL *in vitro* and analyzed the influence of different oxidoreductases of the anaerobic respiratory chain on NifL reduction.

K. pneumoniae NifL is reduced by NADH in the presence of a redox-mediator or anaerobic inside-out vesicles

In general, NifL was synthesized and purified fused to the maltose binding protein (MBP) to keep NifL in a more soluble state. In order to demonstrate whether NADH is a

potential electron donor *in vivo*, reduction of purified MBP–NifL was studied *in vitro* at room temperature.

In the absence of a redox mediator, the FAD cofactor of oxidized MBP–NifL was not reduced by the addition of NADH (data not shown). However, in the presence of methyl viologen, a slow but significant decrease in the flavin-specific A_{450} value was observed (Fig. 1). This indicates that the flavin-moiety of NifL was reduced by electrons derived from NADH with a slow rate, that may be based on the low redox potential of methyl viologen ($E'_0 = -450$ mV). The difference spectrum of oxidized MBP–NifL corrected for the spectrum 50 min after NADH addition clearly showed the flavin-specific absorption maximum at 450 nm and the 420 nm absorbance, that is found generally in reduced NifL synthesized under nitrogen sufficiency [19]. These findings strongly indicate that NADH is a potential electron donor for NifL reduction; however, it appears that *in vivo* the reducing equivalents derived from NADH must be transferred to NifL through an oxidoreductase.

We further analyzed the effect of inside-out vesicles on the reduction state of NifL to obtain evidence for NifL reduction by NADH via a membrane-bound oxidoreductase of the anaerobic respiratory chain *in vivo*. In order to exclude the presence of contaminating redox mediators for those experiments, the cuvettes were washed extensively with chromosulfuric acid and control experiments were performed, in which no significant decrease of the NifL absorbance at 450 nm was observed after the addition of NADH. Inside-out vesicles containing the anaerobic respiratory chain were prepared from anaerobic *K. pneumoniae* cells as described in Materials and methods. Three minutes after the addition of vesicles to the fully oxidized MBP-NifL, a constant, significant decrease at 450 nm for approximately 7 min was detectable, suggesting that NifL was reduced by electrons derived from the reduced mem-
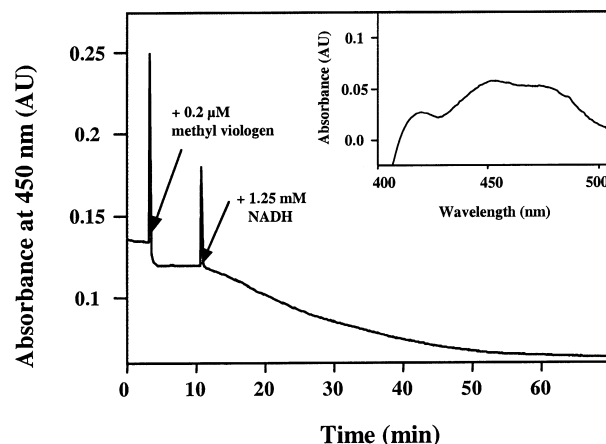
brane-bound oxidoreductases of the inside-out vesicles. The reduction rate then decreased slowly until the unspecific rate of the background decline was again reached (Fig. 2A). Subsequent addition of external NADH to the assay resulted in further reduction of the flavin-specific absorbance at 450 nm (Fig. 2B). These findings suggest that *in vivo*, the NifL-bound FAD cofactor receives reducing equivalents derived from NADH by a component of the anaerobic respiratory chain.
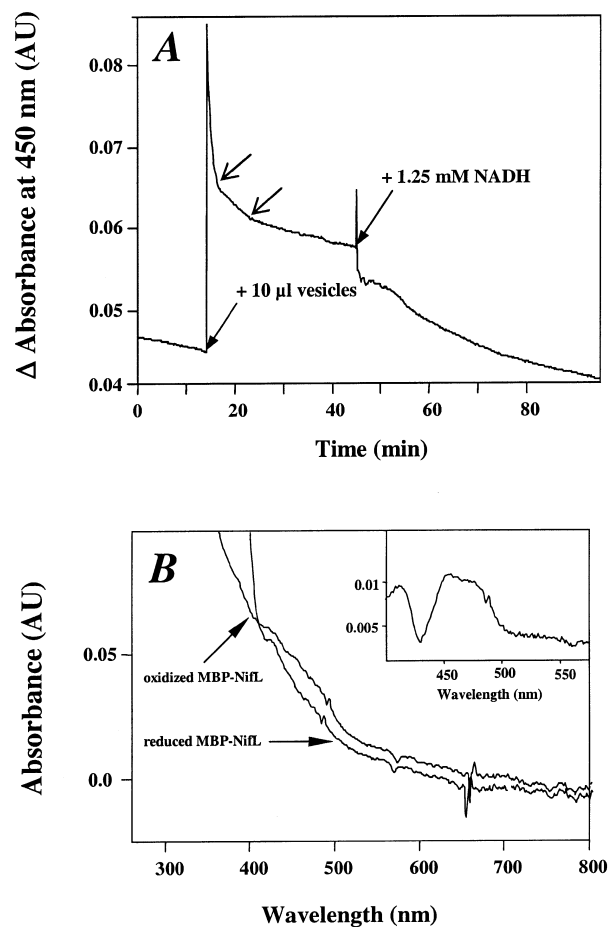

Fig. 2. Reduction of purified MBP-NifL with NADH in the presence of inverted vesicles from *K. pneumoniae*. Purified, fully oxidized MBP-NifL (10 μM) was incubated in an anaerobic cuvette under a N$_2$ atmosphere at 25 °C in a final volume of 400 μL B buffer. Thirty minutes after the addition of 10 μL of inverted vesicles (10 mg·mL^{-1}) of *K. pneumoniae* cells grown under nitrogen-limited and anaerobic conditions, 1.25 mM NADH (final concentration) was added. Changes in absorbance upon the reduction of the flavin cofactor were recorded and monitored using a spectrophotometer with an integrated diode array detector. The absorbance was corrected for the absorbance of B buffer. (A) Time course measurement at 450 nm of the MBP-NifL reduction. The open arrows indicate the time period during which the absorbance decreases due to NifL reduction by electrons derived from the inside-out vesicles. Thirty minutes after the addition of inside-out vesicles, external NADH (1.25 mM) was added. (B) Absorbance spectra of MBP-NifL before (oxidized MBP-NifL) and 45 min after NADH addition (reduced MBP-NifL). The inset shows the corresponding difference spectrum of oxidized MBP-NifL corrected vs. the reduced spectrum.

