Towards understanding the nitrogen signal transduction for nif gene expression in Klebsiella pneumoniae

Jens Glöer, Robert Thummer, Heike Ullrich and Ruth A. Schmitz

Institut für Allgemeine Mikrobiologie, Christian-Albrechts-Universität Kiel, Germany

The free-living Klebsiella pneumoniae is able to use molecular nitrogen as the sole nitrogen source. To avoid unnecessary energy expenditure, the synthesis of nitrogenase is tightly controlled in response to environmental conditions by the regulatory nitrogen fixation operon nifLA [1–4]. Products of the nifLA operon regulate transcription of the other nif genes in response to the two major environmental signals: molecular oxygen and availability of combined nitrogen. NifA is the transcriptional activator of all nif genes and nif operons except the nifLA operon, which is itself regulated in response to the nitrogen status of the cells by the global nitrogen regulatory system, Ntr [5,6]. NifL antagonizes the transcriptional activity of NifA in the presence of ammonium and/or molecular oxygen by direct protein–protein interaction with NifA [4,7–18]. It has been shown that the flavoprotein NifL acts as redox-sensitive regulatory protein by only allowing NifA activity under anaerobic conditions, when the FAD cofactor is reduced [10,19,20]. We recently obtained evidence that NifL-bound FAD cofactor in K. pneumoniae is reduced by electrons derived from the reduced menaquinone pool of the anaerobic respiratory chain, favouring membrane association of NifL.

In the diazotroph Klebsiella pneumoniae, the nitrogen sensory protein GlnK mediates the cellular nitrogen status towards the NifL/NifA system that regulates transcription of the nitrogen fixation genes in response to ammonium and molecular oxygen. To identify amino acids of GlnK essential for this signal transduction by protein–protein interaction, we performed random point mutagenesis by PCR amplification under conditions of reduced Taq polymerase fidelity. Three thousand two hundred mutated glnK genes were screened to identify those that would no longer complement a K. pneumoniae ΔglnK strain for growth under nitrogen fixing conditions. Twenty-four candidates resulting in a Nif− phenotype were identified, carrying 1–11 amino acid changes in GlnK. Based on these findings, as well as structural data, several single mutations were introduced into glnK by site-directed mutagenesis, and the Nif phenotype and the respective effects on NifA-mediated nif gene induction was monitored in K. pneumoniae using a chromosomal nifK′–lacZ fusion. Single amino acid changes resulting in significant nif gene inhibition under nitrogen limiting conditions were located within the highly conserved T-loop (A43G, A49T and N54D), the body of the protein (G87V and K79E) and in the C-terminal region (I100M, R103S, E106Q and D108G). Complex formation analyses between GlnK (wild-type or derivatives) and NifL or NifA in response to 2-oxoglutarate indicated that: (a) besides the T-loop, the C-terminal region of GlnK is essential for the interaction with NifL and NifA and (b) GlnK binds both proteins in the absence of 2-oxoglutarate, whereas, in the presence of 2-oxoglutarate, NifA is released but NifL remains bound to GlnK.

Abbreviation
MBP, maltose binding protein.
under anaerobic conditions [17,21–24]. This sequestration of the inhibitor NifL to the cytoplasmic membrane under anaerobic conditions and simultaneous nitrogen limitation (derepressing conditions) is the key mechanism in _K. pneumoniae_ for regulating cytoplasmic NifA activity by NifL in response to molecular oxygen and ammonium [17,23,24].

PII-like nitrogen sensory proteins have been demonstrated to play a major role in regulating nitrogen metabolism and are considered to be the most widespread signalling proteins in nature [25–27]. Recent studies have revealed that the nitrogen status in the diazotrophs _K. pneumoniae_ and _Azotobacter vinelandii_ is transduced towards the regulatory proteins NifL and NifA by the PII-like protein GlnK, a GlnB paralogue [14,17,28–32]. By contrast to the _glnK_ gene of _A. vinelandii_, enteric _glnK_ genes are under the control of the general nitrogen regulatory system, and are therefore only expressed under conditions of nitrogen starvation [25,33,34]. However, in response to the internal nitrogen status, GlnK is covalently modified by uridylylation, as is GlnB at the conserved tyrosine residue located in the T-loop (Y51), by the uridylytransferase/uridylyl-removing enzyme GlnD [25,35,36]. It has been demonstrated that GlnK mediates the cellular nitrogen status towards the NifL/NifA regulatory system by direct protein interaction; however, the mechanism differs between _K. pneumoniae_ and _A. vinelandii_ [14,17,28–32]. In _A. vinelandii_, the non-uridylylated form of GlnK activates the inhibitory function of NifL under nitrogen excess by direct protein–protein interaction [37,38], whereas, under nitrogen limitation, NifA is relieved from NifL inhibition by elevated levels of 2-oxoglutarate binding to the GAF-domain of NifA [4,14,18,32,37–41]. In _K. pneumoniae_, however, relief of NifA from NifL inhibition under nitrogen limiting conditions depends on the presence of GlnK independent of its uridylylation state [28–31]. We recently demonstrated that _K. pneumoniae_ GlnK interacts with both NifL and NifA, and obtained evidence that the GlnK interaction with the inhibitory NifL/NifA complex under nitrogen limitation results in dissociation of the complex by destabilizing the NifA–NifL interactions [17]. Dissociation of the inhibitory complex under nitrogen limitation allows NifL to contact the cytoplasmic membrane and receive electrons from the menaquinol pool, when oxygen is simultaneously limiting, which subsequently allows cytoplasmic NifA to induce _nif_ gene expression [22,24]. However, under nitrogen sufficient conditions, the cytoplasmic inhibitory NifL/NifA complex is stable because synthesis of GlnK is repressed [29] and residual GlnK is rapidly de-uridylylated and appears to be sequestered to the cytoplasmic membrane in an AmtB-dependent manner [42–44]. To identify the amino acid residues of GlnK that are required for signal transduction to the NifL/NifA complex by protein–protein interaction, we performed a random saturated mutagenesis of _glnK_ and screened for regulatory GlnK derivatives.

