Role of *Escherichia coli* Nitrogen Regulatory Genes in the Nitrogen Response of the *Azotobacter vinelandii* NifL-NifA Complex

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The redox-sensing flavoprotein NifL inhibits the activity of the nitrogen fixation (*nif*)-specific transcriptional activator NifA in *Azotobacter vinelandii* in response to molecular oxygen and fixed nitrogen. Although the mechanism whereby the *A. vinelandii* NifL-NifA system responds to fixed nitrogen in vivo is unknown, the *glnK* gene, which encodes a PII-like signal transduction protein, has been implicated in nitrogen control. However, the precise function of *A. vinelandii* NifL in this response is difficult to establish because of the essential nature of this gene. We have shown previously that *A. vinelandii* NifL is able to respond to fixed nitrogen to control NifA activity when expressed in *Escherichia coli*. In this study, we investigated the role of the *E. coli* PII-like signal transduction proteins in nitrogen control of the *A. vinelandii* NifL-NifA regulatory system in vivo. In contrast to recent findings with *Klebsiella pneumoniae* NifL, our results indicate that neither the *E. coli* PII nor GlnK protein is required to relieve inhibition by *A. vinelandii* NifL under nitrogen-limiting conditions. Moreover, disruption of both the *E. coli* glnB and *ntrC* genes resulted in a complete loss of nitrogen regulation of NifA activity by NifL. We observe that *glnB ntrC* and *glnB glnK ntrC* mutant strains accumulate high levels of intracellular 2-oxoglutarate under conditions of nitrogen excess. These findings are in accord with our recent in vitro observations (R. Little, F. Reyes-Ramirez, Y. Zhang, W. Van Heeswijk, and R. Dixon, EMBO J. 19:6041–6050, 2000) and suggest a model in which nitrogen control of the *A. vinelandii* NifL-NifA system is achieved through the response to the level of 2-oxoglutarate and an interaction with PII-like proteins under conditions of nitrogen excess.

In diazotrophic proteobacteria, the σ^74^-dependent activator NifA activates transcription of the *nif* (nitrogen fixation) genes by a conserved mechanism common to members of the enhancer binding protein family (5). Although NifA proteins have similar domain structures, both transcriptional regulation of *nifA* expression and posttranslational regulation of NifA activity by oxygen and fixed nitrogen vary significantly from one organism to another.

Signal transduction in response to fixed nitrogen status has been best studied in *Escherichia coli* at both the genetic and biochemical levels (26, 27). Cells respond to changes in nitrogen availability by modifying the activity of a key sensory regulatory protein, PII, the product of *glnB*. Under conditions of nitrogen excess, when the internal concentration of glutamine is high, PII is primarily unmodified; in conditions of nitrogen limitation, PII is mainly uridylylated. The enzyme responsible for this covalent modification is an uridylyltransferase/uridylyl-removing enzyme (UTase/UR) encoded by the *glnD* gene. The degree of uridylylation of PII has major physiological implications with respect to both the level and activity of glutamine synthetase (GS), the product of *glnA*. Under nitrogen-excess conditions, native PII protein prevents transcription from the *ntr* promoters by stimulating the histidine protein kinase NtrB to dephosphorylate its cognate response regulator, NtrC (16, 17), leading to decreased expression of *glnA*. In addition, PII stimulates the enzyme adenylyltransferase to adenylylate GS, which decreases its activity (15, 18). Under nitrogen limitation, uridylylation of PII prevents its interaction with NtrB, and thus NtrC is maintained primarily in its phosphorylated form. In addition, PII-UMP stimulates the deadenylation activity of adenylyltransferase, which catalyzes the conversion of the inactive GS-AMP to active GS.

*E. coli* contains a second PII-like protein, which is encoded by the *glnK* gene (36, 37). This PII paralogue is also regulated by the UTase/UR in response to nitrogen availability and also plays a role in nitrogen regulation (2, 3). While expression of the *glnB* gene is constitutive with respect to the intracellular nitrogen status, *glnK* is encoded in the *NtrC*-dependent operon *glnK-amtb*, in which *amtb* encodes a high-affinity ammonium transporter (33). Thus, expression of *glnK* is subject to nitrogen regulation by the NtrB-NtrC two-component regulatory system, and essentially little GlnK is expressed under conditions of nitrogen excess.