Fig. 1. Reduction of purified MBP-NifL with NADH in the presence of methyl viologen. Purified, fully oxidized MBP-NifL (40 μM) in B-buffer (pH 8.0) was incubated in an anaerobic cuvette under a N$_2$ atmosphere at 25 °C. After the addition of methyl viologen, to a final concentration of 0.2 μM, the protein was reduced by the addition of 1.25 mM NADH (indicated by arrows). The spectral changes were recorded using a spectrophotometer with an integrated diode array detector and the reduction of the flavin moiety of the protein was monitored at 450 nm. The inset shows the difference spectrum; the fully oxidized spectrum at 10 min was corrected vs. the reduced spectrum at 60 min.

Effects of chromosomal *ndh* and *frd* null mutations on *nif* induction in a heterologous *E. coli* system

In order to obtain further evidence for NifL reduction by a membrane-bound oxidoreductase system, we studied the influence of *E. coli* NADH dehydrogenaseII (encoded by the *ndh*-gene) and fumarate reductase (encoded by the *frd*-operon) on *nif* regulation in a heterologous *E. coli* system. *E. coli* strain NCM1529 carrying a chromosomal *nifH'-lacZ'* fusion was used as the parental strain [24]. The *K. pneumoniae* regulatory proteins NifL and NifA were synthesized from plasmids pNH3 (*nifLA*) or pJES851 (*nifA*) at induction levels at which NifL function in *E. coli* is regulated normally in response to O_2 and ammonium [20,24]. To study the effect of the two oxidoreductases on NifL regulation of NifA, the respective null alleles, *ndh::tet* and *frd::tet,* were introduced by P1 transduction into the parental strain. After introducing *nifLA* and *nifA* into plasmids, the resulting strains were grown anaerobically under nitrogen-limitation with glutamine as the sole nitrogen source. No significant differences in growth rates or in the NifL and NifA expression levels were obtained for the mutant and the respective parental strains (Table 2). Monitoring NifA-dependent transcription of the *nifH'-'lacZ* fusion during exponential growth showed that the *frd* mutation (RAS54) did not affect *nif*-induction (Table 2). In the absence of a functional NADH dehydrogenaseII (RAS51), expression of *nifH'-'lacZ* significantly decreased resulting in a β-galactosidase synthesis rate that is equivalent to 10% of the synthesis rate in the parental strain (NCM1528). However, the *ndh* mutation does not affect NifA activity in the absence of NifL (Table 2, compare RAS52 with NCM1527). These findings suggest that in the absence of NADH dehydrogenaseII, NifL apparently does not receive the signal for anaerobiosis and consequently inhibits the activity of NifA. It further indicates that in the heterologous *E. coli* system, NADH dehydrogenaseII is responsible for NifL reduction under anaerobic conditions, whereas fumarate reductase appears not to be. This is supported by the findings that the addition of 20 mM

fumarate or trimethylamine *N*-oxide (TMAO) as electron acceptors do not influence *nif* induction in the parental strain (NCM1528) under anaerobic and nitrogen-limiting conditions (Table 2) that is consistent with the findings of Pecher *et al.* [37].

NADH:ubiquinone oxidoreductase and formate dehydrogenase-N affect *nif* regulation in *K. pneumoniae*

Southern blot and PCR analyses showed that, in contrast to *E. coli* and *K. pneumoniae* MGH78578 (ssp. *pneumoniae*), the N_2-fixing strains, *K. pneumoniae* M5a1 and *K. oxytoca* do not exhibit a NADH-dehydrogenaseII. Thus, we decided to examine the influence of two other membrane-bound oxidoreductases involved in anaerobic respiration on *nif* regulation in *K. pneumoniae*.

K. pneumoniae strain UN4495 was used as the parental strain that carries *nifLA* and a *nifK'-lacZ'* fusion on the chromosome and thus allows monitoring of NifA-mediated transcription [30]. Two mutant strains were constructed carrying a chromosomal *nuoCD* null allele (encoding for subunits C and D of the coupling NADH:ubiquinone oxidoreductase) or a chromosomal *fdnG* null allele (encoding for the γ-subunit of formate dehydrogenase-N) as described in Materials and methods. The disruptions in the respective mutant strains were confirmed by PCR and Southern blot analysis (data not shown). The anaerobic cell extracts of the *nuoCD* mutant strain (RAS47) showed a very low NADH-oxidation rate compared to the parental strain (< 4%). This further shows that *K. pneumoniae* M5a1 does not exhibit a NADH-dehydrogenaseII, as the residual NADH-oxidation rate of an *E. coli nuo* mutant strain is equivalent to 20% of the NADH-oxidation rate in the parent strain and is based on the activity of NADH-dehydrogenaseII (this paper and [38]).

The mutant strains were grown in minimal medium under anoxic conditions with glutamine as the limiting nitrogen source. In the absence of NADH:ubiquinone oxidoreductase or formate dehydrogenase-N the doubling

Table 2. Effects of chromosomal *ndh* and *frd* null mutations and external electron acceptors on NifA activity in the heterologous *E. coli* system carrying *K. pneumoniae nifLA* or *nifA* on a plasmid. Cultures were grown at 30 °C under nitrogen-limited and anaerobic conditions and expression of NifL and NifA was induced from the *tac* promoter (Ptac) with 10 μM IPTG. Expression of *nifH'-lacZ'* was monitored by the determination of the β-galactosidase synthesis rates as described [30]. Data presented represent mean values of at least three independent experiments (± SEM).

Strain	Relevant genotype/ electron acceptors	Expression of *nifH'-lacZ'* (U·min^{-1} $D_{600}$$^{-1}$)	Doubling time (h)
NCM1528	Wild-type/Ptac-*nifLA*	3000 ± 100	5.0
NCM1527	Wild-type/Ptac-*nifA*	5000 ± 200	4.8
RAS51[a]	*ndh*/Ptac-*nifLA*	300 ± 20	5.5
RAS52[a]	*ndh*/Ptac-*nifA*	4500 ± 150	5.2
RAS54[b]	*frd*/Ptac-*nifLA*	3500 ± 100	5.0
RAS55[b]	*frd*/Ptac-*nifA*	5400 ± 150	4.9
NCM1528	Wild-type/Ptac-*nifLA*	3000 ± 100	5.0
NCM1528	20 mM fumarate	3200 ± 100	5.5
NCM1528	20 mM TMA	2950 ± 150	5.1

[a] Strain contains the *ndh::tet* allele from ANN001 (T. Friedrich, unpublished results). [b] Strain contains the *frdABCD::tet* allele from JI222 [25].