**Results**

To achieve new insights into the molecular mechanism of nitrogen signal transduction for _nif_ gene expression in _K. pneumoniae_, the present study aimed to identify amino acids of the nitrogen sensory protein GlnK that are crucial for signal transduction to the NifL/NifA-complex by protein–protein interaction.

**Random mutagenesis and genetic screen for _glnK_ mutations affecting nitrogen signal transduction**

The gene _glnK_ under the control of the _tet_ promoter was randomly mutated by PCR amplification under conditions of reduced _Taq_ polymerase fidelity using pRS154 as template (see Fig. S1). The resulting amplified DNA fragments were cloned into pCR®2.1-TOPO® to give a pool of randomly mutated _glnK_ genes (pRS171*); three independent plasmid pools were generated as described in Experimental procedures. To screen for _glnK_ alleles encoding GlnK derivatives that would no longer transduce the nitrogen signal to the NifL/NifA complex, a _glnK_ mutant strain of _K. pneumoniae_ M5a1 (RAS31) was used, which is unable to grow under nitrogen fixing conditions in the absence of a functional GlnK protein [28,29]. Expression of _glnK_ under the control of the _tet_ promoter from pRS171 resulted in complementation with a restoration of anaerobic growth using molecular nitrogen as the sole nitrogen source (Fig. 1A). To identify mutant GlnK derivatives that no longer complement for growth under nitrogen fixing conditions, the mutant plasmid pools were transferred into the _glnK_ mutant strain and approximately 3200 individual clones (RAS31/pRS171*) were screened for Nif– mutants on solid minimal medium with molecular nitrogen as the sole nitrogen source (see Fig. S2). During this screen, 71 clones carrying a derivative of pRS171 were identified that were unable to grow under oxygen and nitrogen limitation. DNA sequence analysis of the p(tet)_glnK_ inserts demonstrated that 25 plasmids carried a nonsense mutation and 22 carried a frame-shift mutation within the _glnK_ gene, resulting in the expression of nonfunctional C-terminal deleted GlnK derivatives in the respective clones. Twenty-four pRS171 derivatives carried point mutations in the _glnK_
gene resulting in 1–11 amino acid changes (see Table S1). Sequence analysis further revealed that wild-type Glk encodes for alanine at position 19, as is the case for Escherichia coli GlnK, indicating that the published sequence [29] with an arginine at position 19 is not correct. In principle, the mutations identified can be divided into three general classes: (a) changes of amino acids, which are located within the T-loop of Glk (amino acids 37–55), that has been proposed to interact with regulatory proteins [28–31,45]; (b) amino acids located in the C-terminal region (amino acids 100–112); and (c) amino acids located within the body of the protein (Fig. 2A).

To verify the Nif’ phenotype, the respective clones were grown in liquid minimal medium, which showed that all identified 24 Glk derivatives failed to complement for growth with molecular nitrogen as the sole nitrogen source (data not shown). Further analysis in the E. coli ΔglnK/ΔglnB background HS9060 [46] by native PAGE followed by western blot analysis demonstrated that all of the Glk derivatives are present in their trimeric state (see Table S1). Thus, the inability of these clones to grow under nitrogen fixing conditions may result from either the failure of the Glk variant to activate NifA from inhibitory NifL/NifA complexes, or its failure to properly bind 2-oxoglutarate and/or ATP, which may affect binding NifL and NifA. Moreover, despite Glk derivatives no longer transducing the nitrogen signal to the NifL/NifA complex, the failure of global or indirect effects of the central nitrogen regulator Glk under nitrogen limitation may also negatively affect growth under nitrogen limiting conditions.

