Activation of Klebsiella pneumoniae and Rhizobium meliloti nitrogenase promoters by gln (ntr) regulatory proteins

(nitrogen metabolism regulation/symbiotic nitrogen fixation/lac fusions)

VENKATESAN SUNDARESAN*†, DAVID W. OW‡†, AND FREDERICK M. AUSUBEL*††

*Committee on Biophysics and †Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138

Communicated by Harold J. Evans, March 11, 1983

ABSTRACT We have studied the expression, in different Escherichia coli gln (ntr) mutants, of fusions (constructed in vitro) of the nifHDK (nitrogenase) promoters from Klebsiella pneumoniae and Rhizobium meliloti to E. coli lacZ. Derepression of the K. pneumoniae nif: lacZ fusion requires the glnF (ntrA) gene product in addition to the K. pneumoniae nifA gene product, indicating that regulation of the K. pneumoniae nif genes is more closely integrated with the overall nitrogen control system than previously demonstrated. Derepression of the R. meliloti nifH:: lacZ fusion in E. coli by the K. pneumoniae nifA gene product (which we had previously shown) exhibits the same requirement for glnF. Derepression of the R. meliloti nifH:: lacZ fusion, but not the K. pneumoniae nifH:: lacZ fusion, can be mediated by the glnC (ntrC) gene product, suggesting that the gln regulatory genes might directly regulate the symbiotic nitrogen fixation genes in Rhizobium.

The free-living nitrogen-fixing bacterium Klebsiella pneumoniae utilizes the enzyme nitrogenase to reduce N2 to NH4+ under conditions of NH4+ starvation and low O2 tension. Nitrogenase is composed of polypeptides encoded by genes nifH, nifD, and nifK, which are located within an operon transcribed in the direction nifH to nifK. The nifHDK operon is itself located within a larger cluster of at least 17 contiguous nif genes, which are organized into seven or eight operons. One nif operon, the nifLA operon, codes for regulatory proteins (Fig. 1; reviewed in refs. 1 and 2). The nifA gene is involved in activation of all the other nif operons, whereas the nifL product is involved in repression of these operons under certain physiological conditions (3, 4).

Recent studies of nitrogen assimilation in enteric bacteria have shown that the process is under the control of a central regulatory system. The products of three genes, glnF (or ntrA), glnL (or ntrB), and glnC (or ntrC), have been identified as the regulatory proteins involved in this process (refs. 6–9). Under conditions of nitrogen limitation, the glnG product appears to act in concert with the glnF product to activate a variety of nitrogen catabolism genes such as those involved in histidine utilization (hut) and proline utilization (put). Under conditions of nitrogen excess, the glnG product has been postulated to act in concert with the glnL product to repress the transcription of these same genes (reviewed in ref. 10). The nif genes of the enteric bacterium K. pneumoniae are indirectly under the control of the gln regulatory system due to the fact that the nifLA operon is regulated by glnG and glnF (11–13). Recently, our laboratory has shown that nifA can substitute for glnG in vivo; i.e., the nifA protein can activate the same genes as the glnG protein (13). In this study, we have examined whether the gln regulatory system can regulate K. pneumoniae nif genes in ad-

*The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

FIG. 1. The nif genes of K. pneumoniae, showing the transcription units and the current model of their regulation by the products of the nifLA operon (1–5).

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>Repression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>HDK</td>
<td>Y</td>
<td>EN</td>
<td>X</td>
<td>USV</td>
<td>MF</td>
<td>LA</td>
<td>BQ</td>
</tr>
<tr>
<td>glnF (ntrA)</td>
<td>glnG (ntrB)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Construction of pVSAA2. A 0.294-kilobase (kb) EcoRI/Hga I fragment containing the nifH promoter of K. pneumoniae (17) was inserted into the lacZ-carrying plasmid pMC1403 (18) as shown in Fig. 2A, so that the ATG start codon of nifH was in the same reading frame as the lacZ gene.

