Autoregulation of nitrogenase expression in *Klebsiella pneumoniae*

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An investigation into the influence of N₂ 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 (C₂H₂-reducing) activity, of NifD and K polypeptides, and of *nifH-lac* expression, following the removal of excess NH₄⁺, were greater under N₂ than Ar. This enhancement occurred in Nif⁺ but not in Nif⁻ strains, and in Nif⁺ strains was prevented by C₂H₂, an inhibitor of N₂ fixation. Thus N₂ 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 N₂ or Ar in a Nif⁺ strain were measured to investigate the regulation. The mechanism regulating the enhancement under N₂ neither involved the MoFe protein of nitrogenase, as proposed by Dixon *et al.* (1980, *Nature* 286, 128–132), 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 N₂ 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 N₂ under anaerobic or microaerobic conditions. The latter is beneficial as regards the efficiency of the utilization 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 *nifLA* operon have regulatory functions. Although

the structural genes (*nifHDK*) 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 *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 σ factor (Merrick, 1992; Kustu *et al.*, 1989; Buck & Cannon, 1992). Transcription from the *nifLA* 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 O₂ (Merrick *et al.*, 1982; Merrick, 1992; Hill, 1988). During derepression tran-

Table 1. Strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or derivation
<i>K. pneumoniae</i>		
M5a1	Wild type	
UNF619	Δlac MuhP ₁ <i>recA</i> <i>hisD</i>	Dixon <i>et al.</i> (1980)
UNF921	Δlac MuhP ₁ <i>recA</i> $\Delta 2639$ (<i>his-nifH</i>) <i>nifH2783::Mud(Aplac)</i>	Filser <i>et al.</i> (1983)
UNF686	Δlac MuhP ₁ <i>recA</i> $\Delta(his-nif)2633$	Dixon <i>et al.</i> (1980)
<i>E. coli</i>		
JC5466	<i>trp his rpsE recA</i>	N. Willetts, Sidney, Australia
J62-1	<i>trp his pro nal lac</i>	N. Datta, London, UK
Plasmids		
pRD1	Km ^R Cb ^R Tc ^R His ⁺ Nif ⁺	Merrick <i>et al.</i> (1980)
pRD191	<i>nifH2191::Tn7</i>	
pRD212	<i>nifH2212::Tn7</i>	
pRD213	<i>nifH2213::Tn7</i>	
pMF100	His ⁺ Nif ⁺	
pMF253	<i>nifB2553::Tn5</i>	
pMF263	<i>nifD2563::Tn5</i>	
pMF281	<i>nifD2581::Tn5</i>	
pMF337	<i>nifL5037::MunifL2401</i>	
pMF183	<i>nifH2783::Mud(Aplac)</i>	
pMF182	<i>nifL2782::Mud(Aplac)</i>	
pHSG415	Km ^R Ap ^R Cm ^R	Paul & Merrick (1989)
pWPH6B	Km ^R Ap ^R <i>nifH,Z,M</i>	
pWPH7B	Km ^R Ap ^R <i>nifH,M</i>	

scription of the *nifLA* operon precedes that of *nifHDKY* and other *nif* operons tested (Cannon *et al.*, 1985), but at least an hour elapses after *nifHDKY* transcription and translation are detected and before nitrogenase activity can be measured. This lag probably reflects the complexity of the processing of the polypeptides (Govezensky *et al.*, 1991).

Two additional phenomena apparently control nitrogenase synthesis: hyperderepression, which occurs when the supply of N₂ 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 N₂ 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⁺- or kanamycin- (Km) (30 µg ml⁻¹) 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⁻¹). 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⁻¹), and were checked for the

presence of the desired plasmids by *Eco*R1 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 (NH₄)₂SO₄. Cultures were bubbled gently with 1% (v/v) CO₂ in N₂. 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⁻¹ in NFDm. When required, IPTG (1 mM) was added immediately after resuspension. Where the effect of C₂H₂ 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⁻¹) or vitamin-free casamino acids (Difco) (400 µg ml⁻¹) 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 N₂ or Ar, both containing 1% (v/v) CO₂, 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 stained 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 (C_2H_2 -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) C_2H_2 in either Ar or N_2 was the gas phase. Biomass was measured, routinely, by OD_{600} , 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 N_2 /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.

