Studies on the roles of GlNK and GlNB in regulating Klebsiella pneumoniae NifL-dependent nitrogen control

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Abstract

In Klebsiella pneumoniae, nitrogen fixation (nif) genes are regulated in response to fixed nitrogen and oxygen. The activity of the nif-specific transcriptional activator NifA is modulated by NifL, which mediates both oxygen and nitrogen control. The signal transduction protein GlNK is required to relieve the inhibitory effect of NifL on NifA that occurs when the intracellular N status is high and in a wild-type cell, the action of GlNK cannot be substituted by the structurally related protein PI. We have studied the modulation of NifA activity by NifL in an heterologous system in which the host organism is Escherichia coli. Using a vglB, vglK mutant, we have shown that the modulation of NifA activity by NifL is dependent on the concentration of GlNK in the cell and that when overproduced, PI can substitute for GlNK. Furthermore, our data suggest that PI can counteract the positive action of GlNK in relieving NifL-dependent inhibition of NifA activity. This negative effect of PI may be physiologically important in establishing repression of nif gene expression when the intracellular nitrogen status rises. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: glnB; glnK; Nitrogen regulation; Nitrogen fixation; Escherichia coli

1. Introduction

In Klebsiella pneumoniae, transcription of the nitrogen fixation (nif) genes is dependent on the activator protein NifA. The nifA gene is co-transcribed with nifL whose product modulates NifA activity in response to oxygen and fixed nitrogen. Expression of nifL and nifA is coupled at the translational level and NifL and NifA are expressed stoichiometrically. The two proteins form a protein complex in vitro and in vivo and stoichiometric levels of the two proteins are required for effective modulation of NifA activity [11,14,23].

Studies in K. pneumoniae have shown that the response of NifL to fixed nitrogen levels is independent of the signal transduction protein PI (encoded by glnB) [16] but is dependent on the structurally similar protein GlNK [18]. The GlNK protein is encoded in a
NtrC-dependent operon, \textit{glnK amtB}, in which \textit{amtB} encodes a proposed high-affinity ammonium transporter [29]. In both \textit{K. pneumoniae} and \textit{Escherichia coli}, \textit{glnB} is apparently constitutively expressed whereas \textit{glnK} expression is controlled by the two-component regulatory system NtrB/NtrC [2,18,29].

In response to nitrogen availability, the activities of both PII and GlnK are regulated by the uridylyltransferase/uridylyl-removing enzyme and by the binding of small effectors molecules [3,29]. However, Edwards and Merrick [9] showed that whilst uridylyltransferase was required for derepression of ntr-regulated promoters in \textit{K. pneumoniae}, it was not involved in the \textit{nif}-specific response to changes in nitrogen status mediated by NifL [9]. The involvement of GlnK and the independence of uridylylation was also shown by studying the inhibition of NifA activity by NifL in an heterologous system using \textit{E. coli} as the host organism [13].

Alignment of amino acid sequences of both PII and GlnK indicates that the two proteins are approximately 70% identical and are characterised by differences at just five of their 112 residues [18]. Indeed, PII and GlnK are capable of substituting for each other with respect to some of their targets and can even form heterotrimers under some circumstances [10] (van Heeswijk, unpublished results). It has previously been shown that in the absence of PII, GlnK can regulate the adenyllylation of glutamine synthetase (GS) but cannot substitute for PII in regulation of \textit{glnA} expression [2,29]. Experiments with PII have indicated that residues at the apex of the T-loop (residues 37–54) are essential for the interaction of PII with different protein receptors [19,21] and two residues in this region (52 and 54) have been identified as characteristically distinguishing PII and GlnK [18].

Given the potential interplay between PII and GlnK and the fact that \textit{glnK} expression is regulated by NtrC and hence by PII [2,18,29], we considered that it was important to study the role of each parologue when constitutively expressed in a background lacking both PII and GlnK. Using such a system, we have examined the effects on NifL/NifA interaction of altering the concentration of GlnK in the cell and the potential for substitution of GlnK by PII. Our data indicate that GlnK concentration is important for the modulation of NifA activity and that PII might play a role in this modulation.