Many proteobacteria express more than one homologue of the PII protein, and current evidence suggests that at least one of the PII-like proteins is involved in nitrogen control of NifA expression and/or activity in several diazotrophs. In *Klebsiella pneumoniae* and *Azotobacter vinelandii*, which are members of the gamma subdivision of Proteobacteria, *nifA* is coordinately transcribed with a second gene, *nifL*, whose product inhibits NifA activity in response to oxygen and fixed nitrogen (9, 10). Transcription of the *K. pneumoniae* *nifL* operon is activated by the phosphorylated form of NtrC and is therefore influenced by nitrogen availability, whereas in *A. vinelandii* *nifL* expression is constitutive. The identification of two PII-like proteins in enteric bacteria has facilitated analysis of nitrogen control of nitrogen fixation in *K. pneumoniae*. Recent evidence

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suggests that the alternative PII-like protein GlnK is required to relieve inhibition of NifA activity by *K. pneumoniae* NifL under nitrogen-limiting conditions (12, 14). This conclusion is based on the observation that NifL inhibits NifA activity irrespective of the nitrogen status in enteric *glnK* mutants. Furthermore, the uridylylation state of GlnK is apparently irrelevant for relief of inhibition by NifL in *K. pneumoniae* (11) and in *E. coli* (12). Although GlnK clearly has a role in maintaining NifL in an inactive state under derepressing conditions for nitrogen fixation, it is not obvious how the *K. pneumoniae* NifL-NifA system responds readily to changes in nitrogen status.

Although *K. pneumoniae* and *A. vinelandii* NifL have similar functions, there is some evidence that *A. vinelandii* NifL may use a different mechanism to respond to the cellular nitrogen status. Previous genetic studies in *A. vinelandii* revealed that a mutation in *nifX* (now called *glnD*), which encodes a homologue of *E. coli* UTPase/UR, gave rise to a Nif phenotype which could be suppressed by a secondary mutation in *nifL* (8). These results suggested that in contrast to *K. pneumoniae*, uridylylation of a regulatory component may be required to prevent inhibition by NifL. Current evidence indicates that *A. vinelandii* contains only a single PII-like protein encoded by a gene designated *glnK*, and in contrast to *E. coli* and *K. pneumoniae*, the *glnK-amtB* operon is expressed under all conditions regardless of fixed nitrogen supply (25). The PII-like protein encoded by *A. vinelandii glnK* (*Av* PII) could therefore be a good candidate for a signal transduction component which could interface between GlnD and NifL, thus controlling the activity of NifL in response to the uridylylation state of PII. However, determination of the role of *A. vinelandii glnK* gene product in nitrogen signaling has been thwarted by the essential nature of this gene since it has not been possible to isolate null mutations in *glnK* (25).

Since it has not been possible to study in vivo regulation of nitrogen fixation in *A. vinelandii* in the complete absence of the PII-like protein, we have resorted to a heterologous system to analyze the response of the *A. vinelandii* NifL-NifA system in *glnB* and *glnK* mutants. We have shown previously that, as is the case with the *K. pneumoniae* NifL-NifA system, *A. vinelandii* NifL modulates NifA activity in *E. coli* in response to oxygen and fixed nitrogen (32). We have used this heterologous system to study the role of the PII-like regulatory proteins in nitrogen regulation of *A. vinelandii* NifL activity. We anticipated that both *A. vinelandii* and *K. pneumoniae* NifL would respond to a common signal transduction pathway for sensing the nitrogen status in *E. coli*. Surprisingly, we found that in contrast to *K. pneumoniae* NifL, neither PII nor GlnK is required to relieve inhibition by *A. vinelandii* NifL under nitrogen-limiting conditions. Moreover, in mutants lacking both the PII and NtrC proteins, NifL activity is no longer responsive to the nitrogen status. These results reveal striking differences in the mechanism of nitrogen regulation of NifL activity between *A. vinelandii* and *K. pneumoniae*.