Fig. 1. Effect of N_2 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 (○, ●) or the absence of IPTG (△) organisms were exposed to either N_2 (open symbols) or Ar (closed symbols). Derepression of nitrogenase under either N_2 or Ar was not affected by the presence of IPTG. Samples were removed for assays of β -galactosidase (Miller units) or specific nitrogenase (C_2H_2 -reducing) [nmol C_2H_4 produced (mg protein)⁻¹ min⁻¹] activities.

Fig. 2. Effect of N_2 on the derepression of (a) *nifH-lac* in the Nif^+ strain UNF619(pMF183) (○, ●) and in the Nif^- strain UNF686(pMF183) (△, ▲) and of (b) nitrogenase in the Nif^+ strain UNF619(pMF183). During derepression, carried out under either N_2 (open symbols) or Ar (closed symbols), samples were removed for assays of either β -galactosidase (Miller units) or specific nitrogenase [nmol C_2H_4 produced (mg protein)⁻¹ min⁻¹] activities.

RESULTS AND DISCUSSION

Nitrogenase derepression and N starvation

When *K. pneumoniae* is grown anaerobically with excess NH_4^+ , 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, N_2 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 N_2 or Ar. After the appearance of nitrogenase (C_2H_2 -reducing) activity, the subsequent derepression was faster under N_2 than under Ar (Figs 1b, 2b and 3b). The ratios of nitrogenase activities under N_2 /under Ar were greater than 1 after 6 h derepression in

Fig. 3. Effect of N_2 on the derepression of (a) $nifH-lac$ in the Nif^+ strain UNF921(pMF100) ($\circ$, $\bullet$) and the Nif^- strain UNF921(pMF263) ($\triangle$, $\blacktriangle$) and of (b) nitrogenase in strain UNF921(pMF100). During derepression, carried out under N_2 (open symbols) or Ar (closed symbols), samples were removed for assays of β -galactosidase (Miller units) or specific nitrogenase [nmol C_2H_4 produced (mg protein) $^{-1}$ min $^{-1}$] activities.

all the Nif^+ derivatives tested (Table 2). Higher levels of $nifH$ and D polypeptides were found under N_2 compared to that under Ar (Fig. 4). Thus the rate of nitrogenase synthesis was enhanced under N_2 compared to that under Ar.

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 N_2 than under Ar, once nitrogenase activity had been detected (Figs 2a and 3a). The ratios of activities under N_2 /under Ar were greater than 1 after 6 h derepression in the Nif^+ derivatives tested (Table 3). Thus, the enhancement of nitrogenase derepression by N_2 might in part reside at the level of transcription.

If N_2 fixation is essential then C_2H_2 , an inhibitor of N_2 fixation (Hwang *et al.*, 1973), should prevent the enhanced

derepression of nitrogenase under N_2 . The presence of C_2H_2 prevented the greater expression of $nifH-lac$ and of nitrogenase activity in Nif^+ strains during 6 h derepression under N_2 compared to that under Ar (Table 4). The ratios under N_2 /under Ar of both β -galactosidase and nitrogenase were lowered to near unity by the presence of C_2H_2 . Thus N_2 fixation is required for the enhanced derepression of nitrogenase under N_2 .

Regulation of nitrogenase derepression by N_2 fixation

Three possibilities were investigated to determine how N_2 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 & Wootton, 1987). This was ruled out because the behaviour of a $NifL^- Nif^+$ strain, UNF921(pMF337), was similar to that of the isogenic wild type $NifL^+ Nif^+$, UNF921(pMF100). In both strains the levels of $nifH-lac$ and of nitrogenase activity after 6 h under N_2 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 $nifLA$ expression and hence increase nitrogenase derepression. The slightly greater $nifL-lac$ expression we observed after 6 h derepression under N_2 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, N_2 fixation by upgrading the N status might stimulate translation. The kinetics of IPTG-induced expression of wild type lac during nitrogenase derepression under N_2 was compared with that under Ar in the Nif^+ strain M5a1. A marked increase in β -galactosidase activity only occurred under N_2 when nitrogenase was detected (Fig. 1). Thus N_2 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 N_2 , as these fusions are of the transcriptional type. Moreover, because nitrogenase polypeptide synthesis was also greater under N_2 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 N_2 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 re-assessment 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 N_2 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 N₂ or ArResults are mean values $\pm$ SE and the number of different experiments is indicated under *n*.

