Autoregulation of nitrogenase expression in *Klebsiella pneumoniae*

Susan Hill and Eugene P. Kavanagh

An investigation into the influence of $N_2$ on the expression of *Klebsiella pneumoniae* nitrogenase has led to a reassessment of the role of the nitrogenase MoFe protein in autoregulation. Anaerobic derepression of nitrogenase ($C_2H_4$-reducing) activity, of NifD and K polypeptides, and of $nifH$-$lac$ expression, following the removal of excess $NH_3$, were greater under $N_2$ than Ar. This enhancement occurred in Nif$^{-}$ but not in Nif$^{+}$ strains, and in Nif$^{+}$ strains was prevented by CZH$_3$, an inhibitor of $N_2$ fixation. Thus $N_2$ fixation is important for maintaining derepression. Derepression of $nifH$-$lac$ under Ar in various Nif$^{+}$ and Nif$^{-}$ strains (including NifH$^{-}$, NifD$^{-}$, NifB$^{-}$ and NifL$^{-}$ mutants) and of wild-type lac under $N_2$ or Ar in a Nif$^{+}$ strain were measured to investigate the regulation. The mechanism regulating the enhancement under $N_2$ neither involved the MoFe protein of nitrogenase, as proposed by Dixon et al. (1980, *Nature* 286, 125–132) nor the $nifL$ product, but was probably due to a general upgrading of the N status. Moreover, during batch growth limited by a non-repressing fixed N source, the levels of $nifH$-$lac$ expression in the Nif$^{+}$ and Nif$^{-}$ strains suggested that the $nifH$ gene product (or Fe protein) may have a positive autoregulatory function.

**Keywords:** *Klebsiella pneumoniae*, nitrogenase, anaerobic derepression, $nifH$-$lac$ expression, autoregulation

**INTRODUCTION**

The ability to fix $N_2$ occurs widely amongst obligate anaerobic, obligate aerobic, and facultative anaerobic prokaryotes (Young, 1992). The nitrogenase enzymes responsible for this fixation are very similar in structure and function (Eady, 1991). Since all are damaged irreversibly by oxygen they require anaerobic conditions as well as needing a source of reducing power and a large amount of ATP.

The structure of the nitrogen fixation genes ($nif$) and the mode of their regulation have been most studied in the facultative anaerobe *Klebsiella pneumoniae* (Dixon, 1984). Similarities and differences are found in other diazotrophs (Merrick, 1993). *K. pneumoniae* fixes $N_2$ under anaerobic or microaerobic conditions. The latter is beneficial as regards the efficiency of a fermentable carbon source for diazotrophy (Hill, 1976). In *K. pneumoniae*, 20 $nif$ genes are arranged in eight transcriptional units and make up the contiguous $nif$ cluster of 23 kb (Dixon, 1984; Merrick, 1993; Dean & Jacobson, 1992). The products of the $nifL$-$A$ operon have regulatory functions. Although the structural genes ($nif$HDK) are part of a single operon, the synthesis and activity of nitrogenase requires at least 12 other $nif$ gene products, including those required to generate a suitable source of reducing power (Dean & Jacobson, 1992).

