

## Two Residues in the T-loop of GlnK Determine NifL-dependent Nitrogen Control of *nif* Gene Expression\*

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X-ray crystallographic analysis of the *Escherichia coli* P<sub>II</sub> protein paralogues GlnB and GlnK has shown that they share a superimposable structural core but can differ in conformation of the T-loop, a region of the protein (residues 37–54) that has been shown to be important for interaction with other proteins. In *Klebsiella pneumoniae* GlnK has been shown to have a clearly defined function in regulating NifL-mediated inhibition of NifA activity in response to the nitrogen status, and GlnB, when expressed from the chromosome, does not substitute for GlnK. Because the T-loops of *K. pneumoniae* and *E. coli* GlnB and GlnK differ at just three residues, 43, 52, and 54, we have used a previously constructed heterologous system, in which *K. pneumoniae nifLA* is expressed in *E. coli*, to investigate the importance of GlnK residues 43, 52, and 54 for regulation of the NifLA interaction. By site-directed mutagenesis of *glnB* we have shown that residue 54 is the single most important amino acid in the T-loop in the context of the regulation of NifA activity. Furthermore, a combination of just two changes, in residues 54 and 43, allows GlnB to function as GlnK and completely relieve NifL inhibition of NifA activity.

Both *Escherichia coli* and *Klebsiella pneumoniae*, together with many other members of the Proteobacteria, encode two members of the P<sub>II</sub> signal transduction protein family, GlnB and its recently discovered paralogue GlnK (1–3). In response to nitrogen availability, the activities of both GlnB and GlnK are regulated by the uridylyltransferase/uridylyl-removing enzyme (UTase)<sup>1</sup> at the Tyr-51 residue and by the binding of small effector molecules (3–5). The unmodified form of GlnB, which acts as a signal of nitrogen excess, stimulates dephosphorylation of NtrC by NtrB and adenylylation of glutamine-synthetase (GS) by adenylyltransferase (ATase), thereby inhibiting both the activity of GS and the expression of its structural gene *glnA*. Conversely, the modified form GlnB~UMP, which is a signal of nitrogen starvation, stimulates deadenylylation of GS by ATase, which allows full activity of GS. GlnB~UMP does not interact with NtrB so that the predominant activity of NtrB

in nitrogen-limiting conditions is to promote phosphorylation of NtrC (for review see Ref. 6).

*E. coli* GlnK can substitute for GlnB functions both *in vivo* and *in vitro* although the proteins are not completely interchangeable in all circumstances. In the absence of GlnB, GlnK can regulate adenylylation of GS *in vivo* and can substitute for GlnB in NtrC-dependent regulation of *glnA* expression (3, 7). Likewise *in vitro* studies show that both GlnB and GlnK can activate the phosphatase activity of NtrB and the adenylylation of GS by ATase, although GlnK is less effective than GlnB in the latter reaction (4). The distinction between the roles of GlnB and GlnK is confounded still further by the recognition that the proteins can form heterotrimers *in vivo* (8, 9). Hence there are clearly still a number of unexplained properties of these proteins, and the precise physiological roles of GlnB and GlnK in *E. coli* remain to be determined (7, 10). There is, however, one well defined function where GlnK has been shown to have a specific role, namely in regulating the interaction between the *K. pneumoniae* nitrogen fixation regulatory proteins NifL and NifA in response to the intracellular nitrogen status (1, 11). In this case, GlnB can only substitute for GlnK when overproduced to non-physiological levels (12), and consequently this system offers the possibility of investigating how the specificity of interaction might be determined.

Both GlnB and GlnK form homotrimers, and x-ray crystallographic analysis indicates that their structural cores are very similar but that the conformation of the T-, C-, and B-loops can differ significantly (13). In *E. coli*, the apical T-loop of the GlnB protein (residues 37–54), which includes the site of uridylylation (Tyr-51), is necessary for interaction with all three of its known targets (ATase, UTase, and NtrB) (14, 15). Alignment of the amino acid sequences of GlnB and GlnK indicates that in *E. coli* and *K. pneumoniae* the two proteins are 67% and 68% identical, respectively. The *E. coli* and *K. pneumoniae* GlnB T-loops or GlnK T-loops are 100% identical, but in each organism the GlnB and GlnK T-loops differ at just 3 residues (43, 52, and 54) of 18 and exhibit an identical codon change in each case (Fig. 1).