2. Materials and methods

2.1. Bacterial strains and media

The strains, plasmids and phage used are listed in Table 1. Strains were grown in Luria broth or M9 media [24] supplemented with 0.4% glucose as carbon source, thiamine (0.4 µg ml\(^{-1}\)) and antibiotics as required: carbenicillin (100 µg ml\(^{-1}\)), chloramphenicol (15 µg ml\(^{-1}\)), gentamicin (25 µg ml\(^{-1}\)), kanamycin (30 µg ml\(^{-1}\)). The nitrogen source was varied according to the nitrogen status required (see β-galactosidase assays).

A \textit{glnK} in-frame deletion was constructed using the 3-kb \textit{BamHI-PstI} fragment from pWVH141 cloned into \textit{BamHI-PstI}-digested pUC18-Not to give pWVH152. The 214-bp \textit{ClaI-BstEII} fragment within \textit{glnK} was then replaced by a linker containing a \textit{BamHI} site (gtgacGGATCC/CCTAGGgc), resulting in an in-frame deletion within \textit{glnK} (pWVH153) that was confirmed by sequence analysis. A \textit{SmaI} gentamicin Ø cassette [26] was inserted at the \textit{SspI} site 171 bp upstream of \textit{glnK} in pWVH153 to generate pWVH159. The \textit{NotI} fragment from pWVH159 was then recloned into the temperature-sensitive plasmid pWVH115 generating pWVH163. pWVH115 is a derivative of pFC13 [8] in which a linker, containing the restriction sites \textit{SfiI}, \textit{NotI} and \textit{BssHI} (5′-GATCCCGCGCGCGCGCGGAGGCGCGCGCGCGCG-3′), was inserted at the \textit{BamHI} site. pWVH163 was then transformed into the \textit{E. coli} wild-type strain YMC10 and allelic exchange was carried out as described by Hamilton et al. [12]. Nalidixic acid (2 µg ml\(^{-1}\)) was used to facilitate the curing of the plasmid and gentamicin resistant (Gm\(^R\)) and Cm\(^S\) colonies were selected. The structure of the resulting strain WCH30 (Fig. 1) was further confirmed by Southern blotting and PCR. The double mutant \textit{aglnBK} was made by transducing the mutation from strain WCH30 into strain RB9060 using phage P1 [22], resulting in strain UNF3435. Gm\(^R\) clones were tested by PCR to verify the presence of the \textit{glnK} in-frame. 
deletion. To check the presence of the glnB deletion in UNF3435 (and RB9060), PCR was performed with various primer sets. The size of the deletion in ΔglnB2306 was estimated as 350 bp and the restriction sites for BsrEII, BglII, SacII and DraI, all of which are within the glnB gene, are deleted. The location of the 5’ end of ΔglnB2306 is estimated as 50 bp from the start codon [30].

A derivative of the glnK plasmid pWVH149 carrying a glnK Y51F mutation was constructed by replacement of the 430-bp BsrEII-SacI fragment (encoding most of glnK and the 5’ end of amtB) with a PCR fragment generated as follows. Oligonucleotides K5 and K6 (5’-GATCTATGAGCTGGTGA3’ and 5’-ATGGACGCTGAATTCCGCCC-3’, respectively) were used to generate a 160-bp fragment covering the 5’ end of glnK and oligonucleotides K7 and M13−20 (5’-CGGAATTCACTCGCA-3’ and 5’-GTAAAACGACGGCCAGT-3’, respectively) to generate a 460-bp fragment covering the 3’ end of glnK and the 5’ end of amtB. Oligonucleotides K6 and K7 incorporate a codon change TAC to TTC and the 5’ end of glnK with a codon change TAC to TTC. Oligonucleotides K5 and M13−20 were designed to introduce a novel EcoRI site. The two PCR fragments then served as templates for a single PCR reaction with glnK5 and M13−20 to generate the mutant fragment carrying the new glnK allele, glnK51, which was confirmed by sequencing.

The translational fusion pnifH-lacZ was constructed by cloning a 400-bp EcoRI-BamHI fragment from pRT22 into pRS552 resulting in pTA34. Conversion of the multicopy fusion to single-copy chromosomal fusion, by in vivo recombination between pTA34 and JRS45, was performed as described [27]. The recombinant phage carrying pnifH-lacZ fusion was then inserted in different genetic backgrounds (YMC10, WCH30 and UNF3435).

2.2. β-Galactosidase assays

 Cultures were grown for 24 h in Luria broth before subculture in M9 medium supplemented with either 0.5 mM glutamine for N limitation or 20 mM (NH4)2SO4 for N sufficiency anaerobically. β-Galactosidase assays were performed as described [22]. The results in Table 2 are the means of at least three independent experiments for each strain in which the S.D.s were not greater than ±10%.