Derepression of $nif$ in *K. pneumoniae* occurs under N limitation (Tubb & Postgate, 1973) and is associated with anaerobiosis (Eady et al., 1978), or very low oxygen concentrations (Hill et al., 1984). Transcription of the $nif$ is under the general nitrogen regulatory control ($ntr$) of enterics, and is mediated by a form of RNA polymerase that contains the rpoN-encoded $\sigma$ factor (Merrick, 1992; Kustu et al., 1989; Buck & Cannon, 1992). Transcription from the $nifL$-$A$ promoter is activated by the NtrC protein (Merrick, 1992; Kustu et al., 1989) as well as being influenced by the superhelical topology of the DNA (Whitehall et al., 1992). Transcription from all other $nif$ operons is activated by the $nifA$ product (Merrick, 1992; Buck, 1990). The activity of the $nifA$ product is inhibited by the $nifL$ product in response to repressive levels of environmental fixed N or O$_2$ (Merrick et al., 1982; Merrick, 1992; Hill, 1988). During derepression tran-
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or derivation</th>
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<tr>
<td><strong>K. pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M5a1</td>
<td>Wild type</td>
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</tr>
<tr>
<td>UNF619</td>
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<td>Dixon et al. (1980)</td>
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<tr>
<td>UNF921</td>
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<td>Filset et al. (1983)</td>
</tr>
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<td>JCS466</td>
<td>trp his rpsE recA</td>
<td>N. Willetts, Sidney, Australia</td>
</tr>
<tr>
<td>J62-1</td>
<td>trp his pro nal lac</td>
<td>N. Datta, London, UK</td>
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<td><strong>Plasmids</strong></td>
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</tr>
<tr>
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<td>Km&lt;sup&gt;R&lt;/sup&gt; Ch&lt;sup&gt;R&lt;/sup&gt; Te&lt;sup&gt;R&lt;/sup&gt; His&lt;sup&gt;+&lt;/sup&gt; Nif&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pRD191</td>
<td>nifH2191:: :Tn7</td>
<td>pRD1 derivative</td>
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<td>nifH2212:: :Tn7</td>
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<td>pHS415 derivative</td>
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<tr>
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<td>Km&lt;sup&gt;R&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt; nifH,Z,M</td>
<td>pHS415 derivative</td>
</tr>
<tr>
<td>pWPH7B</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt; nifH,M</td>
<td>pHS415 derivative</td>
</tr>
</tbody>
</table>

Two additional phenomena apparently control nitrogenase synthesis: hyperderepression, which occurs when the supply of N<sub>2</sub> limits growth (Postgate, 1982; Hill, 1992), and autoregulation, which is hypothetically effected by the MoFe protein of nitrogenase at the nifH promoter (Dixon et al., 1980). The data presented here show that the maintenance of nitrogenase synthesis during nif derepression requires N<sub>2</sub> fixation. This observation has led to a reassessment of the role of the nitrogenase MoFe protein in autoregulation. Some of this work was summarized earlier (Hill & Kavanagh, 1988).

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids are listed in Table 1. His<sup>+</sup>- or kanamycin- (Km) (30 µg ml<sup>-1</sup>) resistant transconjugants of *K. pneumoniae* were selected from matings (Cannon, 1980) with an *Escherichia coli* strain (JC5466 or J62-1), which carried the desired plasmid. Strains receiving plasmids carrying Tn7 were checked for resistance to trimethoprim (20 µg ml<sup>-1</sup>). Transformations of strain UNF686 with pHSG415, pWPH6B or pWPH7B were performed by the method of Merrick et al. (1987). Transformants were selected by resistance to Km (30 µg ml<sup>-1</sup>), and were checked for the presence of the desired plasmids by EcoR1 restriction analysis (Maniatis et al., 1982).

**Growth and derepression conditions.** Strains were maintained on either nutrient agar or minimal glucose medium (Cannon, 1980) containing Km when required. Anaerobic growth (at 28°C) for the derepression experiments was initiated with a 5% (v/v) inoculation from an 8 h aerobic nutrient broth culture in 50 ml of a nitrogen-free medium (NFDM) (Cannon, 1980) supplemented with 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Cultures were bubbled gently with 1% (v/v) CO<sub>2</sub> in N<sub>2</sub>. Cultures were harvested at room temperature by centrifugation for 15 min at 800 g. After decanting the supernatant, the walls of the vessel were wiped with a kleenex tissue to remove residual liquid. The concentrated cells were resuspended under Ar to a biomass density of about 0.45 mg protein ml<sup>-1</sup> in NFDM. When required, IPTG (1 mM) was added immediately after resuspension. Where the effect of C<sub>6</sub>H<sub>12</sub>O on nitrogenase derepression was being examined the procedure was as described previously (Hill et al., 1984). Anaerobic N-limited growth was established in NFDM supplemented with either serine (100 µg ml<sup>-1</sup>) or vitamin-free casamino acids (Difco) (400 µg ml<sup>-1</sup>) and Km, when required, that had been inoculated (5%, v/v) from an 8 h aerobic nutrient broth culture. Anaerobic conditions were achieved by bubbling 5 ml cultures in universal bottles under either N<sub>2</sub> or Ar, both containing 1% (v/v) CO<sub>2</sub>, at 28°C for 18 h. N-limited growth in bijou bottles capped with Subaseal closures was as described by Dixon et al. (1980). The conditions during this growth (18 h at 28°C) were likely to be microaerobic rather than anaerobic, because there was a small amount of air trapped above the medium.