**MATERIALS AND METHODS**

**E. coli** mutant strain constructions. All strains used were derivatives of *E. coli* ET8000 (Table 1). To construct the ΔglnB1 mutation, the 0.31-kb *AgrI-EcoNI* fragment from plasmid pAH5 which carries the *E. coli glnB* region was deleted, and the 5′ extensions from the digested plasmid were trimmed by incubation with mung bean nuclease. The blunt-end extensions were ligated, producing plasmid pglnB1. A *SalI-BglII* fragment carrying the deleted *glnB* gene from this plasmid was cloned into the gene replacement vector pK03 digested with *SalI* and *BomHI*, generating plasmid pglnBO3. *E. coli* ET8000 was transformed with pglnBO3, and homologous recombination between the cloned fragment and bacterial chromosome was carried out as described previously (19), resulting in strain PT8000. The absence of wild-type sequences in the recombinant was confirmed by PCR analysis using primers 5′-TGAAGGCGCTGAGGAC G3′ and 5′-CTTACCCGGATCGCCTG G3′, which flank the *glnB* region.

Strain GT1002 carrying a glnK in-frame deletion was made by transducing the ΔglnK1 mutation from strain WCH30 into ET8000 using phage P1. In addition to the glnK deletion, WCH30 contains a gentamicin resistance cassette in a 171 bp upstream of *glnK*; hence, the transductant colonies were selected by their resistance to gentamicin and were tested by PCR to verify the presence of the glnK deletion (1). Strain GT1001 containing the *amtB* mutation has been described previously (34). The double-mutant *glnB* and *glnK* strains were made by transforming pglnBO3 into strains WCH30 into ET8000 using phage P1. In addition to the glnK deletion, WCH30 contains a gentamicin resistance cassette in a 171 bp upstream of *glnK*; hence, the transductant colonies were selected by their resistance to gentamicin and were tested by PCR to verify the presence of the glnK deletion (1).

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Reference for source</th>
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<tbody>
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<td></td>
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<tr>
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<td>This work</td>
</tr>
<tr>
<td>GT1002</td>
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<td>Gravin Thomas</td>
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<td><strong>Plasmids</strong></td>
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<td>pRT22</td>
<td>pmrH-lacZ in pACYC184</td>
<td>35</td>
</tr>
<tr>
<td>pPR34</td>
<td><em>A. vinelandii</em> nifL4 translated from the natural ribosome binding site of nifL in pT7-7</td>
<td>32</td>
</tr>
<tr>
<td>pPR34</td>
<td>Derivative of pPR34 expressing NifL(147–519) and NifA</td>
<td>32</td>
</tr>
<tr>
<td>pPR39</td>
<td>Derivative of pPR34 expressing NifL(454–519) and NifA</td>
<td>32</td>
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<tr>
<td>pK03</td>
<td>Gene replacement plasmid</td>
<td>19</td>
</tr>
<tr>
<td>pAH5</td>
<td><em>E. coli</em> glnB in pUC18</td>
<td>13</td>
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<tr>
<td>pGlnB19</td>
<td>ΔglnB in pUC19</td>
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<tr>
<td>pGlnBO3</td>
<td>ΔglnB in pK03</td>
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E. coli strains were transformed with plasmid pRT22, which carries a nifH-lacZ translational fusion. NifA activity was measured by determining the level of expression from the nifH promoter. To monitor the activity of wild-type NifL or the truncated NifL(147–519) protein (which lacks the redox response) to inhibit NifA activity, *E. coli* strains were transformed with plasmids pRT22 and pPR34 or pRT22 and pPR34, respectively. The activity of NifA alone was determined using a nifH-lacZ translational fusion. NifA activity was measured by determining the level of expression from the nifH promoter.
assayed by transforming E. coli strains with plasmids pRT22 and pPR39 (Table 1) (32).