2.3. Western blot analysis

Five μg of total protein extract was loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the gel was blotted onto a Hybond C membrane (Amersham), probed with an antisera directed against E. coli PII (which also cross-reacts with GlnK) and detected using the ECL system (Amersham). Protein concentrations of cellular extracts were determined using Pierce Coomassie Plus Protein Assay Reagent.

3. Results and discussion

3.1. Construction of ΔglnK, ΔglnBK and the heterologous system

We first constructed an in-frame deletion, ΔglnK1, within the glnK coding sequence, therefore minimising polar effects on the downstream amtB gene. An Ω gentamicin cassette was inserted upstream of glnK and downstream of the mdl gene to facilitate subsequent transfer of the ΔglnK1 allele by transduction. To avoid any potential read-through, the gentamicin gene was also cloned in the opposite orientation to the glnK amtB operon. The ΔglnK1 mutation in strain WCH30 has no polar effect on the amtB gene as judged by methylamine transport activity (Gavin Thomas, personal communication). The double mutant UNF3435 (ΔglnB2306 ΔglnK1) grew aerobically at about half the rate of the wild-type strain YMC10 in liquid minimal medium supplemented with 20 mM (NH4)2SO4 (μ = 0.33 h−1 vs. μ = 0.6 h−1) or 0.5 mM glutamine (μ = 0.33 h−1 vs. μ = 0.65 h−1).

Despite its reduced growth rate, the phenotype of this strain is quite distinct from that of the ΔglnBK strain (BK) described by Atkinson and Ninfa [2]. That strain displays a severe growth defect on solid defined media, such that it does not form single colonies, and an even more severe defect in liquid minimal media. In strain BK, a region including a large part of the 3’ end of the mdl gene upstream of glnK, the mdl-glnK intergenic region and part of glnK is replaced by a kanamycin resistance gene cassette. This insertion may be polar on the expression of amtB. Nevertheless, Atkinson and Ninfa report [2]...
that the phenotype is completely suppressed by the introduction of multicopy glnB or glnK, suggesting that neither mdd nor amtB affect the phenotype.

For this study, the nifLA operon was constitutively expressed from the lac promoter (pCC46) so that nitrogen regulation of p\textsubscript{nifH} is dependent only on the interaction of NifL with NifA. To maintain nifLA expression at a low level characteristic of its normal expression in K. pneumoniae, p\textsubscript{lac} was not induced by addition of IPTG. We also confirmed, by monitoring \(\beta\)-galactosidase expression from a wild-type lac\textsubscript{Z} gene carried on the same plasmid vector as pCC46, that plac expression was not significantly altered either by the different growth media (\(-N, +N\)) or by the different host strain genotypes (data not shown). In some experiments, a derivative of pCC46 (pCC47) containing an in-frame deletion in nifL was also used. Finally, to avoid possible titration of transcription factors and to simplify complementation studies, a K. pneumoniae\textsubscript{p}nifH\textsubscript{-}\textsubscript{lacZ} translational fusion was inserted in the E. coli chromosome.

Fig. 1. Genetic organisation of the \(\Delta\text{glnK}\) mutant. A gentamicin cassette was inserted at the SspI site upstream of glnK and a linker was used to construct the in-frame deletion and coincidentally to introduce a BamHI site.

Table 1
Strains and plasmids

<table>
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<th>Strains and plasmids</th>
<th>Genotype</th>
<th>Relevant phenotype</th>
<th>References</th>
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<td>Gln\textsubscript{B}\textsuperscript{−}</td>
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<td>Gln\textsuperscript{K}\textsuperscript{−} AmtB\textsuperscript{+}</td>
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of GlnK and GlnB under anaerobiosis in minimal media plus 20 mM (NH₄)₂SO₄ as nitrogen excess conditions or plus 0.5 mM glutamine as nitrogen-limiting conditions. Constitutive overexpression of glnK, glnK₅₁ or glnB was achieved by introduction of plasmids pWVH149, pTA48 or pAH5, respectively. It has been reported by Atkinson and Ninfa [3] that pWVH149 should have the potential to synthesise a LacZ₃-GlnK fusion protein and that when grown with IPTG induction of plc, such a protein is observed. In all our experiments, no IPTG induction was used and we could only detect synthesis of a native GlnK polypeptide (see Fig. 2 and [29]). Western blot analysis indicated that pWVH149 causes a 2–3-fold increase in cellular GlnK levels, whereas pAH5 causes a dramatic increase (estimated to be around 90-fold) in PII levels (Fig. 2A). The presence of the glnK₅₁ mutation (GlnKY₅₁F) did not affect the level of expression of the protein compared to wild-type GlnK (compare pWVH149 and pTA48, Fig. 2B).