**Monitoring the effects of single amino acid changes on growth under nitrogen fixing conditions and nif gene induction in K. pneumoniae**

Based on the nonfunctional Glk derivatives obtained in the general mutation approach and taking the available structural data into account [47–49], selected single amino acid mutations were introduced by site-directed mutagenesis into glk on pRS319 (see Fig. S1). The resulting respective plasmids were introduced into K. pneumoniae glnK mutant strain (RAS31) (\(\bigcirc\)) or derivatives with single amino acid mutations E18G (\(\bigtriangleup\)), A43G (\(\bigcirc\)), N54D (\(\bigcirc\)), K79E (\(\bigtriangleup\)), G87V (\(\bigtriangleup\)) and D108G (\(\bigtriangleup\)).
The level of expression of the respective glnK alleles under the control of the tet promoter from the low copy vector (0.85 µg GlnK-mg⁻¹ total cell extract protein) was in the range of chromosomal glnK expression under nitrogen limitation in K. pneumoniae (0.7 µg GlnK-mg⁻¹ total cell extract protein) as evaluated by quantitative western blot analysis (data not shown). To achieve induction of nif transcription, cultures were grown anaerobically in minimal medium with 4 mM glutamine as the sole (limiting) nitrogen source (see Experimental procedures), which comprise conditions that allow full induction of nif expression in K. pneumoniae [50]. In general, anaerobic growth of K. pneumoniae UN4495 ΔglnK on glutamine as the sole limiting nitrogen source was neither affected by the absence or presence of glnK, nor by the presence of mutated glnK alleles expressed from pACYC184 or by the presence of pACYC184 (data not shown). Monitoring NifA-dependent transcription of the nifK'-lacZ fusion during exponential growth demonstrated that glnK expression from pRS319 in K. pneumoniae UN4495 resulted in nifHDK induction of approximately 1400 ± 100 U·mL⁻¹·D₆₀₀⁻¹, which is slightly lower than nifHDK induction in K. pneumoniae UN4495 under the same growth conditions with 4 mM glutamine as the sole limiting nitrogen source [50]. GlnK derivatives that are no longer able to prevent NifL inhibiting NifA under nitrogen limiting conditions were detectable and quantified by the extent of the decrease in nifHDK induction (Fig. 2B). The
Table 1. Growth analysis of *K. pneumoniae* mutant strains under nitrogen fixing conditions. *K. pneumoniae* ΔglnK carrying pRS319 with single point mutations were grown at 38 °C under anaerobic conditions with molecular nitrogen as the sole nitrogen source. -, failure to complement for growth; +, significant reduced growth; ++, growth restoration to approximately 70%; +++, wild-type.

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strongest effects were obtained upon the change of position 87 in the body of the protein (4 ± 1% *nifHDK* induction), which is in the range of the ΔglnK mutant, and in position 43 of the T-loop (7 ± 1% *nifHDK* induction). Further mutations resulting in a significant effect on *nif* induction were at position 79 located in the body of the protein, positions 49 and 54 within the T-loop, and positions 100, 103, 106 and 108 within the C-terminal region (Fig. 2). To demonstrate that the failure of the GlnK derivatives to express *nifHDK* under derepressing conditions could not be accounted for by a significant decrease in the amount of NiFA and NiFL, we compared the amounts of the NiFL and NiFA proteins in the mutant strains with those in the congenic wild-type strain by immunological means. This analysis clearly demonstrated that the protein levels of NiFA and NiFL are not significantly affected (see Fig. S3), indicating that the observed effects on *nif* gene induction are directly due to altered interactions between the GlnK derivatives and NiFA and/or NiFL. The finding that variants V7T, E18G, E32G, D62N and R101C did not show reduced *nif* gene induction, but resulted in a Nif− phenotype under nitrogen fixing conditions (Table 1), strongly indicates that the obtained Nif− phenotype of those GlnK derivatives results exclusively from an effect on other general functions of the global nitrogen regulator under nitrogen limitation. Most interestingly, changing positions 29 and 60 resulted in a significant increase of *nif* induction (Fig. 2B).

To exclude the possibility that the observed effects on NiFA activity are based on the inability of the GlnK derivatives to form functional trimers, a *tet* resistance cassette was inserted into the blunted SaII site of the respective mutated plasmids, allowing the expression of the mutated glnK genes in the *E. coli* ΔglnK/ΔglnB background (HS9060). Native PAGE and western blot analysis demonstrated that, except for variant E32G, all GlnK derivatives appear to form trimers (Fig. 2C). However, SDS/PAGE and subsequent western blot analysis demonstrated that the variant E32G is expressed in equal amounts but appears to be unable to enter the native gel, indicating a major change of the total charge or overall protein conformation. Compared with the wild-type protein, variants E18G, T29M, D62N and D108G showed lower mobility, whereas variants N54D, K79E and R101C showed a higher mobility. These differences in mobility indicate that the respective amino acid changes affect the overall structure of the trimeric GlnK variants and/or the overall charge of the trimers. These changes may influence the binding of the effectors 2-oxoglutarate and ATP and/or interaction with the modifying protein GlnD; however, they do not necessarily correlate with the negative effects on *nif* gene induction (e.g. GlnK wild-type compared to derivatives T29M, D62N or R101C). With the exception of the variants Y51F and G87V, all GlnK derivatives and the wild-type showed the typical or a partial modification pattern of enteric PII-like proteins under initial nitrogen limitation, with completely unmodified trimers (GlnK3) and trimers with one, two or three monomers [GlnK3-({UMP}), GlnK3-({UMP})2 and/or GlnK3-({UMP})3] uridylylated at position 51 [46] (Fig. 2C). The finding that, under nitrogen limitation, G87V is not uridylylated at all indicates that, in contrast to all other GlnK derivatives, the variant G87V may be unable to interact with GlnD and/or the binding of 2-oxoglutarate and that ATP is significantly affected. To exclude a potential sequestration of the nonmodified derivative G87V to the cytoplasmic membrane under nitrogen limitation [42–44], which would strongly interfere with *nif* regulation, the localization of variant G87V and GlnK expressed from plasmids was analysed by membrane preparations under nitrogen limitation in *K. pneumoniae* UN4495 ΔglnK (see Experimental procedures). This analysis clearly
demonstrated that, under nitrogen limitation, variant G87V is not sequestered to the cytoplasmic membrane; 97 ± 1% of the protein was located in the cytoplasm as was the wild-type protein (data not shown).