Construction of pVSP9. The 0.72-kb Sal I fragment containing the R. meliloti nifH promoter and the first 29 amino

Abbreviation: kb, kilobase(s).
Fig. 2. Construction of the lac fusion plasmids described in the text. (A) pVSA2 carrying the \textit{K. pneumoniae} \textit{nifH}:\textit{lacZ} fusion. (B) pVSP9 carrying the \textit{R. meliloti} \textit{nifH}:\textit{lacZ} fusion. (C) pDO202 carrying the \textit{P_{lacUV5}}-\textit{nifA} fusion (\textit{nifLA} is not drawn to scale). Details are described in the text. Ap\textsuperscript{R}, ampicillin resistance; Km\textsuperscript{R}, kanamycin resistance; bp, base pairs; DNA Pol I, DNA polymerase I; RI or R, EcoRI; H, HindIII; B or Bam, BamHI; S, Sal I.

pgln53Y (13) is an ampicillin-resistant tetracycline-sensitive derivative of pgln53 (9). Like pgln53, it is a \textit{glnG}\textsuperscript{+} plasmid that carries a fusion of the \textit{glnA} promoter to \textit{glnG} on a pBR322 vector; it synthesizes the \textit{glnG} protein from the low-level-constitutive \textit{glnA} promoter.

Measurement of \textit{B}-Galactosidase Activity. Five-milliliter cultures of strains harboring lacZ fusions were grown anaerobically to saturation at 30°C in \textit{nif}-depensation medium (3) with 0.2% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} supplemented with 0.2% L-glutamine and the appropriate antibiotics (kanamycin at 10 \mu g/ml, ampicillin at 50 \mu g/ml, or both) and resuspended with nitrogen-rich or nitrogen-limiting media as indicated in Table 1 (with NH\textsubscript{4}\textsuperscript{+} and without NH\textsubscript{4}\textsuperscript{+}, respectively). Nitrogen-limiting medium was \textit{nif}-depensation medium containing antibiotics as above, but L-glutamine at only 100 \mu g/ml. Nitrogen-rich medium was nitrogen-limiting medium supplemented with 0.2% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and 0.2% L-glutamine. The cultures were incubated for 10 hr anaerobically at 30°C and centrifuged, and the \textit{B}-galactosidase activity was determined as described by Miller (21).

Strains. \textit{E. coli} YMC11 is \textit{supE44 glnA-~/G2000 LacU169} (22); YMC10 is \textit{supE44 LacU169} (22); TH1 is \textit{supE44 LacU169 glnF} (from T. Hunt); DO1413 is \textit{supE44 LacU169 GlnD} (13).
RESULTS

*nifA* Activation of *nifH* Promoters Requires *glnF*. The *nifH::lacZ* fusions constructed as described in Materials and Methods were used to monitor the activity of the *nifH* promoters by measuring β-galactosidase activities in various *E. coli* strains containing different mutations in *gln* (*ntr*) regulatory genes. The *K. pneumoniae* *nifA* protein synthesized constitutively from a lac promoter on plasmid pDO202 activated the *K. pneumoniae* *nifH::lacZ* fusion on plasmid pVSA2 both in the absence and in the presence of *NH₄⁺* and glutamine (experiments 1-1 and 1-2, Table 1); this activation could also be carried out in a Δ*gln*(ALG) background (experiments 1-3 and 1-4), confirming previously published results that the *glnG* gene product is not necessary for activation by *nifA* (4). The lower levels of β-galactosidase measured in the presence of *NH₄⁺* could be due to the shorter half-life (50% of normal) of mRNAs in cells growing on *NH₄⁺* (23). Because the decrease was more pronounced in a *glnL* *G*⁺ strain, it is also possible that the *glnL* product could be mediating some repression of *nifH* in high concentrations of *NH₄⁺*.

The *nifA*-dependent activation of the *K. pneumoniae* *nifH::lacZ* fusion was tested in the Δ*glnF* strain, TH1. It was clear from experiments 1-5 and 1-6 that *nifA* could not activate the *nifH::lacZ* fusion in this strain, either in the presence or in the absence of *NH₄⁺*. This result was confirmed by using the *K. pneumoniae* *nifH::lacZ* fusion carried on a phage F4 vector and the *Pₐ₉₃₂α*-*nifA* fusion carried on plasmid pPMM17, which also carried a copy of the *lacF* gene to repress the *lacUV5* promoter. Addition of isopropyl thiogalactoside induced synthesis of β-galactosidase in a *glnF⁻* strain (experiments 1-1 and 1-2), but not in a *glnF⁺* strain (experiments 4-3 and 4-4). Thus, in addition to *nifA*, *glnF* product appears to be required for activation of the *K. pneumoniae* *nifHDK* promoter.