For β-galactosidase assays and for determination of intracellular pools of 2-oxoglutarate, E. coli strains were grown to late exponential phase in Luria-Bertani medium at 30°C in the presence of appropriate antibiotics. Aliquots (50 μl) of these cultures were then inoculated into 4 ml of NFDM medium (30) supplemented with casein hydrolysate (200 μg ml⁻¹) and glutamine (25 μg ml⁻¹) for nitrogen-limiting conditions or with (NH₄)₂SO₄ (1 mg ml⁻¹) plus glutamine (25 μg ml⁻¹) for nitrogen-excess conditions. Cultures were grown in a plastic vial (7-ml internal volume) sealed with a rubber closure for anaerobic conditions. When conditions required aerobicosis, 5-ml cultures were grown with vigorous shaking in 25-ml conical flasks.

Determination of intracellular 2-oxoglutarate. Extracts for measurements of 2-oxoglutarate were prepared as described previously (21), with minor amendments. Portions of cultures (8 ml) were filtered through 0.45-μm-pore-size membrane filters (25-mm diameter) with vacuum suction. As soon as the liquid was removed (30 s), the filter was transferred to an Eppendorf centrifuge tube containing 1 ml of 0.3 M HClO₄ plus 1 mM EDTA at 0°C. Once the tube contents were mixed thoroughly, the filter was removed from the Eppendorf tube and the content was centrifuged to remove debris. Then 500 μl of the extract was removed and neutralized by the addition of 75 μl of 2 M K₂CO₃. The resulting KClO₄ precipitate was removed by centrifugation, and the supernatant fluid was stored at −80°C for further analysis.

2-Oxoglutarate was determined by fluorometric procedures (22), with some modifications. Reaction mixes contained 0.06 ml of 0.5 M imidazole acetate buffer (pH 7.0), 0.03 ml of 0.005 M ammonium acetate, 0.0075 ml of 0.4 mM NADH, 0.01 ml of glutamate dehydrogenase (1 mg/ml), and sample (0.05 to 0.19 ml). Distilled water was added to a final volume of 0.3 ml. The disappearance of glutamate in the reaction mix measured against standards containing 0.6 to 3 nmol of 2-oxoglutarate, prepared using the same neutralization procedure as used for the cell extracts. Protein was determined by the Lowry method (23), with bovine serum albumin as the standard.

Western blotting. Western blotting was performed as described previously (30), using polyclonal antiserum against A. vinelandii NifL and NifA raised in rabbits and the Amersham enhanced chemiluminescence system for detection.

RESULTS AND DISCUSSION

Activity of A. vinelandii NifL and NifA in E. coli. We have shown previously that when the A. vinelandii nifL and nifA genes are expressed from a constitutive promoter in E. coli, transcriptional activation of the nifH promoter, measured as β-galactosidase activity in Miller units, in cultures grown under the conditions indicated with either casein hydrolysate and glutamine (−N) or (NH₄)₂SO₄ and glutamine (+N) as nitrogen sources. Numbers in parentheses indicate data obtained in the absence of glutamine.

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* All strains contain the nifH-lacZ reporter plasmid pRT22 and the indicated plasmid expressing the appropriate Nif regulatory proteins.

β-Galactosidase activity* (mean ± SD)

Activity of A. vinelandii NifL and NifA in E. coli. We have shown previously that when the A. vinelandii nifL and nifA genes are expressed from a constitutive promoter in E. coli, transcriptional activation of the nifH promoter, measured as β-galactosidase activity in Miller units, in cultures grown under the conditions indicated with either casein hydrolysate and glutamine (−N) or (NH₄)₂SO₄ and glutamine (+N) as nitrogen sources. Numbers in parentheses indicate data obtained in the absence of glutamine.

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β-Galactosidase activity* (mean ± SD)
intracellular level of phosphorylated NtrC under these conditions, as suggested by the observation that this strain displayed an apparent increased growth rate in liquid glucose-arginine medium, a demanding test for NtrC activity (3). This can be explained in the context that GlkK is required for fine control of the level of phosphorylated NtrC as previously suggested (3). The observation that the activity of neither NifL nor NifA was significantly influenced by the glkK mutation was remarkably different from what is observed with the A. vinelandii NifL-NifA system. In this case no activation of the glnB glnK mutation was observed as in the wild-type strain. Simultaneously, the same relief of inhibition of NifA activity in the glnB glnK double-mutant strain. Atkinson and Ninfa have reported that such strains exhibit severe growth defects in minimal medium containing ammonia and glutamine as nitrogen sources, presumably as a consequence of the influence of these mutations on the expression and activity of GS. Surprisingly, under conditions of nitrogen limitation, the nitrogen repression ratio is considerably lower in this strain than in the wild type (3.6 and 65.8, respectively), although this result is difficult to interpret as a consequence of the high levels of β-galactosidase observed with the reporter plasmid.