Studying the interaction of GlnK variants with NifA and NifL by pull-down experiments

Selected amino acid substitutions of GlnK that showed significantly reduced NifA mediated nif gene induction were further analysed concerning their ability to interact with NifA and NifL. The glnK point mutations were introduced into pRS180 (malE-nifL and glnK) and pRS158 (malE-nifA and glnK) by site-directed mutagenesis to allow complex analysis after purification by affinity chromatography [17]. GlnK or derivatives were co-expressed in K. pneumoniae with maltose binding protein (MBP)-NifL or MBP-NifA by approximately the same amount using 100 μM isopropyl thiogalactoside under aerobic and nitrogen sufficient growth conditions to exclude the expression of chromosomal glnK and nifLA. After purification in the absence or presence of 2-oxoglutarate (in the range 0.1–2 mM), the complexes were formed and the protein ratios determined by quantitative western blot analysis, as previously described [17]. In the absence of 2-oxoglutarate, significantly higher amounts of variant G87V compared to wild-type GlnK were bound to MBP-NifA (approximately 250%), whereas significantly lower amounts were obtained in case of the variants A43G (approximately 40%) and R103S and D108G (approximately 10%) (Fig. 3A). Complex formation between MBP-NifA and wild-type GlnK was significantly inhibited in the presence of 2-oxoglutarate; there was a 10-fold reduction in the amount of complexes obtained in the presence of 1 mM 2-oxoglutarate (Fig. 4). Negative but significantly smaller effects on complex formation in the presence of 2-oxoglutarate were also obtained for variants A43G and A49T (approximately 25% decrease in complex formation at 1 mM 2-oxoglutarate; Fig. 4A), both of which bound MBP-NifA with significantly lower affinity than wild-type GlnK in the absence of 2-oxoglutarate (Figs 3A and 4A). By contrast, only minor or negligible effects on 2-oxoglutarate on complex formation between MBP-NifA and the GlnK variants K79E, G87V, R103S and D108G were detectable (Fig. 4A). Concerning complex formation with NifL, significantly higher amounts of GlnK derivative G87V (190%) bound to MBP-NifL, whereas significantly lower amounts were obtained for the variants R103S and A43G (Fig. 3B). Thus, variant A43G and R103S apparently bind both regulators, NifL and NifA, with significantly lower affinities (Fig. 3), strongly indicating that, besides the T-loop, the C-terminal region of GlnK is essential for contacting both proteins. By contrast to complex formation with NifA no significant effects of 2-oxoglutarate on complex formation between NifL and GlnK or the respective variants were obtained (data not shown).

Furthermore, the potential effects of ATP on complex formation were studied by including additional ATP (3.5 mM) and MgCl2 (2 mM) in the buffer of pull-down experiments between GlnK and MBP-NifA.

Fig. 3. Complex formation between GlnK or derivatives and NifL or NifA after co-expression in K. pneumoniae. Co-expressed proteins were purified from cell extracts by affinity chromatography and analysed by SDS/PAGE and subsequent western blot analysis using polyclonal antibodies raised against GlnK, MBP-NifA or MBP-NifL. The protein bands of the complexes were quantified using DIANAIII software (Raytest) and known amounts of the respective purified proteins, which were simultaneously quantified on the same membrane for each experiment. Depicted are the relative amounts of GlnK or GlnK variants (in general stated in terms of the trimeric GlnK protein) in complexes with MBP-NifA (A) or MBP-NifL (B), generally stated in terms of monomeric MBP fusion proteins. Data represent the mean ± SD values of at least three independent co-expressions.
or MBP-NifL. However, no significant effects on binding affinities were observed in the presence of additional ATP, neither for the wild-type, nor for several GlnK derivatives (A43G, A49T, K79E, G87V and R103S). Because ATP is known to be an effector molecule of GlnK, these findings indicate that GlnK or derivatives bind ATP with high affinities and are ATP saturated after expressed in $K.\ pneumoniae$ from the respective plasmids prior to the pull-down experiments.

**Discussion**

The nitrogen sensory protein GlnK is known to transduce the cellular nitrogen status to target proteins by direct protein–protein interaction [25–27]. In the case of the regulation of nitrogen fixation in $K.\ pneumoniae$, the target proteins NifA and NifL appear to interact simultaneously with GlnK [17]. In the present study, we investigated the effects of single amino acid changes of GlnK on growth under nitrogen fixing conditions, $nif$ gene induction, and protein interaction with NifL and NifA based on a random saturated mutagenesis of the $glnK$ gene and taking structural data of GlnK into account [47–49].