We have shown elsewhere that the *K. pneumoniae* and *R. meliloti* *nifH* promoters are similar to the extent that the *R. meliloti* *nifH* promoter can be activated by the *K. pneumoniae* *nifA* product (16). This activation does not require the *glnG* product (ref. 16 and Table 1, experiments 1-7 and 1-8). When we repeated this experiment with the fusions in a *glnF⁻* background, we found that the *R. meliloti* *nifH::lacZ* fusion required both the *glnA* and *glnG* gene products (experiments 1-9 and 1-10), as was the case for activation of the *K. pneumoniae* *nifH::lacZ* fusion.

Table 1. β-Galactosidase activity in *nif*-lac fusion strains

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Strain</th>
<th>Relevant host genotype</th>
<th>Relevant plasmid properties*</th>
<th>Without NH₄⁺</th>
<th>With NH₄⁺</th>
<th>Exp.</th>
<th>Strain</th>
<th>Relevant host genotype</th>
<th>Relevant plasmid properties*</th>
<th>Without NH₄⁺</th>
<th>With NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>YMC10/pVSA2</td>
<td></td>
<td>gln⁺</td>
<td>Kp nifH::lacZ</td>
<td>20</td>
<td>8</td>
<td>3-1</td>
<td>YMC10/pVSP9-1;</td>
<td>gln⁺</td>
<td>Kp nifH::lacZ;</td>
<td>24</td>
</tr>
<tr>
<td>1-2</td>
<td>YMC10/pVSA2; pDO202</td>
<td></td>
<td>gln⁺</td>
<td>Kp nifH::lacZ;</td>
<td>3,073</td>
<td>1,800</td>
<td>3-2</td>
<td>DO1413/pVSP9-1;</td>
<td>ΔglnD</td>
<td>Kp nifH::lacZ;</td>
<td>5</td>
</tr>
<tr>
<td>1-3</td>
<td>YMC11/pVSA2; pDO201</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>58</td>
<td>73</td>
<td>3-3</td>
<td>DO1413/pVSP9-1;</td>
<td>ΔglnD</td>
<td>Kp nifH::lacZ;</td>
<td>41</td>
<td>34</td>
</tr>
<tr>
<td>1-4</td>
<td>YMC11/pVSA2; pDO202</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>4,226</td>
<td>3,306</td>
<td>3-4</td>
<td>YMC11/pVSP9-1;</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>1-5</td>
<td>TH1/pVSA2; pDO202</td>
<td>Δ(glnF)</td>
<td>Kp nifH::lacZ;</td>
<td>12</td>
<td>10</td>
<td>3-5</td>
<td>YMC11/pVSP9-1;</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>99</td>
<td>60</td>
</tr>
<tr>
<td>1-6</td>
<td>TH1/pVSA2; pDO202</td>
<td>Δ(glnF)</td>
<td>Kp nifH::lacZ;</td>
<td>14</td>
<td>10</td>
<td>3-6</td>
<td>DO1413/pVSA3;</td>
<td>ΔglnD</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1-7</td>
<td>YMC11/pVSP9; pDO201</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>55</td>
<td>49</td>
<td>3-7</td>
<td>DO1413/pVSA3;</td>
<td>ΔglnD</td>
<td>Kp nifH::lacZ;</td>
<td>270</td>
<td>264</td>
</tr>
<tr>
<td>1-8</td>
<td>YMC11/pVSP9; pDO201</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>1,721</td>