Strain	Relevant genotype	Plasmid	Relevant genotype	<i>n</i>	Ratio of activities under N ₂ /under Ar	Specific activity under Ar [nmol C ₂ H ₄ produced (mg protein) ⁻¹ min ⁻¹]
M5a1	Wild type	—		5	2.5 $\pm$ 0.5	4.9 $\pm$ 0.7
UNF921	$\Delta(bis-nifH)$ <i>nifH</i> : <i>lac</i>	pRD1	Wild type	4	2.3 $\pm$ 0.1	10.0 $\pm$ 2.5
UNF921	$\Delta(bis-nifH)$ <i>nifH</i> : <i>lac</i>	pMF100	Wild type	8	2.1 $\pm$ 0.1	15.2 $\pm$ 0.8
UNF921	$\Delta(bis-nifH)$ <i>nifH</i> : <i>lac</i>	pMF337	<i>nifL2401</i>	1	2.5	13.0
UNF619	Wild type	pMF183	<i>nifH</i> : <i>lac</i>	2	1.4 $\pm$ 0.2	3.3 $\pm$ 0.1
UNF619	Wild type	pMF182	<i>nifL</i> : <i>lac</i>	2	2.5 $\pm$ 0.3	6.9 $\pm$ 3.2

Fig. 4. A Western blot of wild-type (M5a1) bacterial protein harvested at the end of 6 h derepression under either N₂ or Ar. Polypeptides, separated by SDS-PAGE, were blotted, stained by Aurodyne 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 N₂; 5, NH₄⁺-grown M5a1 harvested before derepression; 6, MoFe protein of *K. pneumoniae* nitrogenase. Lanes 1, 2 and 6 were loaded with 1.5 μ g protein and lanes 3, 4 and 5 were loaded with 3 μ g protein.

in *nifH-lac* expression was observed in these Nif⁺ and Nif⁻ strains after N-limited growth on 100 μ g serine ml⁻¹. Thirdly, this greater expression required the presence of Mo. Fourthly, the levels of *nifH-lac* expression during N-limited growth in two out of three *nifH* insertion mutants were atypical.

The first observation of Dixon *et al.* (1980) might be accounted for by our evidence indicating that the expression of *nifH-lac* during derepression in Nif⁺ strains is enhanced by N₂ fixation. The kinetics of derepression under Ar and under N₂ 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 N₂, which showed that the rate of *nifH-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 *nifH-lac* de-repression enhanced by N₂. A comparison of the kinetics under Ar, where the N status could not be improved by N₂ 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 *nifH-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 *nifLA* in UNF619(pMF183) could have boosted *nifH-lac* expression.

To eliminate the possible involvement of gene dosage by *nifLA* a similar experiment was performed with the Nif⁺ strain UNF921(pMF100) and the isogenic Nif⁻ strain UNF921(pMF263), which carries a Tn5 insertion in *nifD*. Again the results showed that in the Nif⁺ strain, but not in the Nif⁻ strain, *nifH-lac* expression was faster under N₂ 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 [UNF921(pMF100)] (Fig. 3a). This result was confirmed in a number of other strains with Tn5 insertions in the *nifH*, *nifD* or *nifB* (Table 3). Thus, we were unable to detect autoregulation of *nifH-lac* expression by the Fe or MoFe proteins of nitrogenase under conditions of N starvation, during derepression under Ar.