**Immunoblot analysis.** Polypeptides, separated by SDS-PAGE using 12% (w/v) polyacrylamide gels, were transferred to Immobilon-P membrane (Millipore) (0.45 µm) by the semi-dry
blot method (Tovey & Baldro, 1987). Blots were strained with Auro Dye forte (Janssen) and then developed with rabbit antiserum raised to K. pneumoniae NifDK polypeptides (Serotec) and sheep anti-rabbit conjugate peroxidase (Serotec) using the ECL Western-blotting detection system (Amersham International) with HRG X-ray film (Fuji).

**Assays.** β-Galactosidase activity was measured as described by Miller (1972). Nitrogenase (C₂H₂-reducing) activity was measured either after N-limited growth as described by Dixon et al. (1980), or anaerobically in 1 ml samples removed from derepression treatments or from N-limited anaerobic growth by the method described previously (Hill et al., 1990), except that the time course was from 20 to 30 min, substrates were not added and 10% (v/v) C₂H₂ in either Ar or N₂ was the gas phase. Biomass was measured, routinely, by OD₆₀₀, and protein content was calculated from a determined relationship between OD and bacterial protein (see Smith et al., 1988). A ratio for a particular parameter, under N₂/under Ar, or the percentage value related to the relevant wild type was calculated for each experiment, and then the values from the replicated experiments were used to compute the mean and se.

### RESULTS AND DISCUSSION

**Nitrogenase derepression and N starvation**

When K. pneumoniae is grown anaerobically with excess NH₄⁺, harvested and then resuspended anaerobically in NFDM, nif is derepressed, and nitrogenase activity is detected 2–3 h later (Eady et al., 1978; Cannon et al., 1985). The addition of aspartate, glutamine or casamino acids to NFDM speeds up derepression (see Tubb & Postgate, 1973, and references therein; Nair & Eady, 1984) indicating that the rate of derepression is limited by the availability of fixed N. Likewise, N₂ fixation, once initiated, might influence the derepression kinetics in NFDM.

The kinetics of nitrogenase derepression in the wild type (M5a1) and in various Nif⁺ derivatives were followed in NFDM under either N₂ or Ar. After the appearance of nitrogenase (C₂H₂-reducing) activity, the subsequent derepression was faster under N₂ than under Ar (Figs 1b, 2b and 3b). The ratios of nitrogenase activities under N₂/under Ar were greater than 1 after 6 h derepression in

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**Fig. 1.** Effect of N₂ on the derepression in NFDM of (a) wild-type lac and (b) nitrogenase in strain M5a1. During derepression carried out in either the presence of IPTG (O, ●) or the absence of IPTG (△) organisms were exposed to either N₂ (open symbols) or Ar (closed symbols). Derepression of nitrogenase under either N₂ or Ar was not affected by the presence of IPTG. Samples were removed for assays of β-galactosidase (Miller units) or specific nitrogenase [nmol C₂H₂ produced (mg protein)⁻¹ min⁻¹] activities.

**Fig. 2.** Effect of N₂ on the derepression of (a) nifH-lac in the Nif⁺ strain UNF619(pMF183) (O, ●) and in the Nif- strain UNF686(pMF183) (△, △) and of (b) nitrogenase in the Nif⁺ strain UNF619(pMF183). During derepression, carried out under either N₂ (open symbols) or Ar (closed symbols), samples were removed for assays of β-galactosidase (Miller units) or specific nitrogenase [nmol C₂H₂ produced (mg protein)⁻¹ min⁻¹] activities.

1919
Fig. 3. Effect of N₂ on the derepression of (a) nifH–lac in the Nif⁺ strain UNF921(pMF100) (○, □) and the Nif⁻ strain UNF921(pMF263) (△, ▲) and of (b) nitrogenase in strain UNF921(pMF100). During derepression, carried out under N₂ (open symbols) or Ar (closed symbols), samples were removed for assays of β-galactosidase (Miller units) or specific nitrogenase [nmol C₅H₆ produced (mg protein)⁻¹ min⁻¹] activities.

The kinetics of nifH–lac derepression were also measured in Nif⁺ strains. As with nitrogenase, the rate of appearance of β-galactosidase activity was greater under N₂ than under Ar, once nitrogenase activity had been detected (Figs 2a and 3a). The ratios of activities under N₂/under Ar were greater than 1 after 6 h derepression in the Nif⁻ derivatives tested (Table 3). Thus, the enhancement of nitrogenase derepression by N₂ might in part reside at the level of transcription.