The GlnK variants with single amino acid substitutions for which a significant reduction of $nif$ gene induction was obtained generally appear to be able to form trimers. Furthermore, they appear to interact with UTase because they show a wild-type or partial uridylylation pattern, except the variant G87V (Fig. 2). These findings indicate that, under nitrogen limitation, all GlnK derivatives are localized in the cytoplasm, which has been verified for variant G87V. This strongly suggests that the effects on $nif$ gene induction result from an altered interaction of the GlnK derivatives with the target proteins NifL and NifA or upon changes in binding 2-oxoglutarate and/or ATP, which may also affect the interaction with the target proteins. Moreover, we cannot rule out that, in addition to the obtained reduction of $nif$ gene expression, other general functions of GlnK are affected. Interestingly, some of the variants analysed (V7T, E18G, E32G, D62N and R101V), resulting in a Nif$^-$ phenotype under nitrogen fixing conditions, did not show reduced $nif$ gene induction, strongly indicating that the obtained
NiF\(^{-}\) phenotype of those variants results exclusively from an effect on other general functions of the global nitrogen regulator under nitrogen limitation.

From recent structure–function studies of PII-like proteins, it is known that amino acids of the T-loop are essential for signal transduction, and potentially are involved in direct interaction with target proteins [28,30,31,44,49,51–55]. In accordance with those findings, the general mutagenesis pulled out three changes of amino acids within the T-loop structure at positions A43, A49 and N54, which clearly led to a significant decrease in \(\text{nif}\) gene induction (Fig. 2). These findings are consistent with a previous study on the importance of T-loop residues 54 and 43 in the specificity of GlnK for regulating \(K.\ pneumoniae\) NiF activity in a heterologous \(E.\ coli\) system [31]. Moreover, it has also been demonstrated that two amino acid changes (D54N and T43A) allow \(K.\ pneumoniae\) GlnB, in which the T-loop differs from GlnK at just three residues (43, 52 and 54), to functionally complement a glnK mutant strain and relieve NiF inhibition of NiF [30]. The importance of amino acid A49 for the regulatory interaction with one of its target proteins, NiRII, has also been reported for \(E.\ coli\) GlnB. Variant A49P abolishes the interaction of GlnB with NiRII and shows increased affinity for 2-oxoglutarate compared to the wild-type [51,52]. Although amino acid Y51 of \(E.\ coli\) GlnB and GlnK has been demonstrated to be essential for protein interaction with UTase, ATase and NiRII [36,51], no effect on \(\text{nif}\) gene induction in \(K.\ pneumoniae\) was detectable upon mutation of the site of GlnK uridylylation (Y51F; Fig. 2B). This finding is in accordance with and confirms previous findings indicating that uridylylation of GlnK is not required for relief of NiFL inhibition of NiF activity in \(K.\ pneumoniae\) [28,30]. In addition to crucial amino acids in the T-loop and the core protein (G87V and K79E), further changes of several amino acids in the C-terminal region (I100M, R103S, E106Q and D108G) significantly inhibited \(\text{nif}\) gene induction (Fig. 2B), strongly indicating that the C-terminal region of GlnK is essential for interaction with NiFL and NiF in \(K.\ pneumoniae\). Similar effects have been obtained for GlnB activation of NiF in \(Rhodospirillum rubrum\) [53,56] and have been proposed for \(Herbaspirillum seropedicae\) [57]. Based on structural data, it is accepted that some of the identified crucial amino acids in GlnK (e.g. G87, R101 and E106) are directly or indirectly involved in binding the effector molecule ATP [47]. Thus, changing those amino acids may affect ATP binding and/or directly effect protein interaction with NiFL and NiF. Most interestingly, three amino acid changes in \(K.\ pneumoniae\) GlnK resulted in a significant increase of \(\text{nif}\) induction (T29M, K60T and G105S), which is currently under further investigation.

**Interaction of GlnK with NiF is affected by 2-oxoglutarate**

Pull-down experiments demonstrated that, in the presence of the small effector molecule 2-oxoglutarate, GlnK binds NiF with a significantly lower affinity, which may result from a conformational switch of the GlnK protein induced by binding 2-oxoglutarate. By contrast to NiF, complex formation between GlnK and NiFL is not affected by 2-oxoglutarate. We hypothesize that this difference in complex formation in response to 2-oxoglutarate results in the release of NiF from inhibitory NiFL/NiF complexes under nitrogen limitation or, alternatively, does not allow the formation of the NiFL/NiF complexes under nitrogen limitation in the first place and finally leads to \(\text{nif}\) induction if simultaneously oxygen is limiting.