time increased ($td = 5$ h) compared to the parental strain ($td = 3.5$ h). This decrease in growth rate under anoxic conditions is apparently based on the reduced anaerobic respiration and increased fermentative recycling of NAD^+ from NADH that results in lower ATP yields per saccharose unit and in a change of the quinol/quinone ratio. Unexpectedly, both the *nuoCD* and the *fdnG* mutation affected *nif* induction and showed significantly reduced levels of β-galactosidase synthesis rates under depressing conditions (Fig. 3), though the amounts of NifL and NifA did not change compared to the parental strain. The *nif* induction determined for the *fdnG* mutant strain (RAS48) was in the range of 800 ± 50 $U \cdot mL^{-1} \cdot D_{600}^{-1}$. *nif* induction in the *nuo* mutant strain (RAS47) decreased to levels of ≈ 60 $U \cdot mL^{-1} \cdot D_{600}^{-1}$ (Fig. 3), that indicates that the main part of NifL protein is in the oxidized cytoplasmic conformation. This dramatic effect on *nif* induction in a *nuo* mutant strain was unexpected, as one would expect that formate dehydrogenase-N is present in the *nuo* mutant strain and capable of donating electrons to NifL. However, the absence of NADH:ubiquinone oxidoreductase might have an indirect effect on formate dehydrogenase-N.

Analysis of NifL localization under derepressing conditions confirmed the observed *nif⁻* phenotype of both mutant strains. In contrast to the parental strain, NifL was found mainly in the cytoplasmic fraction of the *fdnG*- and the *nuoCD* mutant strain ($83 \pm 5\%$ of total NifL). This suggests that in both mutant strains, NifL was not reduced and remained in its oxidized conformation in the cytoplasm; this is consistent with the observed significant reduction of *nif* induction. Taken together, these findings indicate strongly that the quinol/quinone ratio appears to be important for providing the electrons for NifL reduction (see Discussion).

Effects of terminal electron acceptors on *nif* regulation in *K. pneumoniae*

In order to obtain additional evidence that NifL receives electrons from the reduced quinone pool at the cytoplasmic membrane depending on the quinol/quinone ratio, we studied *nif* induction with glycerol and in the presence of external terminal electron acceptors.

Cultures of *K. pneumoniae* UN4495 were grown under anaerobic conditions with glutamine as the nitrogen-limiting source, and sucrose, glucose or glycerol as carbon and energy sources. In contrast to *E. coli*, *K. pneumoniae* is able to grow with glycerol under anaerobic conditions in the absence of external electron acceptors with reduced growth rates compared to growth with glucose (Table 3) [39]. When growing with glycerol, *nif* induction was significantly reduced and was equivalent to 25% of the induction level obtained with sucrose (Table 3). As we assayed *nif* induction by determining the rates of β-galactosidase synthesis, the calculated induction levels are normalized for the differences in growth rates. Thus, the observed reduction in *nif* induction when growing with glycerol appears to be based on the altered quinol/quinone ratio resulting from the change from respiratory to fermentous conditions.

When *Klebsiella* cells were growing with sucrose or glucose, supplementing the medium with the terminal electron acceptors nitrate or fumarate neither influenced
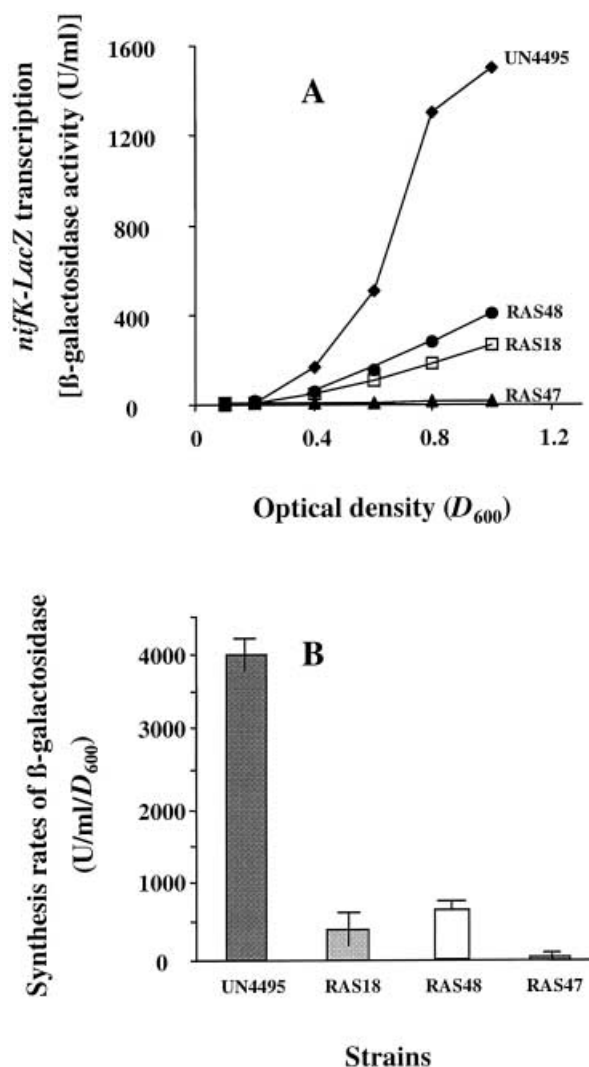

Fig. 3. Effects of chromosomal deletions in gene clusters encoding NADH:ubiquinone oxidoreductase (*nuo*) and formate dehydrogenase-N (*fdn*) on NifA activity in *K. pneumoniae* UN4495. NifA-mediated activation of transcription from the *nifHDK*-promoter in *K. pneumoniae* UN4495 and mutant derivatives was monitored by measuring the β-galactosidase activity during anaerobic growth at 30 °C in minimal medium, with glutamine (4 mM) as the limiting nitrogen source. Activities of β-galactosidase were plotted as a function of D_{600} for *K. pneumoniae* UN4495 (wild-type), the *fnr* mutant strain of UN4495 (RAS18), the *fdnG* mutant strain of UN4495 (RAS48) and the *nuoCD* mutant strain of UN4495 (RAS47) carrying a chromosomal *nifK'-'lacZ* fusion (A). Synthesis rates of β-galactosidase from the *nifHDK* promoter were determined from the slope of these plots from at least five independent experiments and are presented as bars ($\pm$SEM) reflecting *nif*-induction in the respective *K. pneumoniae* strains (B).

the growth rate – as has been also reported for *E. coli* growing in glucose [40] – nor affected *nif* induction (Table 3). The finding that *nif* induction is not affected by nitrate indicates that the presence of nitrate *per se*, that might also potentially serve as an alternative nitrogen source, does not repress *nif* induction. This is further supported by the analysis of the internal glutamine and glutamate pools in

Table 3. Effects of additional electron acceptors on the *nif* induction in *K. pneumoniae* using different carbon and energy sources. Cultures were grown at 30 °C under nitrogen-limited and anaerobic conditions with 0.4% sucrose, 0.8% glucose or 1% glycerol, respectively. Expression of *nifH'-'lacZ* was monitored by the determination of the β-galactosidase synthesis rates as described recently [30]. Data presented represent mean values of at least three independent experiments (± SEM).