If N₂ fixation is essential then C₅H₆, an inhibitor of N₂ fixation (Hwang et al., 1973), should prevent the enhanced derepression of nitrogenase under N₂. The presence of C₅H₆ prevented the greater expression of nifH–lac and of nitrogenase activity in Nif⁻ strains during 6 h derepression under N₂ compared to that under Ar (Table 4). The ratios under N₂/under Ar of both β-galactosidase and nitrogenase were lowered to near unity by the presence of C₅H₆. Thus N₂ fixation is required for the enhanced derepression of nitrogenase under N₂.

**Regulation of nitrogenase derepression by N₂ fixation**

Three possibilities were investigated to determine how N₂ fixation might improve anaerobic derepression of nifH–lac and nitrogenase. Firstly, a modification of the nifL product could increase the effective concentration of the nifA product for nif transcription (Merrick et al., 1982; Filser et al., 1983; Drummond & Wootten, 1987). This was ruled out because the behaviour of a Nif⁻ Nif⁺ strain, UNF921(pMF337), was similar to that of the isogenic wild type Nif⁻ Nif⁺, UNF921(pMF100). In both strains the levels of nifH–lac and of nitrogenase activity after 6 h under N₂ were greater than those under Ar (Tables 2 and 3). Secondly, regulation by the products of the nitrogen regulatory genes ntrBC (Merrick, 1992) might improve nifL–A expression and hence increase nitrogenase derepression. The slightly greater nifL–lac expression we observed after 6 h derepression under N₂ compared to that under Ar in a Nif⁻ background [UNF619(pMF182)], but not in a Nif⁺ background [UNF686(pMF182)] (Table 5) was consistent with this possibility. Thirdly, N₂ fixation by upgrading the N status might stimulate translation. The kinetics of IPTG-induced expression of wild type lac during nitrogenase derepression under N₂ was compared with that under Ar in the Nif⁻ strain M5a1. A marked increase in β-galactosidase activity only occurred under N₂ when nitrogenase was detected (Fig. 1). Thus N₂ fixation by improving the N status probably stimulated lac translation. This stimulation could account for the enhanced expression of nifL–lac and nifH–lac under N₂, as these fusions are of the transcriptional type. Moreover, because nitrogenase polypeptide synthesis was also greater under N₂ compared to that under Ar (Fig. 4) the elevation in N status probably improved translation in general.

**Autoregulation and the MoFe protein of nitrogenase**

The evidence presented above, indicated that the marked difference in nifH–lac expression during derepression under N₂ compared to that under Ar in Nif⁻ strains can be accounted for by an improvement in N status increasing, at least, translation. These results prompted a reassessment of the role of the MoFe protein in autoregulation (Dixon et al., 1980). The evidence of Dixon et al. (1980) for this autoregulation rested upon four observations. Firstly, the expression of nifH–lac during derepression under N₂ in the Nif⁺ strain UNF619(pMF183) was much greater than in the Nif⁻ strain UNF686(pMF183). The latter strain can not make the nifH,D and K products. Secondly, a similar difference
Table 2. Nitrogenase activity in Nif\(^{+}\) strains after 6 h derepression under either N\(_{2}\) or Ar

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>(n)</th>
<th>Ratio of activities under N(_{2})/under Ar</th>
<th>Specific activity under Ar [nmol C(<em>{2})H(</em>{4}) produced (mg protein(^{-1}) min(^{-1})]</th>
</tr>
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<tr>
<td>M5a1</td>
<td>Wild type</td>
<td>–</td>
<td>Wild type</td>
<td>5</td>
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<td>49±0.7</td>
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<td>(\Delta(his-nzH)) nzH:: lac</td>
<td>pRD1</td>
<td>Wild type</td>
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<td>23±0.1</td>
<td>100±0.5</td>
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<tr>
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<td>Wild type</td>
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<td>21±0.1</td>
<td>152±0.8</td>
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<tr>
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<td>pMF337</td>
<td>nifL240</td>
<td>1</td>
<td>25</td>
<td>130</td>
</tr>
<tr>
<td>UNF619</td>
<td>Wild type</td>
<td>pMF183</td>
<td>nifH:: lac</td>
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<td>14±0.2</td>
<td>3.3±0.1</td>
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<td>UNF619</td>
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<td>nifL:: lac</td>
<td>2</td>
<td>25±0.3</td>
<td>6.9±0.3</td>
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</table>

Results are mean values±se and the number of different experiments is indicated under \(n\).