Previous studies showed that substitutions of residues Gly-41 and Ala-49 in the T-loop of *E. coli* GlnB significantly affect the interactions of the protein with UTase and NtrB, respectively, without exhibiting effects on other targets (16). This suggests that the conformation of the T-loop can determine specific interactions of P<sub>II</sub> proteins and that a single amino acid within the loop could have important effects on this conformation. Given these observations we wished to investigate whether the differences between the T-loops of GlnB and GlnK account for the specific requirement for GlnK in regulating the interaction between *K. pneumoniae* NifL and NifA. We have done this using a previously constructed heterologous system in which *K. pneumoniae nifLA* is expressed in *E. coli* (12) and have then used site-directed mutagenesis of GlnB to

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<sup>1</sup> The abbreviations used are: UTase, uridylyltransferase/uridylyl-removing enzyme; GS, glutamine-synthetase; ATase, adenylyltransferase; bp, base pair.

	37	55
<i>E. coli</i> or <i>K. pneumoniae</i> GlnB	GRQKGHTELYRGAEY <b>M</b> VDF	
<i>E. coli</i> or <i>K. pneumoniae</i> GlnK	GRQKGHAELYRGAEY <b>S</b> VNF	
<i>A. vinelandii</i> GlnK	GRQKGHTELYRGAEY <b>V</b> VDF	

FIG. 1. **T-loop sequence comparison.** Alignment of the amino acid sequences of the T-loops (residues 37–55) of *E. coli* or *K. pneumoniae* GlnB and GlnK and *A. vinelandii* GlnK. Residues that differ between the T-loops are indicated in **bold**.

examine the importance of residues 43, 52, and 54 in NifL-dependent regulation of *nif* gene expression.

#### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Media**—Bacterial strains and plasmids are listed in Table I. A derivative of the *E. coli* *glnK* plasmid pWVH149 in which the *glnK* coding sequence is precisely replaced by that of *glnB* was constructed by replacement of the 435-bp *EcoRI-SacI* fragment with a 435-bp *EcoRI-SacI* fragment generated by polymerase chain reaction using BK1 (5'-CCGAATTCTGACCGGAGGGGATCTATGAAA-AAGATTGATGCG-3') and BK2 (5'-GACTGAGCTCATTACGAATGCT-TTGGC-3') oligonucleotides with pAH5 as a template. In the resulting plasmid, pTA49, *glnB* is transcribed from *plac* and translated from the *glnK* ribosome binding site. We then used site-directed mutagenesis with a variety of primer pairs and pTA49 as a template to create single point mutations altering GlnB residues 43, 52, and 54 and all combinations of the three mutations. The 164-bp *EcoRI-SacII* fragment of pTA49 was replaced by a 164-bp *EcoRI-SacII* fragment generated with the mutagenic BK3 oligonucleotide (5'-CGCGCCGCGGTACAGCTCG-GCATGGC-3') and the reverse primer, generating pTA52 (*glnB* T43A). The 285-bp *SacII-SacI* fragment of pTA49 was replaced by the 285-bp *SacII-SacI* fragment generated with the universal M<sub>13-20</sub> primer and mutagenic oligonucleotides BK4 (5'-TGTACCGCGCGCGGAGTAT-TCGGTGG-3'), BK5 (5'-TGTACCGCGCGCGGAGTATATGGTGAATTTTC-3'), or BK6 (5'-TGTACCGCGCGCGGAGTATTCGGTGAATTTTC-3'), generating pTA53 (*glnB* M52S), pTA54 (*glnB* D54N), and pTA55 (*glnB* D54N;M52S), respectively. The replacement of the 164-bp *EcoRI-SacII* fragment of pTA53, pTA54, or pTA55 with the 164-bp *EcoRI-SacII* fragment of pTA52 then generated pTA58 (M52S;T43A), pTA56 (D54N;T43A), and pTA57 (M52S;T43A;D54N), respectively. All new *glnB* alleles were confirmed by DNA sequencing.