Carbon and energy source	Additional electron acceptor (20 mM)	β-galactosidase activity (U·mL^{-1}·$D_{600}$$^{-1}$)	Doubling time (h)
Sucrose	–	4000 ± 100	3.5
Sucrose	Fumarate	4100 ± 150	3.5
Sucrose	Nitrate	3900 ± 150	3.5
Glucose	–	3000 ± 90	3.5
Glucose	Fumarate	2850 ± 85	3.5
Glucose	Nitrate	3100 ± 90	3.5
Glycerol	–	1000 ± 40	5.5
Glycerol	Fumarate	1100 ± 60	5.7
Glycerol	TMAO	980 ± 50	5.4
Glycerol	Nitrate	200 ± 20	5.5

K. pneumoniae that showed that in the presence of nitrate under nitrogen-limitation, the glumatine pool is decreased to the same amount as it is in the case for nitrogen-limiting growth conditions [41] (R. A. Schmitz, unpublished results). However, when growing with glycerol, the addition of nitrate as a terminal electron acceptor resulted in a significant decrease of *nif* induction (200 ± 20 U·mL^{-1}· $D_{600}$$^{-1}$) as compared to cells growing with glycerol in the absence of nitrate (1000 ± 40 U·mL^{-1} ·$D_{600}$$^{-1}$) (Table 3). This is consistent with early reports on negative effects of nitrate on nitrogenase synthesis, when growing with glycerol, an effect, that is not observed for nitrate reductase mutants [37,42]. Taken together, these findings indicate that growing anaerobically with glycerol in the presence of nitrate, electrons from the reduced quinone pool are transferred preferentially onto nitrate via respiratory nitrate reductase to obtain higher energy yields; this in turn changes the quinol/quinone ratio even more dramatically than anaerobic growth with glycerol in the absence of nitrate. It appears that this quinol/quinone ratio does not allow NifL reduction by the quinone pool, resulting in high amounts of oxidized cytoplasmic NifL and thus in the inhibition of NifA activity. Fumarate or TMAO respiration do not apparently change the quinol/quinone ratio to the same amount, as no effect on *nif* induction was observed when fumarate or TMAO were used as terminal electron acceptor (Table 3). This is consistent with the findings of Pecher *et al.* [37] and indicates that the repressive effect of an electron acceptor depends on the size of its redox potential [E′$_0$(TMAO$_{ox}$/TMAO$_{red}$) = + 130 mV, E′$_0$(fumarate/succinate) = + 30 mV, E′$_0$(NO$_3$$^-$/NO$_2$$^-$) = + 420 mV, reviewed in [43]).

Reduced soluble quinone derivatives are able to reduce the flavin cofactor of MBP-NifL

In order to obtain additional evidence that under depressing conditions NifL receives electrons from the reduced quinone pool, we examined *in vitro* whether reduced soluble quinone

derivatives can transfer electrons onto NifL. Dimethylnaphthoquinone (DMN) and menadione (MD) were reduced with molecular H$_2$ in the presence of platin oxide. After the addition of DMNH$_2$ to oxidized MBP-NifL in the absence of a redox mediator, the flavin specific absorbance at 450 nm decreased significantly, indicating that electrons were transferred from DMNH$_2$ to the FAD-cofactor of NifL (Fig. 4A). The reduction of NifL-bound FAD by a quinol derivative was confirmed using menadiol that also resulted in reduction of the flavin cofactor (Fig. 4B). The finding that DMNH$_2$ (E′$_0$ = −80 mV [44]); and MDH$_2$ (E′$_0$ = −1 mV [44]); transfer electrons onto NifL-bound FAD further supports our model that *in vivo* NifL is reduced at the cytoplasmic membrane and receives electrons from the quinone pool.
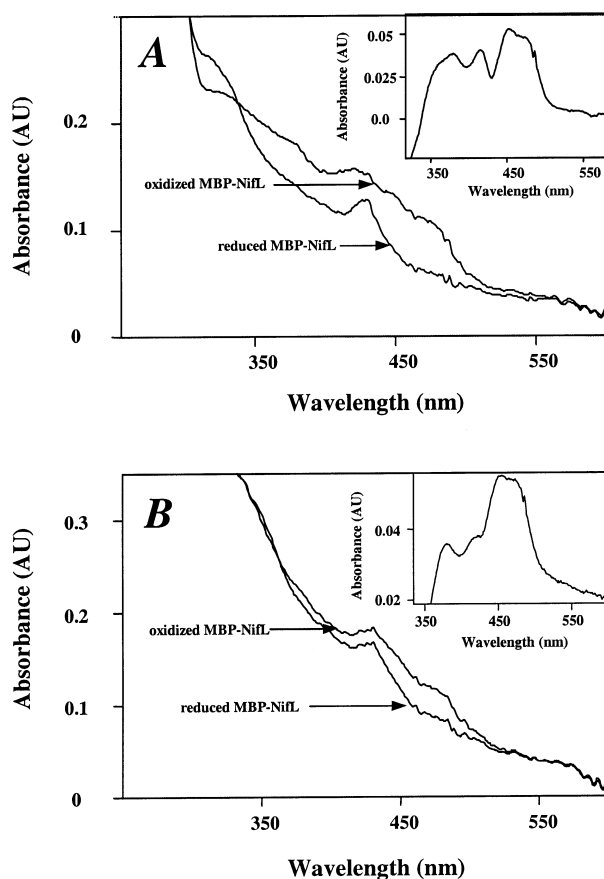

Fig. 4. Reduction of MBP-NifL using reduced dimethylnaphthoquinone or menadione as artificial electron donors. Fully oxidized MBP-NifL (40 μM) was incubated in B buffer under a N$_2$ atmosphere at room temperature. Dimethylnaphthoquinol (DMNH$_2$) (A) or menadiol (MDH$_2$) (B) were added to a final concentration of 120 μM or 100 μM, respectively, and the changes in absorbance were recorded using a spectrophotometer with an integrated diode array detector. Absorbance spectra of MBP-NifL before (oxidized MBP-NifL) and 60 min after the addition of the reduced quinone derivatives (reduced MBP-NifL) are shown. The corresponding difference spectrum of oxidized MBP-NifL corrected vs. the reduced spectrum after addition of DMN$_{red}$ is visualized in the insets, respectively.