<td>1,968</td>
<td>3-8</td>
<td>DO1413/pVSA3;</td>
<td>ΔglnD</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1-9</td>
<td>TH1/pVSP9; pDO201</td>
<td>Δ(glnF)</td>
<td>Kp nifH::lacZ;</td>
<td>110</td>
<td>98</td>
<td>3-9</td>
<td>DP1413/pVSP9-1;</td>
<td>ΔglnD</td>
<td>Kp nifH::lacZ;</td>
<td>155</td>
<td>137</td>
</tr>
<tr>
<td>1-10</td>
<td>TH1/pVSP9; pDO201</td>
<td>Δ(glnF)</td>
<td>Kp nifH::lacZ;</td>
<td>111</td>
<td>107</td>
<td>4-1</td>
<td>YMC11/pVSA3;</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>1-11</td>
<td>YMC10/pVSP9; pDO202</td>
<td>gln⁺</td>
<td>Km nifH::lacZ;</td>
<td>436</td>
<td>49</td>
<td>4-2</td>
<td>YMC11/pVSA3;</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>192</td>
<td>149</td>
</tr>
<tr>
<td>1-12</td>
<td>YMC10/pVSP9; pDO202</td>
<td>gln⁺</td>
<td>Km nifH::lacZ;</td>
<td>1,151</td>
<td>795</td>
<td>4-3</td>
<td>TH1/pVSA3;</td>
<td>ΔglnF</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2-1</td>
<td>YMC10/pVSP9-1</td>
<td>gln⁺</td>
<td>Km nifH::lacZ</td>
<td>83</td>
<td>48</td>
<td>4-4</td>
<td>TH1/pVSA3;</td>
<td>ΔglnF</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2-2</td>
<td>YMC11/pVSP9-1; pBR322</td>
<td>Δ(glnALG)</td>
<td>Km nifH::lacZ;</td>
<td>21</td>
<td>18</td>
<td>2-3</td>
<td>YMC11/pVSP9-1; glnG/glnF</td>
<td>Km nifH::lacZ;</td>
<td>278</td>
<td>209</td>
<td>4-3</td>
</tr>
<tr>
<td>2-3</td>
<td>YMC11/pVSP9-1; pBR322</td>
<td>glnG</td>
<td>Km nifH::lacZ;</td>
<td>9</td>
<td>8</td>
<td>2-4</td>
<td>YMC10/pVSA2; pBR322</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2-4</td>
<td>YMC10/pVSA2; pBR322</td>
<td>gln⁺</td>
<td>Km nifH::lacZ;</td>
<td>10</td>
<td>9</td>
<td>2-5</td>
<td>YMC10/pVSA3; pBR322</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2-5</td>
<td>YMC10/pVSA3; pBR322</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>10</td>
<td>9</td>
<td>2-6</td>
<td>YMC10/pVSA3; pBR322</td>
<td>Δ(glnALG)</td>
<td>Kp nifH::lacZ;</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Activation of the *K. pneumoniae* and *R. meliloti* *nifH::lacZ* fusions by *nifA*, *glnF*, and *glnG* gene products. See text for details. The experiments are divided into four sets, and the absolute values of β-galactosidase activity should be compared only within the same set. It is also necessary to keep in mind that the vectors carrying the *nifH::lacZ* fusions are not the same in all experiments—i.e., both pBR322 and phage F4 were used. The data in Exp. 1-3/1-4 and 1-7/1-8 are being published elsewhere (16) and are shown here only for comparison.