**$\beta$ -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 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> for N-sufficiency. Cells were grown anaerobically at 30 °C for 18 h, and  $\beta$ -galactosidase assays were performed as described (17).

**Western Blot Analysis**—5  $\mu$ g of total protein extract from cells grown in N-limitation (see " $\beta$ -Galactosidase Assays") were loaded onto a 15% SDS-polyacrylamide gel. After electrophoresis the gel was blotted onto a HybondC membrane (Amersham Pharmacia Biotech), probed with an antiserum directed against *E. coli* PII (which also cross-reacts with GlnK), and detected using the ECL system (Amersham Pharmacia Biotech). Protein concentrations of cellular extracts were determined using Pierce Coomassie Plus protein assay reagent.

#### RESULTS

In *K. pneumoniae*, the activity of the *nif*-specific transcriptional activator NifA is modulated in response to nitrogen availability by NifL. The signal transduction protein GlnK is specifically required in nitrogen-limiting conditions to relieve the inhibitory effect of NifL on NifA, and this effect of GlnK cannot be substituted by GlnB (1, 11). The rationale for these experiments was that if the GlnK specificity for regulation of the NifLA interaction resides in the T-loop, then it might be possible to convert GlnB (which differs from GlnK at a total of 37 residues) to GlnK by changing just one or more of the three T-loop-specific residues 43, 52, and 54. For this study, we used a previously constructed heterologous system in which a *K. pneumoniae* *pnifH-lacZ* fusion is inserted in the *E. coli* chromosome, and the *nifLA* operon is constitutively expressed from the *lacZ* promoter on plasmid pCC46. In this situation nitrogen regulation of *pnifH* is dependent only on the interaction of NifL with NifA as modulated by GlnK (12).

**Construction of *glnB* Mutants**—Normal NifL-dependent regulation of NifA activity can be achieved when *glnK* is expressed from *plac* (pWVH149) in a  $\Delta$ *glnBK* background (12). The initial step was to construct a derivative of pWVH149 in which the *glnK* coding sequence was replaced precisely with *glnB* (pTA49). Site-directed mutagenesis of *glnB* on pTA49 was then used to change the threonine 43, methionine 52, and aspartate 54 codons so as to code for alanine 43 (pTA52), serine 52 (pTA53), and asparagine 54 (pTA54), respectively. In addition, we constructed all possible double mutants, *i.e.* M52S,D54N (pTA55); M52S,T43A (pTA58); and D54N,T43A (pTA56) and the triple mutant M52S,T43A,D54N (pTA57). By Western blotting we then showed that the presence of the different mutations did not significantly affect the steady state level of the protein compared with wild type GlnB in either nitrogen excess (+N) (data not shown) or starvation -N conditions (Fig. 2).

**Modulation of NifA Activity by GlnB Variants**—We had previously shown that GlnB expressed under normal physiological conditions (*i.e.* from its own promoter on the chromosome) is unable to relieve NifL-mediated inhibition and indeed might have an antagonistic effect on GlnK in some situations (12). We then studied NifL inhibition of NifA activity in a  $\Delta$ *glnBK* (pCC46) background carrying the *glnB* wild type or mutant genes. Cells were grown under anaerobiosis in minimal media in +N or -N conditions. Constitutive expression of the *glnB* wild type or mutant genes was achieved by introduction of the relevant plasmids (pTA49, pTA52, pTA53, pTA54, pTA55, pTA56, pTA57, and pTA58).

When either *glnB* or *glnK* were constitutively expressed in an identical context, GlnB was again far less effective than GlnK in relieving NifL-mediated inhibition, giving just 17% of the GlnK-mediated activity (compare pTA49 and pWVH149, Table II). The activity that is observed with GlnB is due to the relative overexpression of *glnB* compared with the expression levels obtained with the chromosomal gene (compare  $\Delta$ *glnK* (pCC46) with  $\Delta$ *glnBK* (pCC46, pTA49), Table II). The assay can therefore distinguish readily between the activities of GlnK and GlnB in regulating the NifLA interaction and can be used to examine the activities of the different GlnB variants.