Nitrogen regulation of NifL activity is absent in strains carrying both nrc and glkB mutations. Since the data obtained with the glkB glkK mutant could be influenced by its slow growth and the presence of high levels of phosphorylated NtrC, we examined the effect of introducing a nrc::Tn5 mutation into the double-mutant strain. As expected, this mutation rendered cells auxotrophic for glutamine, presumably as a consequence of the influence of these mutations on the expression and activity of GS. Surprisingly, under conditions of nitrogen excess, NifL inhibition was substantially relieved in the triple-mutant strain in the case of both native NifL and the truncated variant, and in both cases the repression ratio was close to 1 (Table 3, MT8000). Moreover, in this background, greater relief of NifL inhibition was seen under nitrogen-limiting conditions compared with the wild-type strain, regardless of whether native NifL or NifL(147-519) was present (Table 3, compare ET8000 and MT8000). These data clearly demonstrate that neither of the PII paralogues are required to relieve inhibition by A. vinelandii NifL under N-limiting conditions. Since phosphorylated NtrC is required for activation of GlkK expression in E. coli, we suspected that the glkK mutation did not contribute to the phenotype of the glkB glkK nrcC strain. Accordingly, we constructed a glkB nrcC double-mutant strain. The nitrogen response of NifL in this strain was similar to that observed with the triple mutant, although the levels of NifA activity observed in the presence of NifL or NifL(147-519) under nitrogen-limiting conditions were lower than those observed with the glkB glkK nrcC strain (Table 3, RT8000). Western blotting analysis suggested that there were no major differences in the levels of NifL and NifA expression in the mutant strains compared with the wild-type, and the ratios of NifL to NifA were similar in all cases (Fig. 1). Hence, the failure of NifL to inhibit NifA activity in the mutant strains under nitrogen-excess conditions is unlikely to be a consequence of de-
creases in the level of NifL protein or to the presence of excess NifA in these strains.

To ensure that the loss of NifL inhibition was specific to the nitrogen response, we also tested the redox response of NifL under aerobic conditions in the triple-mutant strain (Table 4). As expected, native NifL was responsive to oxygen inhibition in the wild-type and mutant strains (compare Tables 3 and 4), whereas the truncated NifL(147-519) protein was insensitive to aerobicism in the wild-type and glnB strains but nevertheless was responsive to the nitrogen source (Table 4). In contrast, when expressed in the triple glnB glnK ntrC mutant, the truncated NifL protein was unable to inhibit NifA activity regardless of the growth conditions, and the level of NifA activity remained significantly higher than in the wild-type strain even in nitrogen-limiting conditions (Table 4). These results are similar to those obtained under anaerobic conditions and suggest that the presence of NtrC in combination with PII influences the nitrogen but not the oxygen response of the A. vinelandii NifL-NifA system in E. coli.

Since ntrC mutants have pleiotropic effects on nitrogen metabolism, the results observed in the mutant strains could potentially be physiological or perhaps due to the lack of expression of a specific gene or operon which is subject to NtrC control. We therefore studied the activity of NifL in a strain containing an ntrC null mutation. Although this strain gave slightly greater relief of NifL inhibition under nitrogen-limiting conditions compared with the wild type (around 1.5-fold), the NifL and the NifL(147-519) proteins remained responsive to fixed nitrogen in this background, although the nitrogen repression ratio was lower than in the wild-type strain (Table 3, NT8000). Hence, the absence of NtrC by itself is not sufficient to inactivate the nitrogen response of A. vinelandii NifL.

As relief of NifL inhibition under nitrogen-excess conditions was not observed in either the glnB or ntrC single-mutant strain, inactivation of the nitrogen response apparently requires mutations in both glnB and ntrC. Since NtrC is required for transcription of the glnk-amtB operon, we also considered the possibility that the methylammonium transporter amtB might be required for the nitrogen response. However, neither an amtB or a double-mutant glnB amtB background appeared to influence NifL activity (Table 3, GT1001 and AT8000).