The second observation of Dixon *et al.* (1980) showed that *nifH-lac* expression in the Nif⁺ strain UNF619(pMF183) was much greater than in the Nif⁻ strain UNF686(pMF183) during N-limited growth on 100 μ g serine ml⁻¹. We also found a similar difference in *nifH-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 *nifLA* 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

Table 3. *nifH-lac* derepression in UNF921 [$\Delta(his-nifH)nifH2783::Mud(Aplac)$] carrying a Nif⁺ or a Nif⁻ plasmid under either N₂ or Ar

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

Plasmid	Nif phenotype	Relevant genotype	<i>n</i>	β -Galactosidase activity at 6 h	
				Ratio under N ₂ /under Ar	Under Ar (%)
pMF100	+	Wild type	8	3.16 $\pm$ 0.25	100 (206 $\pm$ 10)
pMF281	-	<i>nifD2581::Tn5</i>	2	1.06 $\pm$ 0.01	127 $\pm$ 4
pMF263	-	<i>nifD2563::Tn5</i>	3	1.05 $\pm$ 0.02	121 $\pm$ 6
pMF253	-	<i>nifB2553::Tn5</i>	2	1.00 $\pm$ 0.04	106 $\pm$ 5
pMF337	+	<i>nifL2401</i>	2	3.73 $\pm$ 0.22	78 $\pm$ 9
pRD1	+	Wild type	4	2.94 $\pm$ 0.53	100 (229 $\pm$ 56)
pRD191	-	<i>nifH2191::Tn7</i>	2	0.98 $\pm$ 0.02	156 $\pm$ 25
pRD212	-	<i>nifH2212::Tn7</i>	3	0.87 $\pm$ 0.07	128 $\pm$ 21
pRD213	-	<i>nifH2213::Tn7</i>	3	1.05 $\pm$ 0.04	100 $\pm$ 9

Table 4. Influence of C₂H₂ on the N₂-enhanced derepression of *nifH-lac* and nitrogenase in UNF921 [$\Delta(his-nifH)nifH2783::Mud(Aplac)$] carrying a Nif⁺ plasmid

Results are mean values $\pm$ SE [*n* = 2 for UNF921(pMF100); *n* = 1 for UNF921(pRD1)]. Figures in parentheses show the activities (mean $\pm$ SE) for the parameters under Ar in Miller units (β -galactosidase) and C₂H₄ produced (mg protein)⁻¹ min⁻¹ (nitrogenase).

Plasmid	Presence of C ₂ H ₂	Ratio of parameters at 6 h under N ₂ /under Ar	
		β -Galactosidase	Nitrogenase
pMF100	-	3.07 $\pm$ 0.18 (193 $\pm$ 26)	1.97 $\pm$ 0.10 (15.8 $\pm$ 2.3)
	+	1.07 $\pm$ 0.03 (147 $\pm$ 6)	0.69 $\pm$ 0.11 (14.9 $\pm$ 4.1)
pRD1	-	2.39 (395)	1.96 (15.1)
	+	1.06 (342)	1.10 (14.8)

Table 5. *nifL-lac* derepression in a Nif⁺ or a Nif⁻ [$\Delta(his-nif)2633$] strain carrying pMF182 [*nifL2782::Mud(Aplac)*] under either N₂ or Ar

Results are mean values $\pm$ SE (*n* = 3). Figures in parentheses show the activity in Miller units (mean $\pm$ SE).

Strain	Nif phenotype	β -Galactosidase activity at 6 h	
		Ratio under N ₂ /under Ar	Under Ar (%)
UNF619	+	1.41 $\pm$ 0.06	100 (232 $\pm$ 77)
UNF686	-	0.97 $\pm$ 0.13	205 $\pm$ 97

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 μ g 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 [$\Delta(his-nif)2633$] after growth on serine (100 $\mu\text{g ml}^{-1}$) under either air (microaerobiosis) or ArResults are mean values $\pm$ SE and the number of experiments is indicated under *n*.

Strain	Nif phenotype	Potential to synthesize:*			β -Galactosidase (Miller units) under:			
		FeMoco	NifH product	NifDK products	Air	(<i>n</i>)	Ar	(<i>n</i>)
UNF619	+	+	+	+	7948 $\pm$ 921	(2)	6036 $\pm$ 1323	(4)
UNF686	-	-	-	-	536 $\pm$ 72	(3)	1828 $\pm$ 386	(4)

* See Table 7.