Neither the T43A or M52S GlnB variants showed any increase in *pnifH* expression compared with wild type GlnB, but the single D54N GlnB protein exhibited 56% of the activity obtained with GlnK (Table II). Analysis of the efficiency of the *glnB* double mutants in relieving NifL-mediated inhibition showed that combinations of T43A and M52S or of M52S and D54N, although slightly better than wild type GlnB, were not nearly as effective as the single D54N GlnB variant (Table II). However, the combination of D54N and T43A in either the double (pTA56) or the triple mutant (pTA57) resulted in a further significant increase up to 92% of the maximal *pnifH* activation seen with GlnK (compare pWVH149 with pTA56 or pTA57, Table II).

When GlnK is overexpressed (pWVH149) during nitrogen sufficiency, the expression from *pnifH* increases significantly compared with a wild type strain (Table II). By contrast, when GlnB is overexpressed from pTA49, the expression level from *pnifH* in +N does not exceed that seen with a  $\Delta$ *glnBK* (pCC46) background. This intracellular concentration of GlnB is therefore not sufficient to allow relief of NifL inhibition in nitrogen sufficiency, although nitrogen-limiting conditions result in an 8-fold induction. With those GlnB proteins that mimic GlnK, we observed an increase in both the -N and the +N *pnifH* expression level such that the +N level almost reaches that obtained with GlnK overexpression (compare pWVH149 with pTA56 or pTA57, Table II). Hence the elevation of NifA-mediated expression from *pnifH* that we observe in +N is correlated

TABLE I  
Strains and plasmids

Strains and plasmids	Genotype	References
<i>E. coli</i> strains		
YMC10	$\Delta lacU169 endA1 thi-1 hsdR17 supE44$	24
WCH30	$\Delta lacU169 endA1 thi-1 hsdR17 supE44 \Delta glnK1$	12
UNF3435	$\Delta lacU169 endA1 thi-1 hsdR17 supE44 \Delta glnB2306 \Delta glnK1$	12
Plasmids		
pAH5	<i>E. coli glnB</i> expressed from its own promoter in pUC18	25
pCC46	<i>K. pneumoniae nifLA</i> expressed from <i>placZ</i> in pHSG575	26
pMM78	<i>pnifL-lacZ</i> fusion cloned in pACYC184	21
pTA49	<i>E. coli glnB</i> expressed from <i>placZ</i> in pBluescript-II SK+	This work
pTA52	pTA49 derivative carrying <i>glnBT43A</i> mutation	This work
pTA53	pTA49 derivative carrying <i>glnBM52S</i> mutation	This work
pTA54	pTA49 derivative carrying <i>glnBD54N</i> mutation	This work
pTA55	pTA49 derivative carrying <i>glnBD54N,M52S</i> mutations	This work
pTA56	pTA49 derivative carrying <i>glnBD54N,T43A</i> mutations	This work
pTA57	pTA49 derivative carrying <i>glnBD54N,M52S,T43A</i> mutations	This work
pTA58	pTA49 derivative carrying <i>glnBM52S,T43A</i> mutations	This work
pWVH149	<i>E. coli glnK</i> expressed from <i>placZ</i> in pBluescript-II SK+	3

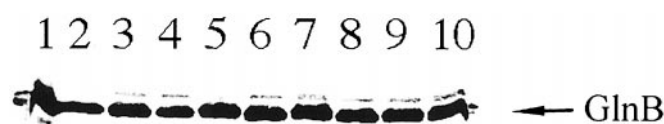


FIG. 2. Western blot of GlnB variants. Lane 1, purified GlnB; lanes 2–10, extracts from cells grown in  $-N$ . The host strain was  $\Delta glnBK$  (pCC46) carrying: lane 2 (pWVH149), lane 3 (pTA49), lane 4 (pTA52), lane 5 (pTA53), lane 6 (pTA54), lane 7 (pTA55), lane 8 (pTA56), lane 9 (pTA57), lane 10 (pTA58).

directly with the efficiency of the variant GlnB proteins to relieve NifL-specific inhibition in  $-N$ . Likewise, the induction ratio ( $-N/+N$ ) is in nearly all cases between 6 and 8.