To eliminate the possible involvement of gene dosage by nif\(L\)\(A\) a similar experiment was performed with the Nif\(^{+}\) strain UNF921(pMF100) and the isogenic Nif\(^{-}\) strain UNF921(pMF263), which carries a Tn5 insertion in nif\(D\). Again the results showed that in the Nif\(^{+}\) strain, but not in the Nif\(^{-}\) strain, nif\(H\)–lac expression was faster under N\(_{2}\) than under Ar (Fig. 3a). When the kinetics under Ar were compared, the level of expression was not higher in the strain making the MoFe protein of nitrogenase, whereas the chromosome of UNF686(pMF183) carries none. The additional copy of nif\(L\)\(A\) in UNF619(pMF183) could have boosted nif\(H\)–lac expression.

The second observation of Dixon et al. (1980) showed that nif\(H\)–lac expression in the Nif\(^{+}\) strain UNF619(pMF183) was much greater than in the Nif\(^{-}\) strain UNF686(pMF183) during N-limited growth on 100 µg serine ml\(^{-1}\). We also found a similar difference in nif\(H\)–lac expression in these strains when grown under the microaerobic conditions (see Methods) used by Dixon et al. (1980), and when grown under Ar (Table 6). The extra copy of nif\(L\)\(A\) in the Nif\(^{-}\) strain UNF619(pMF183) (as indicated above) may have contributed, in part, to the greater expression in this Nif\(^{-}\) strain compared to that in the Nif\(^{+}\) strain UNF686(pMF183). When this experiment was performed with isogenic strains, the expression of

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**Fig. 4.** A Western blot of wild-type (M5a1) bacterial protein harvested at the end of 6 h derepression under either N\(_{2}\) or Ar. Polypeptides, separated by SDS-PAGE, were blotted, stained by Aurodye forte (a) and then this blot was analysed for NifD and K polypeptides by ELISA (b) (see Methods). Lanes: 1 and 3, derepressed under Ar; 2 and 4, derepressed under N\(_{2}\); 5, N\(_{2}\)-grown M5a1 harvested before derepression; 6, MoFe protein of K. pneumoniae nitrogenase. Lanes 1, 2 and 6 were loaded with 15 µg protein and lanes 3, 4 and 5 were loaded with 3 pg protein.

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in nif\(H\)–lac expression was observed in these Nif\(^{+}\) and Nif\(^{-}\) strains after N-limited growth on 100 µg serine ml\(^{-1}\). Thirdly, this greater expression required the presence of Mo. Fourthly, the levels of nif\(H\)–lac expression during N-limited growth in two out of three nif\(H\) insertion mutants were atypical.

The first observation of Dixon et al. (1980) might be accounted for by our evidence indicating that the expression of nif\(H\)–lac during derepression in Nif\(^{+}\) strains is enhanced by N\(_{2}\) fixation. The kinetics of derepression under Ar and under N\(_{2}\) were compared in the strains used by Dixon et al. (1980) [the Nif\(^{+}\) strain UNF619(pMF183) and the Nif\(^{-}\) strain UNF686(pMF183)] (Fig. 2). We confirmed their kinetics under N\(_{2}\), which showed that the rate of nif\(H\)–lac expression was much faster in the Nif\(^{+}\) than that in the Nif\(^{-}\) strain (Fig. 2a). As expected, our data showed that only in the Nif\(^{+}\) strain was nif\(H\)–lac derepression enhanced by N\(_{2}\). A comparison of the kinetics under Ar, where the N status could not be improved by N\(_{2}\) fixation, revealed that the level of expression was higher in the Nif\(^{+}\) strain. This result suggested that the Nif\(^{-}\) strain UNF686(pMF183) lacked a component which helps to drive nif\(H\)–lac expression in UNF619(pMF183). However, these strains differ not only in the ability of the Nif\(^{-}\) strain UNF619(pMF183) to make the MoFe protein of nitrogenase, but also the latter carries all the nif genes on the chromosome, whereas the chromosome of UNF686(pMF183) carries none. The additional copy of nif\(L\)\(A\) in UNF619(pMF183) could have boosted nif\(H\)–lac expression.