Studying the interaction of selected GlnK variants conferring a decreased \(\text{nif}\) gene induction with NiF in the presence or absence of 2-oxoglutarate allowed further insight into the nitrogen signal transduction. First, for several GlnK derivatives conferring a decreased \(\text{nif}\) gene induction, only minor or negligible effects of 2-oxoglutarate on complex formation with NiF were obtained (K79E, G87V, R103S and D108G; Fig. 4). This may be the result of the inability of those GlnK variants to bind 2-oxoglutarate or to perform the conformational switch upon binding 2-oxoglutarate. Because changing amino acid 79 (located in the \(\alpha\beta\) strand on the trimer surface) did not affect binding affinity to NiF or NiFL, the inability to respond to 2-oxoglutarate results in an effective simultaneous binding of NiF and NiFL under nitrogen limitation and thus in reduced \(\text{nif}\) gene induction (Fig. 2B). Variant G87V binds both target proteins, NiF and NiFL, with a significant higher affinity than GlnK (Fig. 3); consequently, based on its inability to respond to 2-oxoglutarate, no NiF is released from the inhibitory NiFL/NiF complexes under nitrogen limitation, resulting in maximal \(\text{nif}\) repression. Because amino acid G87 is involved in ATP binding [47,48], and the change to valine presumably impairs ATP-binding (Fig. 5), this finding strongly indicates that ATP binding is crucial for integrating the 2-oxoglutarate signal (i.e. the signal for nitrogen limitation) by GlnK, potentially leading to a conformational switch of the protein. This is consistent with the recent finding obtained for GlnK\(_J\) from *Methanococcus jannaschii* showing that ATP binding to GlnK causes the T-loop to assume a compact conformation, which, in turn, creates a 2-oxoglutarate...
arate binding site at the apex of the T-loop [49]. Upon changing the amino acid residue at position 103 or 108 in the C-terminal region (R103S and D108G), GlnK almost lost its ability to interact with NifA and NifL at all (Fig. 3); consequently, inhibitory NifL/NifA complexes are more stable under nitrogen limitation and most NifA is not released from the complex.

Second, changing position 43 or 49 in the T-loop, which has been demonstrated to be crucial for the interaction with the target proteins for several PII-like proteins [28,30,31,44,49,51–55], significantly decreased the ability of GlnK to bind to NifA in the absence of 2-oxoglutarate and also, in the case of position 43, the ability to bind to NifL (Fig. 3). The combination of decreased binding of the NifL/NifA complex in the absence of 2-oxoglutarate and a significantly less efficient response to 2-oxoglutarate (Fig. 4) appears to be responsible for the significantly reduced nif gene induction of A43G and A49T because most of the inhibitory NifL/NifA complexes do not interact with the GlnK derivatives and thus remain stable, even under nitrogen limitation.

Hypothetical model of regulatory interaction of GlnK with NifA and NifL in K. pneumoniae

Overall, the results obtained in the present study do not allow the discrimination or identification of the amino acid residues that are responsible for binding exclusively NifA or NifL. Furthermore, the different single mutations mostly affect the binding of GlnK to both target proteins in the absence of 2-oxoglutarate, suggesting that the GlnK binding site for NifA might be close to the GlnK binding site for NifL. This finding excludes the possibility that NifA and NifL each contact GlnK at two different sites of the core protein (e.g. top and bottom), which would result in a sandwich conformation, as has been proposed, for example, for GlnK interacting with the nitrogenase regulatory enzyme DraG and the ammonium transporter AmtB in Azospirillum brasilense [58] and as has been discussed as one possibility for Bacillus subtilis GlnK interacting with the transcriptional regulator TnrA and the ammonium transporter AmtB [59]. Moreover, we obtained evidence that the C-terminal region of GlnK is essential for the interaction with both target proteins, NifL and NifA. Finally, the present study provides support for the idea that the release of NifA from the NifL/NifA inhibitory complexes bound to GlnK is dependent on GlnK binding the effector molecule 2-oxoglutarate, which appears to induce a conformational switch of the protein. Based on these findings, we hypothesize that, besides the T-loop, the C-terminal region of GlnK is essential for signal transduction, which contacts both regulatory proteins, NifL and NifA, exclusively under nitrogen sufficiency. Under nitrogen limitation, however, binding of 2-oxoglutarate appears to induce a conformational switch of the overall protein structure, which may rearrange the C-terminal region, resulting in NifA release from the inhibitory NifL/NifA complex. Because, under nitrogen and simultaneously oxygen limiting conditions, approximately 97% of chromosomal expressed GlnK is localized in the cytoplasm (J. Glöer and R. A. Schmitz, unpublished data), whereas approximately 90% of chromosomal expressed NifL is reduced and sequestered to the cytoplasmic membrane [23,24], we assume that, after the reduction of NifL at the cytoplasmic membrane, GlnK is no longer able to bind to the reduced NifL conformation located at the cytoplasmic membrane and thus dissociates into the cytoplasm.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids employed in the present study are listed in Table 2. Plasmid DNA was transformed into E. coli cells as previously described [60] and into K. pneumoniae cells by electroporation.