**Effects of T-loop Changes on GlnB Interaction with NtrB**—The GlnB and GlnK proteins regulate the activities of other proteins involved in nitrogen assimilation, namely ATase and NtrB. In response to the nitrogen status of the cell, NtrB promotes phosphorylation or dephosphorylation of the transcriptional activator NtrC, and dephosphorylation of NtrC is modulated in accordance with the uridylylation state of either GlnB or GlnK (7). To determine whether the alterations in GlnB that facilitated its interaction with the NifLA system had altered its ability to function with a different target protein, namely NtrB, we studied the expression of an NtrC-dependent promoter by using a *pnifL-lacZ* fusion (carried on pMM78) in a  $\Delta glnBK$  background. As previously predicted by Atkinson and Ninfa (7), we observed overexpression in  $+N$  and  $-N$  of the *nifL-lacZ* fusion in a  $\Delta glnBK$  background. Wild type regulation was restored by the introduction of a wild type *glnB* (pTA49) or *glnK* (pWVH149) gene but also by each of the *glnB* mutants (Table III). These results indicate that, as might be expected, all of the GlnB variants were still competent to interact with one of their natural targets, namely NtrB.

**Comparison of the T-loop Structures in GlnB and GlnK**—A superposition of the *E. coli* GlnB and GlnK structures in which the T-loops are not disordered (Protein Data Bank (PDB) (18) accession codes 2PII and 1GNK, respectively) shows a good agreement in the core regions, but their T-loop conformations differ greatly (Fig. 3). Residues 43 and 52 reside in the middle portion of the loop and occupy entirely different positions in space, their C $\alpha$ s being separated by distances of around 11.7 and 7.5 Å, respectively. By contrast, residue 54 lies at the C-terminal end of the loop where the agreement between the two structures resumes. Analysis of the main chain torsion angles for this residue gives values of  $\phi = -93^\circ$ ,  $\psi = -11^\circ$  for GlnB and  $\phi = -112^\circ$ ,  $\psi = 152^\circ$  for GlnK, suggesting that the main chain configuration adopted by this residue is a major determinant of the T-loop conformation. In the GlnB structure,

the side chain of Asp-54 is directed toward the N-terminal end of the T-loop and forms hydrogen bonds through its O<sup>δ2</sup> with the main chain NH group of Arg-47 and the side chain of Arg-17 in a neighboring subunit of the trimer. Conversely, in the GlnK structure, the side chain of Asn-54 is directed outward and forms crystal contacts through its O<sup>δ2</sup> to the main chain NH groups of residues 26 and 27 in a neighboring trimer.

#### DISCUSSION

The results reported in this study indicate that residue 54 in the T-loop is the single most important amino acid in determining the discrimination between the GlnK and GlnB proteins in the context of the regulation of NifA activity. Furthermore, the T43A change significantly enhances the major effect seen with D54N such that either GlnB D54N,T43A or GlnB D54N,T43A,M52S mimic almost perfectly the GlnK protein in relieving NifL inhibition of NifA activity.

It was therefore of interest to consider these results in the context of our present knowledge of the relevant protein structures. Such considerations need to take into account the facts that neither of the structures currently available are complexed with 2-ketoglutarate and that only the GlnK structure has ATP bound. Because binding of these small molecules is synergistic and likely to affect the conformation of the proteins, the present structures may not reflect the “active” forms of the proteins (10). Nevertheless comparison of the available structures does suggest that the main chain conformation of the residue at position 54 is potentially a major determinant of the T-loop conformation. In GlnB Asp-54 forms hydrogen bonds with the main chain NH group of Arg-47 and the side chain of Arg-17 in a neighboring subunit of the trimer, and although the substitution of Asp by Asn at this position would not preclude these interactions, they would undoubtedly be weaker because of the neutrality of this side chain. It has already been suggested that these T-loop structures are largely a result of their interactions with the crystalline environment and that in solution they do not have a rigid structure (13). This argument is supported by the observation that in both the available GlnK crystal structures (PDB codes 1GNK and 2GNK) there is at least one trimer with disordered T-loops. It seems likely that this flexibility may be essential for the T-loop to interact with different target proteins. We conclude that the D54N,T43A double mutation modifies the characteristics of the T-loop in such a way that the altered GlnB protein is now fully competent to interact with its target, be that NifL or NifA or the complex of both proteins, to relieve the NifL-mediated inhibition of NifA activity. However, the exact role of residues 43 and

TABLE II  
Effects of GlnB T-loop variants on NifL-mediated regulation of NifA activity

NifA activity was assessed by using *pnifH-lacZ* expression for which the  $\beta$ -galactosidase activity is given in Miller units. Values are the average of at least three independent experiments for each strain. Results from duplicate cultures generally differed by <15%. -N, Nitrogen-limiting medium; +N, Nitrogen-sufficient medium.