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Autoregulation of nitrogenase in K. pneumoniae

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1921
Table 3. nifH-lac derepression in UNF921 [(h-is-nifH)nifH2783::Mud(ApLac)] carrying a Nif+ or a Nif- plasmid under either N₂ or Ar

Results are mean values ± SE and the number of different experiments is indicated under n. Figures in parentheses show the activities in Miller units (mean ± SE).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Nif phenotype</th>
<th>Relevant genotype</th>
<th>n</th>
<th>β-Galactosidase activity at 6 h</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>Ratio under N₂/under Ar Under Ar (%)</td>
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<td>pRD213</td>
<td></td>
<td>nifJ2213::Tn7</td>
<td>3</td>
<td>1.05±0.04</td>
</tr>
</tbody>
</table>

Table 4. Influence of C,H₂ on the N₂-enhanced derepression of nifH-lac and nitrogenase in UNF921 [(h-is-nifH)nifH2783::Mud(ApLac)] carrying a Nif+ plasmid

Results are mean values ± SE [n = 2 for UNF921(pMF100); n = 1 for UNF921(pRD1)]. Figures in parentheses show the activities (mean ± SE) for the parameters under Ar in Miller units (β-galactosidase) and C,H₂ produced (mg protein)^⁻¹ min⁻¹ (nitrogenase).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Presence of C,H₂</th>
<th>Ratio of parameters at 6 h under N₂/under Ar</th>
<th>β-Galactosidase</th>
<th>Nitrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMF100</td>
<td>-</td>
<td>3.07±0.18 (193±26)</td>
<td>1.97±0.10 (15.8±2.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.07±0.03 (147±6)</td>
<td>0.69±0.11 (14.9±4.1)</td>
<td></td>
</tr>
<tr>
<td>pRD1</td>
<td>-</td>
<td>2.39 (395)</td>
<td>1.96 (15.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.06 (342)</td>
<td>1.10 (14.8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. nifL-lac derepression in a Nif+ or a Nif− [(h-is-nif)263] strain carrying pMF182 [nifL2782::Mud(ApLac)] under either N₂ or Ar

Results are mean values ± SE (n = 3). Figures in parentheses show the activity in Miller units (mean ± SE).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nif phenotype</th>
<th>β-Galactosidase activity at 6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ratio under N₂/under Ar Under Ar (%)</td>
</tr>
<tr>
<td>UNF619</td>
<td>+</td>
<td>1.41±0.06</td>
</tr>
<tr>
<td>UNF686</td>
<td></td>
<td>0.97±0.13</td>
</tr>
</tbody>
</table>

nifH-lac in the NifD⁻ or NifB⁻ strains was not lower than that in the relevant wild type (Table 7). Thus, we were unable to demonstrate autoregulation by the MoFe protein of nitrogenase after N-limited growth on 100 μg serine ml⁻¹ under the conditions used by Dixon et al. (1980) or under Ar.

We did not investigate the third observation of Dixon et al. (1980) regarding the requirement for Mo.

The fourth observation of Dixon et al. (1980) concerned the atypical levels of nifH-lac expression during N-limited growth in two out of three nifH insertion mutants. Whereas the expression of nifH-lac in one NifH⁻ merodiploid (carrying a mutation on pRD213) was lower than in the relevant Nif⁻ wild type, the expression was similar to the wild type in two other NifH⁻ derivatives (these carried mutations on either pRD191 or pRD212). Dixon et al. (1980) suggested that in the latter two mutants the insertions were non-polar, so that synthesis of the MoFe protein could occur. The expression of nifH-lac in strains carrying the same nifH mutations as used by Dixon et al. (1980) was compared with that in the relevant Nif⁺ strain during N-limited batch growth. Under their growth...
Table 6. nifH–lac expression from pMF183 [nifH2783::Mud(Aplac)] in either the Nif+ strain UNF619 or the Nif- strain UNF686 [Δ(his–nif)2633] after growth on serine (100 μg ml⁻¹) under either air (microaerobiosis) or Ar

Results are mean values ± se and the number of experiments is indicated under n.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nif phenotype</th>
<th>Potential to synthesize:*</th>
<th>β-Galactosidase (Miller units) under:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FeMoco</td>
<td>NifH product</td>
</tr>
<tr>
<td>UNF619</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UNF686</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* See Table 7.