Discussion

In *K. pneumoniae* the NifL-bound FAD receives electrons from the reduced quinone pool at the cytoplasmic membrane under depressing conditions

In order to verify that in our model the FAD cofactor of NifL is reduced by electrons derived from the reduced quinone pool resulting in a conformation of NifL that stays membrane-associated, we studied the process of NifL reduction. First-line evidence was provided by biochemical analyses of the purified MBP-NifL protein. Spectral analysis showed clearly that NifL reduction by NADH only occurs in the presence of a redox mediator or inside-out vesicles derived from *K. pneumoniae* cells grown under anaerobic conditions and thus containing the anaerobic respiratory chain (Figs 1 and 2). Three other lines of evidence derived from *in vivo* and *in vitro* studies of *nif* regulation further supported our model: first, analysis of mutant strains indicated that the absence of formate dehydrogenase-N or NADH:ubiquinone oxidoreductase in *K. pneumoniae* and the absence of NADH dehydrogenaseII in the heterologous *E. coli* system affect *nif* regulation significantly. In the absence of the respective membrane-bound oxidoreductases, *nif* induction was low under depressing conditions (Table 2 and Fig. 3). This indicates clearly that the majority of the flavoprotein NifL in the mutant strains was not reduced at the cytoplasmic membrane resulting in high amounts of cytoplasmic NifL and thus in significant inhibition of NifA in the cytoplasm. Localization analysis of NifL in the *K. pneumoniae nuoCD*- and *fdnG*-mutant strains confirmed that under depressing conditions, NifL was indeed found mainly in the cytoplasmic fraction. Second, studies of *nif* induction in *K. pneumoniae* grown anaerobically with glycerol under nitrogen-limitation revealed that the presence of nitrate as a terminal electron acceptor resulted in a significant decrease in *nif* induction (Table 3). This negative effect of nitrate on synthesis of nitrogenase when grown anaerobically with glycerol has been reported earlier by Böck and coworkers [37]. As no *nif* repression was obtained in chlorate resistant mutants that do not respire in the presence of nitrate, it is nitrate respiration, rather than nitrate *per se*, that abolishes *nif* expression [37,42]. It appears that during anaerobic growth with glycerol, electrons of the quinone pool are transferred preferentially onto nitrate [$E'_0(NO_3^-/NO_2^-) = 420$ mV], allowing energy conservation by the respiratory nitrate reductase [45] (reviewed in [46]). Thus, during the unfavourable ratio between quinone reduction and quinol oxidation a high percentage of NifL protein does not receive electrons from the reduced quinone pool, and consequently remains in its oxidized conformation in the cytoplasm and thereby inhibits NifA activity. Third, we demonstrated that the reduced soluble quinone derivatives, dimethylnaphthoquinol (DMNH$_2$) and menadiol (MDH$_2$) are able to reduce the FAD cofactor of purified NifL in the absence of a redox mediator (Fig. 4). Taken together, these data indicate strongly that under anaerobic conditions and at a favourable quinol/quinone ratio, the FAD-cofactor of NifL receives electrons from the reduced quinone pool generated by different membrane-bound oxidoreductases of the anaerobic respiratory chain. As the most hydrophobic regions of NifL-protein are located in the N-terminal domain [31] that binds the FAD-cofactor [17], one can speculate that the N-terminal domain of NifL might enter the lipid bilayer and contact the quinones dissolved within the bilayer of the cytoplasmic membrane. The reduction of NifL by electrons derived from the quinone pool, rather than by a single specific membrane-bound enzyme is a particularly attractive model as it explains that NADH dehydrogenaseII in the heterologous *E. coli* system significantly effects *nif* regulation, although a homologous oxidoreductase does not appear to be present in *K. pneumoniae*. Potentially, it further allows for the simultaneous signal integration of the cell's energy status for *nif* regulation.

In contrast to *K. pneumoniae* NifL, no membrane association for *A. vinelandii* NifL has been reported to date [1,16,47]. In *in vitro* experiments, *A. vinelandii* NifL is reduced by NADH when catalyzed by the *E. coli* cytoplasmic flavoheme protein (HMP). However, the functional and physiological relevance of NifL reduction by HMP, that is proposed to be a global O$_2$ sensor, or an oxidoreductase, preventing cells from endogenous O$_2$ stress, has not been demonstrated *in vivo* [18,48,49]. It is hypothesized currently that the reduction of *A. vinelandii* NifL occurs nonspecifically and is dependent on the availability of reducing equivalents in the cell [1,18].

The anaerobic metabolism of the N$_2$-fixing *K. pneumoniae* M5a1 and *E. coli* differ in some aspects

Interestingly, the significant effect of *fdnG* on *nif* induction in *K. pneumoniae* M5a1 was observed in the absence of nitrate. This indicates that in *K. pneumoniae* M5a1, a basal induction of the *fdn*-operon occurs even in the absence of nitrate; this is in contrast to the *E. coli* system [50,51]. However, the effect of nitrate reductase obtained in *K. pneumoniae* in the absence of nitrate is consistent with the findings of Böck and collaborators, who demonstrated a basal level of formate dehydrogenase-N in *K. pneumoniae* in the absence of nitrate by ^{75}Se incorporation into macromolecules [52]. In addition to this difference in expression regulation of respiratory nitrate reductase, *E. coli* and *K. pneumoniae* M5a1 also differ concerning their NADH:oxidoreductase systems. *E. coli* contains two NADH:oxidoreductase systems. One enzyme, NADH:ubiquinone oxidoreductase (NDH-I), encoded by the *nuo*-operon and expressed primarily under anaerobic respiratory conditions, couples NADH oxidation to proton translocation and thus conserves the redox energy in a proton gradient [45,53–58]. The second enzyme, NADH dehydrogenaseII (NDH-II) encoded by *ndh*, does not couple the redox reaction to proton translocation [54,59] and is significantly induced under aerobic conditions [60–62]. In contrast to the situation in *E. coli*, we have obtained evidence that the N$_2$-fixing *K. pneumoniae* M5a1 strain does not exhibit a homologous NADH-dehydrogenaseII in addition to the coupling of NADH:ubiquinone oxidoreductase encoded by the *nuo* operon. However, the non-N$_2$-fixing *K. pneumoniae* ssp. *pneumoniae* strain appears to contain both NADH:oxidoreductase systems as is the case for *E. coli*. These findings indicate that the presence of a single coupling NADH:ubiquinone

oxidoreductase in *K. pneumoniae* M5a1 may be due to the high energy requirement of N_2-fixation. We propose that in the absence of external terminal electron acceptors, the electrons derived from NADH and transferred by the NADH:ubiquinone oxidoreductase to the quinone pool in *K. pneumoniae* M5a1 are transferred mainly onto internally produced fumarate, resulting in higher ATP yields by anaerobic fumarate respiration. Thus, under anaerobic conditions in the absence of external terminal electron acceptors, *K. pneumoniae* M5a1 does not grow completely in a fermentative manner but also in a partial respiratory manner.