* Kp, *K. pneumoniae*; Rm, *R. meliloti*.
ginG Product Activates the R. meliloti nifH Promoter. In a ginG+ background, nifA protein activated the R. meliloti nifH::lacZ fusion (experiments 1-11 and 1-12); however, we observed derepression of the R. meliloti nifH::lacZ fusion even in the absence of nifA, when the cells were starved for NH₄⁺ (experiment 1-11). On the other hand, no derepression occurred in a Δgln(ALG) strain (experiments 1-7 and 1-8) or in a ΔglnF strain (experiments 1-9 and 1-10). It is likely, therefore, that ginG plus glnF products were activating R. meliloti nifH in the ginG+ E. coli strain when the cells were under conditions of nitrogen deficiency.

The above results—i.e., the requirement for glnF for the activation of the K. pneumoniae and R. meliloti nifH promoters and the activation of the R. meliloti nifH promoter by the ginG product under derepressing conditions—were unexpected in the light of existing models of nif regulation (1, 2); they are, however, consistent with recent results from our laboratory demonstrating that nifA can function like ginG (13). We decided, therefore, to test directly whether ginG could replace nifA in the activation of nifH::lacZ fusions (as suggested by experiment 1-11). To do this we used a multicopy plasmid, pgln53Y, that carries the ginG gene fused to the glnA promoter; it does not carry intact glnA or glnG and synthesizes ginG product constitutively (9). Experiments 2-2 and 2-3 showed that the ginG product activated the R. meliloti nifH::lacZ fusion in an E. coli background. The activation of R. meliloti nifH::lacZ by multicopy ginG was of the same magnitude as that observed with multicopy nifA (not shown), but direct comparisons cannot be made without determining the amounts of glnG and nifA proteins present. In contrast, the K. pneumoniae nifH::lacZ fusion was not activated by ginG product, even when ginG product was being overproduced by the multicopy plasmid pgln53Y (experiments 2-5 and 2-6).

The experiments demonstrating ginG activation of R. meliloti nifH::lacZ (2-1 to 2-6), were repeated with the fusions in a glnD− background. The glnD product is involved in the derepression of the glnALG operon and glnD− mutants synthesize only low levels of glnG product (24). As expected, in a glnD− strain, the R. meliloti nifH::lacZ fusion was not derepressed upon NH₄⁺ starvation (experiments 3-1 and 3-2). This effect was due to low levels of glnG product and not to a direct requirement for glnD; when the glnG constitutive plasmid, pgln53Y, was introduced into the same strain, activation was restored (experiment 3-3). However, the level of activation was lower than in a parallel experiment using a Δgln(ALG) strain (experiments 3-4 and 3-5), possibly due to repression mediated by glnL or non-uridylylated glnB product (9, 25). As before, we found that ginG could not activate the K. pneumoniae nifH::lacZ fusion (experiments 3-6 and 3-8). We also showed that the activation of the R. meliloti and K. pneumoniae nifH::lacZ fusions by nifA did not require glnD product (experiments 3-2 and 3-9, and experiments 3-6 and 3-7).

DISCUSSION

Role of glnF and nifA-Mediated Activation. The current model of nif regulation in K. pneumoniae can be summarized as follows: Under conditions of NH₄⁺ starvation, transcription of glnG is activated and glnG gene product, in concert with the glnF gene product, activates transcription of the nifLA operon (reviewed in refs. 1, 2, and 5). The nifA gene product then activates all the other nif operons, which are also subject to repression by nifL under certain physiological conditions such as high O₂ tension (3). There has been no evidence presented so far that the proteins of the gln regulatory system interact with any nif operons other than nifLA (4). Rather, it has been proposed that gln-mediated regulation of nif expression is due solely to the action of glnG and glnF products on the nifLA promoter (3). Our demonstration that both glnG+ and nifA are required for activation of the K. pneumoniae nifH::lacZ and R. meliloti nifH::lacZ fusions is consistent with our previous discovery that nifA can substitute for glnG in activating a number of genes involved in nitrogen assimilation and with the model that nifA evolved from an ancestral glnG gene (13). Recently, Sibold and Elmerich (26) and Merrick (27) have also found that glnF is required for nifHDK expression even in the presence of a constitutive nifA plasmid.

The mode of action of glnF protein is not known at present. One possible mechanism that has been suggested is that it forms an activating complex with glnG (6), presumably it would function in the same manner in the case of nifA. It has also been proposed that glnF might be acting as the overall nitrogen sensor of the cell by converting glnG to an activator form during NH₄⁺ starvation, either directly or indirectly; it might act indirectly by synthesizing a small effector molecule in response to nitrogen deficiency (6, 10). The latter model is by analogy to the adenylate-cleavage—cAMP—cAMP-binding protein system involved in catabolite repression. Our data are not consistent with this model of glnF action for the following reasons: We find that (i) nifA absolutely requires glnF to activate the nifH::lacZ fusion, and (ii) when nifA is synthesized constitutively from a lac promoter in a glnG− background, it activates nifH::lacZ even in the presence of high levels of NH₄⁺. If glnF were responding to NH₄⁺ levels as proposed, activation under these conditions would not be expected. Our results are in agreement with those of Chen et al. (9), who have suggested that the regulatory responses of the cells to NH₄⁺ starvation are mediated through glnD and glnL. While our results can be interpreted to suggest that glnF is always present in its active form, we cannot rule out some form of modulation of glnF activity or glnF product synthesis in response to changing NH₄⁺ levels.