Construction of plasmids

For construction of plasmid pRS171, a 0.9-kbp PCR fragment carrying glnK under the control of the tet promoter
was generated using the Vent polymerase for PCR amplification (New England Biolabs, Frankfurt am Main, Germany), pRS154 as template [46] and a set of primers, which were homologous to pACYC184 flanking the 5′ and 3′ regions of the P(tet)glnK construct in pRS154 (pACYC1457, 5′-GATGACCCGACCTGACGCCCCATA-3′; pACYC2348, 5′-GATCCAGATACCGCAAGCGACAG-3′) (see Fig. S1A). The 0.9 kbp PCR fragment was cloned into the TOPO TA-cloning vector pCR®.1-TOPO® (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. The DNA sequences of both strands were determined independently and completely. For construction of plasmid pRS319 containing the K. pneumoniae glnK gene inserted into the EcoRV site of pACYC184 and under the control of the tet promoter (see Fig. S1B), chromosomal glnK was amplified by PCR using the primer set 5′-AC-GTTGTAGCTGCCGGAACATAC-3′ and 5′-GCTCAGCATCGCAAGCGACAG-3′. The 0.45 kbp PCR fragment was cloned into the TOPO TA-cloning vector pCR®.1-TOPO® (Stratagene). The EcoRI I fragment of the latter, containing glnK, was purified, blunt ended and ligated into the EcoRV-site of pACYC184. The correct orientation (under the control of the tet promoter) was verified by DNA sequence analysis.

**Random mutagenesis of glnK and subsequent cloning into pCR®.1-TOPO®**

glnK under the control of the tet promoter [P(tet)glnK] was PCR amplified using pRS154 as template, the primer set pACYC1457 and pACYC2348 (see above) and Taq polymerase (MBI Fermentas, St Leon-Rot, Germany) in the presence of 100–500 μM MnCl₂, conditions of reduced Taq polymerase fidelity [61]. The resulting mutated PCR products were purified and TA-cloned into the TOPO TA-cloning vector pCR®.1-TOPO® (Stratagene) according to the manufacturer’s instructions. The resulting plasmids containing mutated glnK (pRS171*) were transformed and amplified in E. coli DH5α on plates. Three independent mutated plasmid pools were purified (RG0, RG4 and HU1), derived from 2100, 3010 and 500 independent clones. The range of mutation frequencies of these pools were determined by DNA sequence analysis of glnK-inserts of 40 randomly chosen plasmids for each pool (RG0 and HUq: 4 ± 1 mutated nucleotides; RG4: 10 ± 3 mutated nucleotides within the glnK-gene).

**Screening system to identify mutated glnK conferring a Nir² phenotype**

Pools of randomly mutagenized plasmid pRS171 were transformed into the glnK mutant K. pneumoniae M5a1 strain (RAS31) [46]. Recombinant K. pneumoniae clones were grown in parallel on rich medium plates (LB medium) and under nitrogen fixing conditions on solid minimal medium supplemented with 0.4% sucrose as the sole carbon source [50]. For the minimal medium, purified agar (Difco Laboratories, Detroit, MI, USA) was used to exclude contamination of organic nitrogen. The minimal medium agar plates were incubated at 30 °C for 6 days in anaerobic pots, which were degassed via a vacuum pump for three cycles prior to incubation and kept under an atmosphere of molecular nitrogen (N₂, grade 4.0; Messer Griesheim, Sulzbach, Germany).

K. pneumoniae wild-type strain and K. pneumoniae ΔglnK carrying the respective plasmids (pRS171), mutated derivatives (pRS171*) or pRS172 were grown anaerobically at 30 °C in liquid minimal medium [50] under nitrogen fixing conditions. The main cultures (25 mL) were grown in closed bottles with 0.4% sucrose as the carbon source and N₂ as the sole nitrogen source in the gas atmosphere (162 kPa). The cultures were inoculated with 0.5 mL precultures grown overnight in minimal medium supplemented with 0.4% sucrose and 4 mM glutamine as the limiting...
nitrogen source under aerobic conditions. Prior to inoculation of the main cultures, the cells were washed twice and concentrated in minimal medium. Samples were taken anaerobically to monitor growth at 600 nm.

DNA sequence analysis

DNA sequences of identified glnK derivatives were determined independently and completely for both strands at the Göttingen Genomics Laboratory using the primers pACYC1457 and pACYC2348. Sequence analysis was performed with ccc software package (Genetics Computer Group, Tampa, FL, USA) [62].

Western blot analysis

Mutated plasmids (pRS171* and pRS319*) were transformed into an E. coli glnK/glnB background constructed by Verena Weiss (HS9060). Cells were grown in 400 mL of LB medium in the absence of tetracycline at 37 °C. When $D_{600}$ of 0.5–0.6 was reached for exponentially growing cultures, tetracycline was added to a final concentration of 0.25 μg/mL to additionally induce expression of glnK*. After 4 h of induction, cell extracts were prepared by disruption of the cells in B-buffer [20 mM potassium N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid, pH 8.0, 125 mM potassium glutamate, 10% (v/v) glycerol, 1.5 mM dithiothreitol] using a French press cell. Native gel electrophoresis was performed as previously described [46] using 12.5% polyacrylamide gels (29 : 1, acrylamide : bisacrylamide) with 5% stacking gels, running gel buffer (187.5 mM Tris/HCl, pH 8.9), stacking gel buffer (62.5 mM Tris/HCl, pH 7.5) and running buffer (82.6 mM Tris/HCl, pH 9.4, containing 33 mM glycine). Proteins were subsequently transferred to nitrocellulose membranes (BioTrace®NT, Pall Life Science, Dreieich Germany) for western blot analysis. The membranes were exposed to polyclonal rabbit antisera directed against the GlnK protein of K. pneumoniae and protein bands were detected with secondary antibodies directed against rabbit IgG and coupled to horseradish peroxidase (Bio-Rad Laboratories, München, Germany) using the ECL-Plus system (GE Healthcare, La Jolla, CA, USA) for detection. Purified GlnK from K. pneumoniae was used as standard.