**Relationship between in vivo 2-oxoglutarate pools and NifL activity.** The above observation that the combination of glnB and ntrC mutations eliminates the nitrogen response of A. vinelandii NifL in E. coli suggests that more than one factor is involved in this response. In addition to the potential role of the PII paralogues, the additional factor could be a metabolite whose levels are influenced by the ntrC mutation or a gene product which is expressed under the control of NtrC. It is interesting to consider the former possibility in the light of our recent in vitro data, which demonstrates that the NifL-NifA system is directly responsive to 2-oxoglutarate within the physiological range (20). Under conditions of nitrogen excess, the reported 2-oxoglutarate concentration in E. coli is ~100 μM, which increases to ~1 mM under conditions of nitrogen limitation (31). We therefore considered the possibility that 2-oxoglutarate could be an effector required for relief of inhibition by NifL in vivo. To investigate this hypothesis, we measured the intracellular accumulation of 2-oxoglutarate in E. coli mutant strains grown under conditions identical to those used to determine transcriptional activation by NifA using the reporter system. The 2-oxoglutarate concentration was below the level of detection (>50 μM) in the wild-type strain grown under conditions of nitrogen excess (glucose, ammonia, and glutamine) but increased by a factor of at least 30-fold to ~10 nmol/mg of protein under nitrogen-limiting conditions (Table 5, ETS8000). This value corresponds to ~3 nmol/mg (dry weight), or an intracellular concentration of ~1.5 mM. Similar results were observed with the glnB mutant (Table 5, PT8000). Surprisingly, the level of 2-oxoglutarate increased substantially in the glnB glnK double mutant under nitrogen-excess conditions. This may result from overadenylation of GS in this strain (3, 6) and consequent accumulation of 2-oxoglutarate in the absence of efficient nitrogen assimilation. The ntrC mutation did not appear to influence 2-oxoglutarate accumulation under conditions of nitrogen excess, but a small increase was

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**TABLE 4. Inhibition of NifA activity by NifL and NifL<sub>147-519</sub> under aerobic conditions**

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genotype</th>
<th>β-Galactosidase activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NifL + NifA</td>
<td>NifL&lt;sub&gt;147-519&lt;/sub&gt; + NifA</td>
</tr>
<tr>
<td></td>
<td>(pRT22, pPR34)</td>
<td>(pRT22, pPR54)</td>
</tr>
<tr>
<td></td>
<td>-N</td>
<td>+N</td>
</tr>
<tr>
<td></td>
<td>+N</td>
<td>-N</td>
</tr>
<tr>
<td>ETS8000</td>
<td>Wild type</td>
<td>210 55</td>
</tr>
<tr>
<td></td>
<td>glnB</td>
<td>190 32</td>
</tr>
<tr>
<td></td>
<td>glnB glnK ntrC</td>
<td>360 530</td>
</tr>
<tr>
<td>PT8000</td>
<td>glnB</td>
<td>190 32</td>
</tr>
<tr>
<td></td>
<td>glnB glnK ntrC</td>
<td>16,000 10,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> All strains contained the nifH-lacZ reporter plasmid pRT22 and plasmids expressing the appropriate Nif regulatory proteins.

<sup>b</sup> NifA-mediated activation of transcription from the nifH promoter, measured as β-galactosidase activity in Miller units, in cultures grown in aerobiosis with either casein hydrolysate and glucose (−N) or (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and glutamine (+N) as nitrogen sources. Standard deviations were within 11% of the mean.
observed under nitrogen-limiting conditions. This might be expected if nitrogen is poorly assimilated since the level of GS is significantly decreased in ntc mutants under conditions of nitrogen limitation (28). In contrast, the combination of the ntc with the glnB or glnB and glnK mutations gave rise to a high level of 2-oxoglutarate accumulation under conditions of nitrogen excess, resulting in levels similar to that observed with the wild-type strain under nitrogen-limiting conditions (Table 5, MT8000 and RT8000 compared with ET8000). We interpret this accumulation as a consequence of physiological nitrogen limitation in these strains even when excess external ammonium is present. Notably, the growth rates of these strains were similar in the presence and absence of ammonia. Overall, these results suggest that there is some correlation between the intracellular accumulation of 2-oxoglutarate and the nitrogen repression ratio observed with the nifH-lacZ reporter (Table 3), indicating that 2-oxoglutarate may have a role in modulating the activity of the A. vinelandii NifL-NifA system in vivo.