Differences Between nifA and glnC. If nifA and glnG products interact directly with the nifH promoters, our finding that either nifA or glnG can activate R. meliloti nifH::lacZ but that only nifA can activate K. pneumoniae nifH::lacZ suggests that the nifA and glnG gene products recognize different DNA sequences. In this light, it is interesting to compare the DNA sequences of the two nifH promoters (16) with the DNA sequence of the nifLA promoter (28); all three of these promoters can be activated by nifA. As illustrated in Fig. 3 (i) all three promoters share the sequence T-G-C-A in the −12 region; (ii) for the two promoters activated by glnG (R. meliloti nifH and K. pneumoniae nifLA), the homologous region at −12 is longer—i.e., T-T-G-C-A; (iii) in the case of the two nifH promoters, the homologous sequence at −12 is T-G-C-A-C, but there is also a longer 8-base-pair homologous sequence at −30 (A-C-G-C-T-G-G). Both of these nifH promoters show strong acti-
vation with nifA. It is possible that the sequence T-G-C-A at −12 is a common element required by both nifA and glnG products for activation. However, the glnG product might require the complete sequence T-T-T-G-C-A, which is absent in the K. pneumoniae nifH promoter, explaining why this promoter cannot be activated by glnG. Recently, Beynon et al have shown that a consensus sequence T-G-C-A is found at the same location in all the K. pneumoniae nifH promoters; they suggest that this sequence is involved in RNA polymerase initiation complex formation in promoters that are active under nitrogen limitation conditions (J. L. Beynon, M. C. Cannon, V. Buchan-on-Wollaston, and F. C. Cannon, personal communication).

The observation that the K. pneumoniae nifH promoter shows a high degree of specificity for nifA suggests that there has been an evolutionary selection for K. pneumoniae to develop a regulatory system that is highly specific for the nif gene cluster. Because nitrogen fixation is energy intensive, and because the nitrogenase enzyme is oxygen sensitive, it would be advantageous to keep nif genes repressed under aerobic conditions [a function provided by nifL (3)] while keeping other nitrogen-assimilation pathways open. Under such selection, a secondary regulatory circuit for indirect control of the nif genes by glnG could have evolved.

In contrast to K. pneumoniae, the symbiotic reduction of N₂ by Rhizobium species may not be physiologically as stressful, because both the energy requirement and the O₂-protection system are supplied by the plant. Our finding that glnG can activate the R. meliloti nifH promoter raises the possibility that in R. meliloti the genes for nitrogenase are under the direct control of the gln regulatory proteins. [We should add the caution that an E. coli host was used for these experiments; however, the one Rhizobium RNA polymerase purified, from R. leguminosarum, does recognize the same phase T7 promoters as does E. coli RNA polymerase (29).] Such models have been proposed for nif regulation in Rhizobium "cowpea" sp. 32H1 (30). On the other hand, it is possible that R. meliloti does indeed have a "nifA-like" protein, but that it is intermediate in specificity between the K. pneumoniae glnG and nifA proteins. In this context we note that a putative regulatory gene closely linked to the nifHDK genes of R. meliloti has been recently identified in our laboratory (W. Szeto and L. Zimmerman, personal communication). A transposon insertion into this gene prevents synthesis of the products of all three nitrogenase structural genes (nifHDK) and in this respect it resembles nifA.

Like K. pneumoniae, bacteria in the genus Azotobacter fix nitrogen symbiotically. In two species of Azotobacter, nif regulatory mutations that can be complemented by K. pneumoniae nifA have been found (31). It would be interesting to see if these can also be complemented by glnG. Such studies may clarify whether the evolution of a nifA protein, and of nif promoters that are specifically activated by it, is unique for K. pneumoniae or whether it is common among other organisms that fix nitrogen in the free-living state.

We thank T. Hunt for providing strain TH1, P. McLean for plasmid pFM517, and R. Hyde for typing the manuscript. This research was funded by National Science Foundation Grant PCM-8104193, awarded to F.M.A., and U.S. Department of Agriculture Grant 59-2253-1-1-722-0 awarded to W. J. Orme-Johnson at the Massachusetts Institute of Technology, with a subcontract to F.M.A.