Site-directed mutagenesis

Site-directed mutagenesis was performed to insert single amino acid mutations into the glnK gene on plasmid pRS319, pRS180 and pRS158 using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The respective primers used are provided in the (Table S2). The mutations were generally verified by DNA sequence analysis of both strands. To analyse the oligomeric state of the variant GlnK in HS9060, a tetracycline resistance cassette was inserted into the blunt ended SalI site of the respective mutated pRS319 plasmid.

β-Galactosidase assays

Plasmids carrying the wild-type $\text{P}_{\text{rpoB}}\text{glnK}$ insert (pRS319) or mutated derivatives were transferred into K. pneumoniae UN4495ΔglnK. NifA-mediated activation of transcription from the nifHDK promoter was monitored by measuring β-galactosidase activity and calculating the β-galactosidase synthesis rate (U·mL$^{-1}$·h$^{-1}$) during exponential growth under nitrogen and oxygen limiting growth conditions at 30 °C [50]. The negative effects of glnK mutations on NifA activity were assessed by virtue of a decrease in nifHDK expression.

Analysis of in vivo complex formation by pull-down experiments

Co-expression of malE-nifL or malE-nifA, plus-wild-type glnK or mutant derivatives of glnK was induced with 100 μM isopropyl thio-β-D-galactoside in K. pneumoniae carrying pRS180, pRS158 or the respective mutated derivatives. Main cultures (1 L) were grown in minimal medium under aerobic conditions in the presence of 10 mM ammonium to exclude chromosomal glnK expression and protein synthesis was induced for 2 h when $D_{600}$ of 0.6–0.7 was reached [50]. Purification of complexes in general subsequently followed directly after cell harvest without any storage at lower temperatures. Cell extracts were prepared in 1× B-buffer (see above) in the presence of the protease inhibitor cocktail for bacterial cell extracts (Sigma-Aldrich, Schnelldorf, Germany) and the respective fusion proteins were purified from the 20 000 g supernatant by amylose affinity chromatography as previously described [17]. In the case that the complexes were purified in the presence of physiological concentrations of 2-oxoglutarate (0.1–2.0 mM) or ATP (3.5 mM ATP and 2.0 mM MgCl$_2$), 2-oxoglutarate or ATP/MgCl$_2$ were added to the cell extracts prior the addition of the amylose resin and added to the wash and elution buffers used. The respective wash and elution fractions were analysed by gel electrophoresis and subsequent western blot analysis using polyclonal antibodies raised against K. pneumoniae NifL, NifA or GlnK as described above [63]. Polyclonal GlnK antibodies were used in a very high dilution range, and under conditions under which cross-reaction with GlnB was approximately negligible. The protein bands of the complexes were quantified from at least two independent cultures using DIANAII software (Raytest, Straubenhardt, Germany) and known amounts of the respective purified proteins, which were simultaneously quantified on the same membrane for each experiment [17].

Mutational analysis of K. pneumoniae GlnK

Mutational analysis of K. pneumoniae GlnK
Quantification of purified proteins MBP-NifL and MBP-NifA was linear within absolute amounts in the range 0.03–0.27 µg per lane and GlnK in the range 0.008–0.06 µg. All quantifications of proteins were performed within this linear range of the detection system. The relative amounts of GlnK in complexes are generally stated in terms of the trimeric GlnK protein (GlnK₃).

Membrane preparations

Cytoplasmic and membrane fractions of cell-free cell extracts were separated by several centrifugation steps in the presence of the protease inhibitor cocktail for bacterial cell extracts (Sigma-Aldrich) as previously described [17,23]. The GlnK protein bands of cytoplasmic and membrane fractions were quantified by western blot analyses using known amounts of purified protein as described above. Quantities of GlnK in the cytoplasmic and membrane fractions were calculated relative to total GlnK, respectively, and setting the absolute amounts of the respective protein in both fractions (cytoplasmic and membrane fraction) as 100%.

Protein structure analysis

For structural analysis, the structure of E. coli GlnK (Protein Data Bank: 2GNK) [47] and the program pymol 0.99rc6 (http://www.pymol.org) was used.

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References


**Supporting information**

The following supplementary material is available:

**Fig. S1.** Plasmids pRS154 and pRS319.

**Fig. S2.** Initial screen for Nif- phenotype on solid minimal medium.

**Fig. S3.** Amounts of chromosomally expressed NifA and NifL in wild-type and mutant *K. pneumoniae* strains.

**Table S1.** GlnK derivatives that lost the ability to complement the *glnK* mutant strain for growth with molecular nitrogen as the sole nitrogen source.

**Table S2.** Primer pairs used for for site-directed mutagenesis of *glnK*.

This supplementary material can be found in the online version of this article.

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