Strain	Relevant genotype <sup>a</sup>	$\beta$ -Galactosidase activity	
		-N	+N
YMC10(pCC46)	<i>glnB</i> <sup>+</sup> , <i>glnK</i> <sup>+</sup>	2200	6
WCH30(pCC46)	$\Delta$ <i>glnK</i>	60	50
UNF3435 (pCC46)	$\Delta$ <i>glnBK</i>	145	100
UNF3435 (pCC46)(pWVH149)	$\Delta$ <i>glnBK</i> ( <i>glnK</i> <sup>+</sup> )	4850	800
UNF3435 (pCC46)(pTA49)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> <sup>+</sup> )	810	100
UNF3435 (pCC46)(pTA52)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> T43A)	510	70
UNF3435 (pCC46)(pTA53)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> M52S)	670	110
UNF3435 (pCC46)(pTA54)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N)	2710	215
UNF3435 (pCC46)(pTA55)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N,M52S)	1640	210
UNF3435 (pCC46)(pTA56)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N,T43A)	4450	680
UNF3435 (pCC46)(pTA58)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> M52S,T43A)	1230	220
UNF3435 (pCC46)(pTA57)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N,M52S,T43A)	4010	625

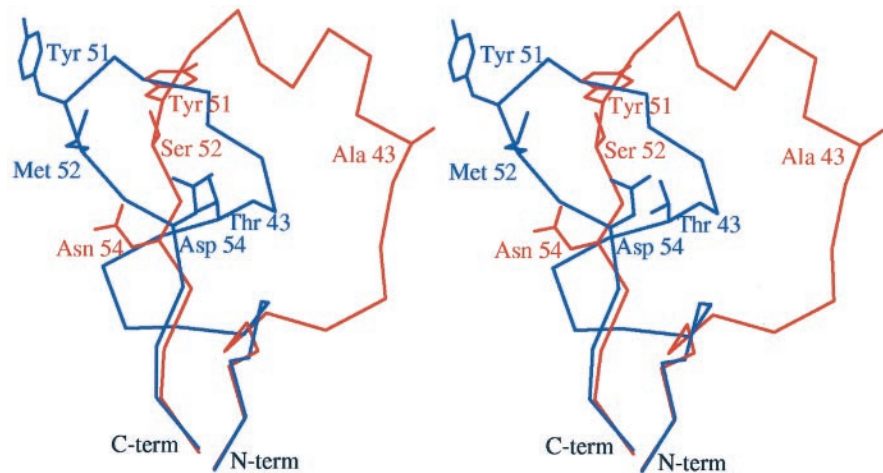
<sup>a</sup> All strains carry a chromosomal recombinant  $\lambda$  phage containing a *pnifH-lacZ* translational fusion (12).

TABLE III  
Effects of GlnB T-loop variants on NtrB activity

NtrB activity was assessed by using *pnifL-lacZ* expression for which the  $\beta$ -galactosidase activity is given in Miller units. Values are the average of at least three independent experiments for each strain. Results from duplicate cultures generally differed by <15%. -N, Nitrogen-limiting medium; +N, Nitrogen-sufficient medium.

Strain	Relevant genotype	$\beta$ -Galactosidase activity	
		-N	+N
YMC10(pMM78)	<i>glnB</i> <sup>+</sup> , <i>glnK</i> <sup>+</sup>	2450	410
RB9060(pMM78)	$\Delta$ <i>glnB</i>	2740	830
WCH30 (pMM78)	$\Delta$ <i>glnK</i>	3090	520
UNF3435 (pMM78)	$\Delta$ <i>glnBK</i>	5230	4680
UNF3435 (pMM78)(pWVH149)	$\Delta$ <i>glnBK</i> ( <i>glnK</i> <sup>+</sup> )	2450	360
UNF3435 (pMM78)(pTA49)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> <sup>+</sup> )	1160	410
UNF3435 (pMM78)(pTA52)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> T43A)	2140	420
UNF3435 (pMM78)(pTA53)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> M52S)	1350	360
UNF3435 (pMM78)(pTA54)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N)	1660	450
UNF3435 (pMM78)(pTA55)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N,M52S)	1870	380
UNF3435 (pMM78)(pTA56)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N,T43A)	2120	410
UNF3435 (pMM78)(pTA58)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> M52S,T43A)	2950	420
UNF3435 (pMM78)(pTA57)	$\Delta$ <i>glnBK</i> ( <i>glnB</i> D54N,M52S,T43A)	2120	250