Conclusions. We have utilized defined E. coli mutant strains to analyze nitrogen regulation of A. vinelandii NifA activity mediated by NifL. This heterologous system has been used previously to examine the role of PII-like proteins in modulating the activity of K. pneumoniae NifL in vivo (1, 12) and hence allows direct comparison of the properties of the A. vinelandii and K. pneumoniae nitrogen fixation regulatory genes.

In contrast to the observations made with the equivalent K. pneumoniae NifL-NifA regulatory components (1, 12, 14), our data demonstrate clearly that the E. coli PII paralogues are not required to prevent A. vinelandii NifL from inhibiting NifA. This conclusion is based on the observation that under nitrogen-limiting conditions, the activity of NifA in the presence of NifL does not decrease in the glnB glnK and glnB glnK ntcC mutant strains, which do not express either of the PII paralogues. Rather, the circumstantial evidence presented here suggests that under nitrogen-limiting conditions, 2-oxoglutarate may be required to alleviate inhibition of NifA activity by NifL, in agreement with our in vitro observations (20). Whereas in the K. pneumoniae system, GlnK is absolutely required to relieve inhibition by NifL, it would appear that the PII paralogues have the opposite role to increase the activity of A. vinelandii NifL under conditions of nitrogen excess. Hence, although both the K. pneumoniae and A. vinelandii NifL-NifA systems are responsive to fixed nitrogen in E. coli, the mechanism for this response is fundamentally different in each case. Although the nitrogen response of A. vinelandii NifL in E. coli could be dependent solely on the level of 2-oxoglutarate, the requirement for the PII proteins to increase the inhibitory activity of NifL under nitrogen-excess conditions would allow a more highly tuned response. Our in vitro experiments show that the nonmodified form of E. coli PII and Av PII can increase the inhibitory activity of NifL, whereas E. coli GlnK is ineffective (20). Thus, E. coli PII is apparently functionally analogous to Av PII with respect to its interaction with the A. vinelandii NifL-NifA system. The involvement of Av PII in increasing the inhibitory function of NifL is also suggested from genetic evidence with A. vinelandii, since glnD mutants which have lost the ability to fully uridylylate Av PII are Nif (8, 29). Furthermore, a mutation in A. vinelandii glnK which prevents uridylylation of the target tyrosine residue in the T loop of Av PII is also Nif”. Both of these mutant classes can be suppressed by insertion mutations in nifL (8, 29; P. Rudnick and C. Kennedy, unpublished results). This is in accord with our in vitro data which show that uridylylation of Av PII prevents it activating the inhibitory function of NifL (20).

Why are the mechanisms of the nitrogen response radically different between A. vinelandii with K. pneumoniae? One possibility lies in the differences between the C-terminal domains of the NifL counterparts (4, 38). The C-terminal domain of A. vinelandii NifL is homologous to the histidine protein kinase family, and although this protein does not exhibit kinase activity, it does bind adenosine nucleotides, particularly ADP, which is required to form the inhibitory NifL-NifA complex (32). In contrast, the C-terminal domain of K. pneumoniae NifL shows only limited homology to the histidine kinase family and has not been shown to interact with nucleotides. Although no extensive in vitro studies have been performed with the K. pneumoniae system, it is possible that K. pneumoniae NifL interacts with NifA irrespective of the presence of metabolites. This may impose the requirement for GlnK to destabilize the complex in order to achieve nitrogen regulation. In contrast in the A. vinelandii system, formation of the inhibitory complex is influenced by metabolite concentrations (ADP and 2-oxoglutarate) and the nonmodified form of PII stabilizes the complex. These differences reveal the considerable versatility of the PII signal transduction proteins in their mode of interaction with receptors.

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