Table 7. nifH–lac expression in UNF921 [Δ(his–nif)2633::Mud(Aplac)] carrying either a Nif+ or a Nif- plasmid after growth on serine (100 μg ml⁻¹) under either air (microaerobiosis) or Ar

Results are mean values ± se and the number of experiments is indicated under n. Figures in parentheses show Miller units (mean ± se).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Nif phenotype</th>
<th>Relevant genotype</th>
<th>Potential to synthesize:*</th>
<th>β-Galactosidase (% of wt) under:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FeMoco</td>
<td>NifH product</td>
</tr>
<tr>
<td>pMF100</td>
<td>+</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pRD1</td>
<td>+</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Capabilities to make the indicated products are represented as: +, able; −, unable; ±, able to make a mutant form (see text). ?, these capabilities are possible, but have not been determined.

Table 8. nifH–lac expression in strain UNF686 [Δ(his–nif)2633] carrying pMF183 [nifH2783::Mud(Aplac)] and either the vector or a recombinant plasmid after growth on serine (100 μg ml⁻¹) or casamino acids (400 μg ml⁻¹) under either air (microaerobiosis) or Ar

Results are mean values ± se [n = 3 for experiments with serine; n = 4 (with pWPH7B) or 6 (with pHSG415 and pWPH6B) for experiments with casamino acids]. Figures in parentheses show Miller units (mean ± se).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>nif genotype of recombinant plasmid</th>
<th>β-Galactosidase (%) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serine under:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>pHSG415</td>
<td>-</td>
<td>100 (676 ± 192)</td>
</tr>
<tr>
<td>pWPH6B</td>
<td>nifH,Z,M</td>
<td>155 ± 27</td>
</tr>
<tr>
<td>pWPH7B</td>
<td>nifH,M</td>
<td>131 ± 28</td>
</tr>
</tbody>
</table>

conditions or under Ar (Table 7) our results were similar to those of Dixon et al. (1980); only in UNF921(pRD213) was the expression lower than in the wild type. The capabilities of these strains to synthesize the nif products needed for the production of the MoFe nitrogenase protein are shown in Table 7. This analysis
assumes that the mutations are all polar, and incorporates
S. HILL and E. P. KAVANAGH
the synthesis of FeMoco. They also demonstrated that a
more recent work with the NifH- mutants (Filler
activity of the Fe protein of nitrogenase, was made from
was a
Table 7 indicates that the only likely factor missing from
strain UNF921(pRD213), but present in the other strains,
a
A
Autoregulation and the

The effect of the nifH product on nifH-lac expression was
tested by introducing a derivative of the low-copy plasmid
phSG415, either pWPH6B or pWPH7B (Paul & Merrick,
1989), into UNF686(pMF183). UNF686 carries a bis-nif
deletion which is complemented by pMF183, except that
the latter is NifH+, D- and K- as it bears the nifH-lac
fusion. The recombinant plasmids carry nifH and M
(pWPH7B) or nifH, M and Z (pWPH6B), and both encode
an active Fe protein of nitrogenase (Paul & Merrick,
1989). The expression of nifH-lac in these strains
was compared with that in the strain carrying the vector
phSG415 after 18 h of N-limited growth on 100 µg
serine ml-1 under either Ar or the assay conditions
(microaerobic) of Dixon et al. (1980) (Table 8). The levels
were not consistently higher in the strains carrying the
recombinant plasmids. The possibility that the in-
consistency arose from a slightly unfavourable environ-
mental condition for nifH-lac expression in this back-
ground was considered. The quality of the fixed N source
was altered to 400 µg casamino acids ml-1, which slightly
improved the expression of nifH-lac (Table 8) without
altering the biomass. The levels in the strains carrying
the recombinant plasmids were then consistently higher
than in that carrying the vector, but only under Ar. This
suggested that the nifH product or the Fe protein could
have a positive autoregulatory role under anaerobic
conditions. The involvement of a Fe protein component
of nitrogenases in the regulation of nitrogenase synthesis
is not novel. The anfA-dependent expression of the
alternative Fe nitrogenase of Azotobacter vinelandii requires
the presence of nifH and nifM (Joerger et al., 1991), and
the nifH can be replaced by A. vinelandii nifH (Joerger et
al., 1991) or the nifH of a K. pneumoniae background (Jacob
& Drummond, 1993).

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