3.2. Modulation of NifA activity is dependent on the GlnK concentration

There was negligible β-galactosidase activity driven from pniH in the absence of active NifA. When NifL and NifA were present, induction of pniH expression occurred in a wild-type or ΔglnB strain grown under N-limiting conditions but no induction occurred in a ΔglnK or ΔglnBK background (Table 2). These data confirm a role for GlnK in relieving the inhibitory effect of NifL on NifA as previously observed by He et al. [13] and Jack et al. [18]. When glnK was expressed from plc under N-limiting conditions, an increase in β-galactosidase activity of 2–4-fold was observed compared to the level in a wild-type strain without pWVH149 (Table 2). This activity was correlated with an increased intracellular level of GlnK (Fig. 2, compare lanes 5 and 7). The highest levels of pniH expression observed with increased GlnK were comparable to the lower values seen when NifA was expressed in the absence of NifL (see pCC47, Table 2). These data suggest that

Table 2
Effects of GlnK and GlnB on pniH-lacZ* expression in different backgrounds

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<td>YMC10 (wild-type)</td>
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<td>25</td>
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<td>50</td>
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<td>50</td>
<td>60</td>
<td>7600</td>
<td>8000</td>
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*β-Galactosidase activity (Miller units).
under the standard derepressing conditions used here, the wild-type strain does not realise the maximum potential level of \( \text{p}n\text{ifH} \) expression.

Our results contrast with those of He et al. [13], who compared the effects of \( \text{glnK} \) expressed from a high or low copy number plasmid (pACYC184-based or miniF-based, respectively) and reported similar levels of expression from \( \text{p}n\text{ifH-}\text{lacZ} \) in each case. It is difficult to compare these experiments directly with ours as there are a number of differences, notably, the \( \text{p}n\text{ifH-}\text{lacZ} \) fusion itself or its chromosomal location, the chromosomal genotype of the host strains and also that He et al. expressed \( \text{nifLA} \) from \( \text{p}t\text{ac} \) induced with 10 \( \mu \) IPTG. All of these factors may be important. In particular, we recognise that the relative levels of expression of all the relevant proteins may have significant effects.

Comparison of the data derived from \( \text{glnB}^{+} \) strains as compared to \( \text{glnB}^{-} \) strains reveals a negative effect of the presence of PII on the levels of derepression from \( \text{p}n\text{ifH} \) (compare YMC10(pCC46) and RB9060(pCC46) or WCH30(pCC46, pWVH149) and UNF3435(pCC46, pWVH149), Table 2). This effect is most pronounced when the GlnK protein is locked in the non-uridylylated form, GlnKY51F (compare WCH30(pCC46, pTA48) and UNF3435(pCC46, pTA48), Table 2). The levels of \( \text{p}n\text{ifH-}\text{lacZ} \) expression are consistently lower with GlnKY51F compared with wild-type GlnK. These data suggest that PII can antagonise the effect of GlnK in relieving NifA inhibition by NifL and that this effect is much more marked when GlnK is in a non-uridylylated form.

### 3.3. Overexpressed PII can substitute for GlnK

In contrast to a GlnK\(^{-} \) mutant, a PII\(^{-} \) mutant has no effect on NifL inhibition of NifA activity (Table 2). This difference is quite striking given the similarity between the two proteins but reflects other specificity differences already recognised between PII and GlnK and described above [2,29]. In order to determine whether PII has any ability at all to substitute for GlnK, we significantly overexpressed \( \text{glnB} \) (using pAH5) in the \( \Delta\text{glnBK} \) background (Table 2). In this case, the \( \text{EcoRI-}\text{SalI} \) fragment containing the \( \text{glnB} \) gene cloned into \( \text{EcoRI-}\text{SalI} \) sites of pUC18 allows for the expression of \( \text{glnB} \) from its own promoter and also from \( \text{plac} \). When PII was overexpressed (Fig. 2, lanes 10 and 11), we observed relief of the NifL-mediated inhibition to levels comparable to those seen in the wild-type strain, indicating that PII is capable of substituting for GlnK. However, in this case, the PII concentration was estimated as some 90 times higher than in the \( \Delta\text{glnK} \) mutant (Fig. 2, lanes 9 and 11). We conclude that the binding coefficient of PII in its presumed interaction with one or more components of the NifLA complex is significantly lower than that of GlnK but that this is overcome when the protein concentration is raised to such levels. Whilst this situation is clearly non-physiological, such a phenomenon has been observed previously in that PII has also been shown to activate the deadenylylation of GS-AMP in vitro when present in a relative high concentration [20].