FIG. 3. **Overlay of GlnB and GlnK T-loop structures.** Stereo figure comparing the backbone conformations of the T-loops from GlnB in blue (PDB code 2PII) and GlnK in red (PDB code 1GNK). Only the side chains of the three amino acids that differ between the two structures and the conserved Tyr-51 residues are shown. The positions of the N- and C-terminal ends of the T-loops are also indicated. This figure was produced using the programs O and OPLLOT (27).



54 cannot easily be determined without knowledge of the T-loop structure under these conditions.

Although residue Tyr-51 in the T-loop is the site of uridylylation in both GlnB and GlnK, it has been shown that the effect of GlnK in modulating NifA activity is independent of the uridylylation state of the protein (11, 12, 19). Hence although Jaggi *et al.* (14) showed that alteration of residue Tyr-46 in the T-loop affects the rate of uridylylation of the GlnB protein, we can exclude the possibility that effects on the uridylylation state of GlnB are responsible for the altered specificity of GlnB D54N,T43A.

*The T-loop as a Discriminator between GlnB and GlnK*—The presence of two P<sub>II</sub>-like proteins, designated GlnB and GlnK, is now recognized as a common feature in many bacteria. The most conserved feature of the related genetic architecture is the linkage of the gene encoding GlnK to that encoding a proposed high affinity ammonium transporter, AmtB (20). It has been suggested that this conserved linkage could reflect a functionally important interaction between these two proteins, but GlnK may well have evolved other important interactions, including that which regulates NifLA interaction in *K. pneumoniae*. As a consequence, if the T-loop of the protein is indeed

important for protein-protein interactions, then the amino acid sequence of the T-loop may have evolved to reflect these diverse functions.

A comparison of P<sub>II</sub> protein sequences from six species of proteobacteria having both GlnB and GlnK identified five residues that apparently serve to distinguish GlnB and GlnK, and just two of these, residues 52 and 54, are in the T-loop (1). Residue 43 is almost invariably threonine in both GlnB and GlnK, and so the fact that this residue is alanine in *E. coli* and *K. pneumoniae* GlnK is not typical. In GlnB proteins, residues 52 and 54 are well conserved; residue 52 is either methionine, isoleucine, or valine, and residue 54 is invariably aspartate. However, in GlnK proteins these residues are much more variable; residue 52 is serine, valine, or alanine, and residue 54 is serine, aspartate, or asparagine, suggesting that the conformation of the T-loop in GlnK proteins could be somewhat more variable than in GlnB proteins.

The designation of P<sub>II</sub> proteins as either GlnB or GlnK on the basis of the genetic linkage of their structural genes may not of course always reflect structural or functional similarity (20). Apart from *K. pneumoniae*, one other diazotroph, namely *Azotobacter vinelandii*, has a well characterized NifLA regulatory system, and in this organism a P<sub>II</sub>-like protein has also been implicated in *nif* gene regulation via NifLA (21, 22). *A. vinelandii* apparently has only one P<sub>II</sub>-like protein that is encoded in a *glnKamtB* operon, but this GlnK protein has a T-loop in which residues 43, 52, and 54 are threonine, valine, and aspartate, respectively (Fig. 1), *i.e.* more characteristic of GlnB-like proteins (23). The conclusion that *A. vinelandii* GlnK has a "GlnB-like" T-loop is supported by studies in a heterologous system in which *E. coli* GlnB rather than GlnK was found to be effective in regulating *A. vinelandii* NifLA function (22). Such considerations suggest that the interactions between GlnK and the NifLA complex are likely to be quite specific to each organism.

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