Hypothetical model for O_2 and nitrogen control of *nif* regulation in *K. pneumoniae*

We obtained strong evidence that NifL is reduced at the cytoplasmic membrane by electrons derived from the reduced quinone pool, resulting in higher membrane affinity. Considering the FNR-requirement for O_2 signal transduction in *K. pneumoniae* [20], it is attractive to speculate that in *K. pneumoniae* M5a1, the membrane-associated oxidoreductases of the anaerobic respiratory chain (that transfer electrons to the quinone pool) are regulated transcriptionally by FNR. As the genes encoding formate dehydrogenase-N in *E. coli* are transcribed in an FNR-dependent manner [63], one can expect that expression of formate dehydrogenase-N in *K. pneumoniae* is also controlled by FNR in the same manner. This is supported by sequence analysis of the *K. pneumoniae fdnG* promoter upstream region that indicates the presence of potential FNR-boxes (data not shown). Transcription of the *E. coli nuo*-operon is regulated by O_2 mainly through the transcriptional regulator, ArcA that represses *nuo* transcription under aerobic conditions [57]. However, as the N_2-fixing *K. pneumoniae* strain contains only a single NADH oxidizing enzyme, one can expect a different regulation of the *nuo*-operon in *K. pneumoniae* M5a1. Based on preliminary sequence analysis of the promoter upstream regions of the *K. pneumoniae nuoA* gene and determination of the NADH oxidation rate in the *K. pneumoniae fnr* mutant strain, we speculate that in *K. pneumoniae*, transcription of the *nuo*-operon is up-regulated by FNR under anaerobic conditions. Thus, in our current working model for O_2 signal transduction in *K. pneumoniae*, we propose that under anaerobic conditions, the primary O_2 sensor FNR activates transcription of membrane-bound oxidoreductases leading to a quinol/quinone ratio that allows electron transfer onto NifL. It is attractive to speculate that the rates of quinone reduction and oxidation, and consequently the quinol/quinone ratio, are important for providing the signal for NifL. As very low amounts of electrons from the reduced quinone pool are required for NifL reduction and the most electrons will flow to the terminal electron acceptors, we propose that the electron flow onto NifL is unspecific. However, at the current experimental stage we cannot rule out completely the possibility of an additional oxidoreductase system mediating electrons from the reduced quinone pool onto NifL. The reduced conformation of the NifL–protein favours membrane association of NifL and thus results in a sequestration of NifL to the membrane, allowing cytoplasmic NifA to activate *nif* genes. In the presence of

O_2, however, NifL appears to be oxidized directly by O_2 and dissociates from the membrane [21].

Concerning the nitrogen signal transduction, it is known that uridylylated GlnK transduces the signal of nitrogen-limitation to the *nif* regulon [8–10,21]. Experimental data indicate that under nitrogen-limitation, GlnK interacts with the inhibitory NifL–NifA complex, resulting in the dissociation of the complex (J. Stips and R. A. Schmitz, unpublished observation). Thus, under anaerobic and nitrogen-limited conditions, NifL would be able to receive electrons from the quinone pool and stay associated with the membrane. However, under anaerobic but nitrogen-sufficient conditions, NifL is not released from the cytoplasmic inhibitory NifL–NifA complex as the synthesis of GlnK is repressed [9] and alreadysynthesized GlnK is sequestered to the cytoplasmic membrane [64], consequently NifL stays in the cytoplasm as demonstrated recently [21].

Acknowledgements

References

1. Dixon, R. (1998) The oxygen-responsive NIFL-NIFA complex: a novel two-component regulatory system controlling nitrogenase synthesis in gamma-proteobacteria. *Arch. Microbiol.* **169**, 371–380.
2. Schmitz, R.A., Klopprogge, K. & Grabbe, R. (2002) Regulation of nitrogen fixation in *Klebsiella pneumoniae* and *Azotobacter vinelandii*: NifL, transducing two environmental signals to the *nif* transcriptional activator NifA. *J. Mol. Microbiol. Biotechnol.* **4**, 235–242.
3. Govantes, F., Andujar, E. & Santero, E. (1998) Mechanism of translational coupling in the *nifLA* operon of *Klebsiella pneumoniae*. *EMBO J.* **17**, 2368–2377.
4. Henderson, N., Austin, S. & Dixon, R.A. (1989) Role of metal ions in negative regulation of nitrogen fixation by the *nifL* gene product from *Klebsiella pneumoniae*. *Mol. General Genet.* **216**, 484–491.
5. Money, T., Jones, T., Dixon, R. & Austin, S. (1999) Isolation and properties of the complex between the enhancer binding protein NifA and the sensor NifL. *J. Bacteriol.* **181**, 4461–4468.
6. Lei, S., Pulakat, L. & Gavini, N. (1999) Genetic analysis of *nif* regulatory genes by utilizing the yeast two-hybrid system detected formation of a NifL-NifA complex that is implicated in regulated expression of *nif* genes. *J. Bacteriol.* **181**, 6535–6539.
7. Money, T., Barrett, J., Dixon, R. & Austin, S. (2001) Protein–protein interactions in the complex between the enhancer binding protein NifA and the sensor NifL from *Azotobacter vinelandii*. *J. Bacteriol.* **183**, 1359–1368.
8. He, L., Soupene, E., Ninfa, A. & Kustu, S. (1998) Physiological role for the GlnK protein of enteric bacteria: relief of NifL inhibition under nitrogen-limiting conditions. *J. Bacteriol.* **180**, 6661–6667.