Table 7. *nifH-lac* expression in UNF921 [$\Delta(his-nifH)nifH2783::Mud(Aplac)$] carrying either a Nif⁺ or a Nif⁻ plasmid after growth on serine (100 $\mu\text{g ml}^{-1}$) under either air (microaerobiosis) or ArResults are mean values $\pm$ SE and the number of experiments is indicated under *n*. Figures in parentheses show Miller units (mean $\pm$ SE).

Plasmid	Nif phenotype	Relevant genotype	Potential to synthesize:*			β -Galactosidase (% of wt) under:	
			FeMoco	NifH product	NifDK products	Air [<i>n</i>]	Ar [<i>n</i>]
pMF100	+	Wild type	+	+	+	100 (941 $\pm$ 38) [4]	100 (1145 $\pm$ 95) [6]
pMF281	-	<i>nifD2581::Tn5</i>	+	+	-	132 $\pm$ 14 [3]	126 $\pm$ 6 [4]
pMF263	-	<i>nifD2563::Tn5</i>	+	+	-	122 $\pm$ 10 [2]	132 $\pm$ 14 [3]
pMF253	-	<i>nifB2553::Tn5</i>	-	+	+	116 $\pm$ 21 [3]	116 $\pm$ 7 [4]
pRD1	+	Wild type	+	+	+	100 (1415 $\pm$ 97) [3]	100 (1410 $\pm$ 190) [5]
pRD191	-	<i>nifH2191::Tn7</i>	+	$\pm$	-	144 $\pm$ 13 [3]	165 $\pm$ 21 [4]
pRD212	-	<i>nifH2212::Tn7</i>	?+	? $\pm$	-	138 $\pm$ 42 [2]	184 [1]
pRD213	-	<i>nifH2213::Tn7</i>	-	-	-	67 $\pm$ 18 [3]	61 $\pm$ 6 [4]

* Capabilities to make the indicated products are represented as: +, able; -, unable; $\pm$, 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 [$\Delta(his-nif)2633$] carrying pMF183 [*nifH2783::Mud(Aplac)*] and either the vector or a recombinant plasmid after growth on serine (100 $\mu\text{g ml}^{-1}$) or casamino acids (400 $\mu\text{g ml}^{-1}$) under either air (microaerobiosis) or ArResults are mean values $\pm$ 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 $\pm$ SE).

Plasmid	<i>nif</i> genotype of recombinant plasmid	β -Galactosidase (%) with:			
		Serine under:		Casamino acids under:	
		Air	Ar	Air	Ar
pHSG415	-	100 (676 $\pm$ 192)	100 (2177 $\pm$ 356)	100 (2121 $\pm$ 333)	100 (3140 $\pm$ 239)
pWPH6B	<i>nifH,Z,M</i>	155 $\pm$ 27	158 $\pm$ 16	121 $\pm$ 19	180 $\pm$ 11
pWPH7B	<i>nifH,M</i>	131 $\pm$ 28	150 $\pm$ 26	108 $\pm$ 12	204 $\pm$ 23

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 more recent work with the *NifH*⁻ mutants (Filler *et al.*, 1986). These authors found that the *nifH* product made from pRD191, but not that from pRD213, could support the synthesis of FeMoco. They also demonstrated that a truncated form of the *nifH* product, which lacked the activity of the Fe protein of nitrogenase, was made from pRD191. They did not examine pRD212. The analysis in Table 7 indicates that the only likely factor missing from strain UNF921(pRD213), but present in the other strains, was a *nifH* product. Therefore the *nifH* product rather than the MoFe protein of nitrogenase seemed a more likely candidate for the 'autoregulator'.

Autoregulation and the *nifH* product

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 inconsistency arose from a slightly unfavourable environmental condition for *nifH-lac* expression in this background 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 *vnfH* and *nifM* (Joerger *et al.*, 1991), and the *vnfH* 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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