### 3.4. The nitrogen response

In a wild-type strain, \( \text{glnK} \) expression is very low or completely absent under nitrogen excess (+N) conditions [2] and the expression of \( \text{p}n\text{ifH} \) is around 1–2% of the induced (−N) level, i.e. hardly above background (Table 2). By contrast, when GlnK is overexpressed in YMC10 or UNF3435, the level of expression in +N increases very significantly, to 20% of the −N level. It would therefore appear that the presence of elevated levels of GlnK even under nitrogen excess conditions is sufficient to allow for relief of NifA inhibition. Nitrogen-limiting conditions induce a further 5-fold increase in \( \text{p}n\text{ifH} \) expression with no apparent increase in GlnK levels (see Fig. 2, lanes 6 and 7). A similar effect is seen when PII is overexpressed in nitrogen sufficiency. \( \text{p}n\text{ifH} \) expression is elevated 4–7-fold compared to the wild-type strain but these activities increase by a further 6–10-fold when nitrogen is limiting (Table 2).

### 4. Conclusions

In the heterologous system that we have used to analyse the effects of GlnK and PII on the relief of NifL-mediated inhibition of NifA activity, we find that the ‘effective’ NifA activity can be increased by an increase in the cellular concentration of GlnK. This suggests that under normal derepressing
conditions in a wild-type cell, a significant proportion of NifA may still be complexed with NifL and therefore inactive. Such a hypothesis is supported by the fact that the induced level of psnH expression in the wild-type is significantly elevated when nifL is inactivated both in E. coli (this work) and in K. pneumoniae [1]. These data also suggest that in wild-type K. pneumoniae, maximal nitrogenase expression might not be achieved under normal nitrogen limitation. This is consistent with the phenomenon of hyperinduction where elevated nitrogenase activity is observed when K. pneumoniae is grown under argon rather than atmospheric nitrogen [25].

Our data also suggest that the relief of NifL-mediated inhibition may involve both changes in the intracellular level of GlnK and changes in its activity. It is known that nitrogen limitation leads to a marked elevation of glnK expression (both in E. coli and in K. pneumoniae) and our data indicate that the consequent increase in the intracellular GlnK concentration is alone sufficient to promote relief of inhibition. Both our data and previous reports [9,13] demonstrate that this effect is independent of the uridylylation of GlnK. Consequently, relief of inhibition is also seen when GlnK is expressed in nitrogen sufficiency. However, it is also clear that the levels of relief achieved are significantly greater in nitrogen limitation than nitrogen sufficiency. With either GlnK or GlnKY51F, activation of psnH expression increases 5–8-fold in nitrogen limitation, indicating that a second component (which could be some other modification of GlnK or a change in the level of a critical metabolite) is required to achieve full induction.

Finally, when we uncouple transcriptional control of glnK from the Ntr system, we observe a marked antagonistic effect of PII on GlnK that is more pronounced when GlnK is not uridylylated. This effect could be direct or indirect but could conceivably occur by the formation of heterotrimers between PII and GlnK with a consequent inactivation of GlnK. It has been reported that, unlike PII, GlnK is not rapidly deuridylylated by the uridylyl-removing activity of uridylyltransferase [3]. Hence, given that both the uridylylated and deuridylylated forms of GlnK are active in relieving NifL-mediated inhibition, the question arises as to how NifL inhibition of NifA activity might be rapidly restored when ammonium is added back to the medium [13]. Given that our data suggest a potential interaction between PII and GlnK, one possibility is that inactivation of GlnK is actually mediated through PII. Such an effect could occur via heterotrimer formation between PII and GlnK, if GlnK-UMP has a greater affinity for PII than for PII-UMP.

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