9. Jack, R., De Zamaroczy, M. & Merrick, M. (1999) The signal transduction protein GlnK is required for NifL-dependent nitrogen control of *nif* gene expression in *Klebsiella pneumoniae*. *J. Bacteriol.* **181**, 1156–1162.

10. Arcondeguy, T., van Heeswijk, W.C. & Merrick, M. (1999) Studies on the roles of GlnK & GlnB in regulating *Klebsiella pneumoniae* NifL-dependent nitrogen control. *FEMS Microbiol. Lett.* **180**, 263–270.

11. Arcondeguy, T., Lawson, D. & Merrick, M. (2000) Two residues in the T-loop of GlnK determine NifL-dependent nitrogen control of *nif* gene expression. *J. Biol. Chem.* **275**, 38452–38456.

12. Little, R., Reyes-Ramirez, F., Zhang, Y., van Heeswijk, W.C. & Dixon, R. (2000) Signal transduction to the *Azotobacter vinelandii* NifL-NifA regulatory system is influenced directly by interaction with 2-oxoglutarate and the PII regulatory protein. *EMBO J.* **19**, 6041–6050.

13. Reyes-Ramirez, F., Little, R. & Dixon, R. (2001) Role of *Escherichia coli* nitrogen regulatory genes in the nitrogen response of the *Azotobacter vinelandii* NifL-NifA complex. *J. Bacteriol.* **183**, 3076–3082.

14. Little, R., Colombo, V., Leech, A. & Dixon, R. (2002) Direct interaction of the NifL regulatory protein with the GlnK signal transducer enables the *Azotobacter vinelandii* NifL-NifA regulatory system to respond to conditions replete for nitrogen. *J. Biol. Chem.* **277**, 15472–15481.

15. Rudnick, P., Kunz, C., Gunatilaka, M.K., Hines, E.R. & Kennedy, C. (2002) Role of GlnK in NifL-mediated regulation of NifA activity in *Azotobacter vinelandii*. *J. Bacteriol.* **184**, 812–820.

16. Hill, S., Austin, S., Eydmann, T., Jones, T. & Dixon, R. (1996) *Azotobacter vinelandii* NIFL is a flavoprotein that modulates transcriptional activation of nitrogen-fixation genes via a redox-sensitive switch. *Proc. Natl. Acad. Sci. USA.* **93**, 2143–2148.

17. Schmitz, R.A. (1997) NifL of *Klebsiella pneumoniae* carries an N-terminally bound FAD cofactor, which is not directly required for the inhibitory function of NifL. *FEMS Microbiol. Lett.* **157**, 313–318.

18. Macheroux, P., Hill, S., Austin, S., Eydmann, T., Jones, T., Kim, S.O., Poole, R. & Dixon, R. (1998) Electron donation to the flavoprotein NifL, a redox-sensing transcriptional regulator. *Biochem. J.* **332**, 413–419.

19. Klopprogge, K. & Schmitz, R.A. (1999) NifL of *Klebsiella pneumoniae*: redox characterization in relation to the nitrogen source. *Biochim. Biophys. Acta.* **1431**, 462–470.

20. Grabbe, R., Klopprogge, K. & Schmitz, R.A. (2001) FNR Is required for NifL-dependent oxygen control of *nif* gene expression in *Klebsiella pneumoniae*. *J. Bacteriol.* **183**, 1385–1393.

21. Klopprogge, K., Grabbe, R., Hoppert, M. & Schmitz, R.A. (2002) Membrane association of *Klebsiella pneumoniae* NifL is affected by molecular oxygen and combined nitrogen. *Arch. Microbiol.* **177**, 223–234.

22. Inoue, H., Nojima, H. & Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**, 23–28.

23. Silhavy, T.J., Bermann, M. & Enquist, L.W. (1984) Experiments with Gene Fusions, pp. 107–112. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

24. He, L., Soupene, E. & Kustu, S. (1997) NtrC is required for control of *Klebsiella pneumoniae* NifL activity. *J. Bacteriol.* **179**, 7446–7455.

25. Imlay, J.A. (1995) A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*. *J. Biol. Chem.* **270**, 19767–19777.

26. MacNeil, D., Zhu, J. & Brill, W.J. (1981) Regulation of nitrogen fixation in *Klebsiella pneumoniae*: isolation and characterization of strains with *nif-lac* fusions. *J. Bacteriol.* **145**, 348–357.

27. Skorupsky, K. & Taylor, R.K. (1996) Positive selection vectors for allelic exchange. *Gene* **169**, 47–52.

28. DeLorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K.N. (1990) Mini-Tn5 Transposon Derivatives for Insertion Mutagenesis, Promoter Probing, and Chromosomal Insertion of Cloned DNA in Gram-Negative Eubacteria. *J. Bacteriol.* **172**, 6568–6572.

29. Sambrock, J., Fritsch, E.F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

30. Schmitz, R.A., He, L. & Kustu, S. (1996) Iron is required to relieve inhibitory effects of NifL on transcriptional activation by NifA in *Klebsiella pneumoniae*. *J. Bacteriol.* **178**, 4679–4687.

31. Narberhaus, F., Lee, H.S., Schmitz, R.A., He, L. & Kustu, S. (1995) The C-terminal domain of NifL is sufficient to inhibit NifA activity. *J. Bacteriol.* **177**, 5078–5087.

32. Mott, J.E., Grant, R.A., Ho, Y.S. & Platt, T. (1985) Maximizing gene expression from plasmid vectors containing the lambda PL promoter: Strategies for overproducing transcription termination factor-ρ. *Proc. Natl. Acad. Sci. USA* **82**, 88–92.

33. Whitby, L.G. (1953) A new method for preparing flavin-adenine dinucleotide. *Biochem. J.* **54**, 437–442.

34. Krebs, W., Steuber, J., Gemperli, A.C. & Dimroth, P. (1999) Na + translocation by the NADH:ubiquinone oxidoreductase (complex I) from *Klebsiella pneumoniae*. *Mol. Microbiol.* **33**, 590–598.

35. Friedrich, T., Hofhaus, G., Ise, W., Nehls, U., Schmitz, B. & Weiss, H. (1989) A small isoform of NADH:ubiquinone oxidoreductase (complex I) without mitochondrially encoded subunits is made in chloramphenicol-treated *Neurospora crassa*. *Eur. J. Biochem.* **180**, 173–180.

36. Laemmli, U.K. (1970) Cleavage of the structural proteins during the assembly of the head of the Bacteriophage T4. *Nature* **227**, 680–685.

37. Pecher, A., Zinoni, F., Jatisatienr, C., Wirth, R., Hennecke, H. & Böck, A. (1983) On the redox control of synthesis of anaerobically induced enzymes in enterobacteriaceae. *Arch. Microbiol.* **136**, 131–136.

38. Falk-Krzesinski, H.J. & Wolfe, A. (1998) Genetic analysis of the *nuo* locus, which encodes the proton-translocating NADH dehydrogenase in *Escherichia coli*. *J. Bacteriol.* **180**, 1174–1184.

39. Lin, E.C.C. (1976) Glycerol dissimilation and its regulation in bacteria. *Ann. Rev. Microbiol.* **30**, 535–578.

40. Tran, Q.H. & Unden, G. (1998) Changes in the proton potential and the cellular energetics of *Escherichia coli* during growth by aerobic and anaerobic respiration or by fermentation. *Eur. J. Biochem.* **251**, 538–543.

41. Schmitz, R.A. (2000) Internal glutamine and glutamate pools in *Klebsiella pneumoniae* grown under different conditions of nitrogen availablity. *Current Microbiol.* **41**, 357–362.

42. Hom, S.S., Hennecke, H. & Shanmugam, K.T. (1980) Regulation of nitrogenase biosynthesis in *Klebsiella pneumoniae*: effect of nitrate. *J. General Microbiol.* **117**, 169–179.

43. Ingledew, W.J. & Poole, R.K. (1984) The respiratory chains of *Escherichia coli*. *Microbiol. Rev.* **48**, 222–721.

44. Unden, G., Hackenberg, H. & Kröger, A. (1980) Isolation and functional aspects of the fumarate reductase involved in the phosphorylative electron transport of *Vibrio succinogenes*. *Biochem. Biophyis. Acta* **591**, 275–288.

45. Tran, Q.H., Bongaerts, J., Vlad, D. & Unden, G. (1997) Requirement for the proton-pumping NADH dehydrogenase I of *Escherichia coli* in respiration of NADH to fumarate and its bioenergetic implications. *Eur. J. Biochem.* **244**, 155–160.

46. Richardson, D.J. (2000) Bacterial respiration: a flexible process for a changing environment. *Microbiology* **146**, 551–571.

47. Austin, S., Buck, M., Cannon, W., Eydmann, T. & Dixon, R. (1994) Purification and *in vitro* activities of the native nitrogen fixation control proteins NifA and NifL. *J. Bacteriol.* **176**, 3460–3465.

48. Poole, R.K. (1994) Oxygen reactions with bacterial oxidases and globins: binding, reduction and regulation. *Antonie Van Leuwenhoek* **65**, 289–310.

49. Stevanin, T.M., Ioannidis, N., Mills, C.E., Kim, S.O., Hughes, M.N. & Poole, R.K. (2000) Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes $b_{o'}$ or b_d, from nitric oxide. *J. Biol. Chem.* **275**, 35868–35875.

50. Berg, B.L. & Stewart, V. (1990) Structural genes for nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. *Genetics* **125**, 691–702.

51. Rabin, R.S. & Stewart, V. (1993) Dual response regulators (NarL and NarP) interact with dual sensors (NarX and NarQ) to control nitrate- and nitrite-regulated gene expression in *Escherichia coli* K-12. *J. Bacteriol.* **175**, 3259–3268.

52. Heider, J., Forchhammer, K., Sawers, G. & Böck, A. (1991) Interspecies compatibility of selenoprotein biosynthesis in Enterobacteriaceae. *Arch. Microbiol.* **155**, 221–228.

53. Weidner, U., Geier, S., Ptock, A., Friedrich, T., Leif, H. & Weiss, H. (1993) The gene locus of the proton-translocating NADH: ubiquinone oxidoreductase in *Escherichia coli*. Organization of the 14 genes and relationship between the derived proteins and subunits of mitochondrial complex I. *J. Mol. Biol.* **233**, 109–122.

54. Calhoun, M.W., Oden, K.L., Gennis, R.B., deMattos, M.J. & Neijssel, O.M. (1993) Energetic efficiency of *Escherichia coli*: effects of mutations in components of the aerobic respiratory chain. *J. Bacteriol.* **175**, 3020–3025.

55. Friedrich, T. (2001) Complex I: a chimaera of a redox and conformation-driven proton pump? *J. Bioenerg. Biomembr.* **33**, 169–177.

56. Wackwitz, B., Bongaerts, J., Goodman, S.D. & Unden, G. (1999) Growth phase-dependent regulation of *nuoA-N* expression in *Escherichia coli* K-12 by the Fis protein: upstream binding sites and bioenergetic significance. *Mol. General Genet.* **262**, 876–883.

57. Bongaerts, J., Zoske, S., Weidner, U. & Unden, G. (1995) Transcriptional regulation of the proton translocating NADH dehydrogenase genes (*nuoA-N*) of *Escherichia coli* electron acceptors, electron donors and gene regulators. *Mol. Microbiol.* **16**, 521–534.

58. Unden, G., Achebach, S., Holighaus, G., Tran, Q.H., Wackwitz, B. & Zeuner, Y. (2002) Control of FNR function of *Escherichia coli* by O_2 and reducing conditions. *J. Mol. Microbiol. Biotechnol.* **4**, 263–268.

59. Matsushita, K., Ohnishi, T. & Kaback, H.R. (1987) NADH-ubiquinone oxidoreductases of the *Escherichia coli* aerobic respiratory chain. *Biochemistry*. **26**, 7732–7737.

60. Green, J. & Guest, J.R. (1994) Regulation of transcription at the *ndh* promoter of *Escherichia coli* by FNR and novel factors. *Mol. Microbiol.* **12**, 433–444.

61. Spiro, S., Roberts, R.E. & Guest, J.R. (1989) FNR-dependent repression of the *ndh* gene of *Escherichia coli* and metal ion requirement for FNR-regulated gene expression. *Mol. Microbiol.* **3**, 601–608.

62. Meng, W., Green, J. & Guest, J.R. (1997) FNR-dependent repression of *ndh* gene expression requires two upstream FNR-binding sites. *Microbiology* **143**, 1521–1532.

63. Li, J. & Steward, V. (1992) Localization of upstream sequence elements required for nitrate and anaerobic induction of *fdn* (Formate Dehydrogenase-N) operon expression in *Escherichia coli* K-12. *J. Bacteriol.* **174**, 4935–4942.

64. Coutts, G., Thomas, G., Blakey, D. & Merrick, M. (2002) Membrane sequestration of the signal transduction protein GlnK by the ammonium transporter AmtB. *EMBO J.* **21**, 536–545.