Influence of Low Oxygen Concentration on Derepression of Nitrogenase in *Klebsiella pneumoniae*

By F. J. BERGERSEN,¹* CHRISTINA KENNEDY² AND SUSAN HILL²

¹ CSIRO, Division of Plant Industry, Canberra, A.C.T. 2601, Australia

² A.R.C. Unit of Nitrogen Fixation, The University of Sussex, Brighton, Sussex BN1 9RQ, U.K.

(Received 17 August 1981)

Effects of oxygen upon derepression of nitrogenase were studied in *Klebsiella pneumoniae*, using oxyleghaemoglobin to supply and monitor very low dissolved O₂ concentrations in a steady-state system. Expression of the *nifH* gene was studied by using a *nifH::lac* fusion strain, which also carried the Nif⁺ plasmid pRD1 so that the production of active nitrogenase could also be monitored. When compared with anaerobic treatments, very low concentrations of dissolved O₂ inhibited derepression of both *nifH::lac* and pRD1 *nif*. Fifty percent inhibition of derepression occurred at 0.1 µM-O₂. The apparent K_s of the dominant terminal oxidase was 0.08 µM-O₂. These results suggest that there is a close relationship between the terminal respiratory system of these bacteria and the repression of nitrogenase by O₂.

INTRODUCTION

Nitrogen fixation by *Klebsiella* spp. is primarily associated with the anaerobic mode of metabolism, although nitrogenase can be produced and is active when O₂ supply limits growth (Klucas, 1972; Hill, 1976; Bergersen & Turner, 1980). Repression by O₂ of synthesis of nitrogenase (the products of the structural genes *nifHDK*) in *K. pneumoniae* was demonstrated by Eady *et al.* (1978), when derepressed cultures were bubbled with either 5% or 20% (v/v) O₂. Further work (Hill *et al.*, 1981) showed that repression occurred with a dissolved oxygen tension of 0.25% (3 µM-O₂), but the lowest concentration needed to cause repression was not established.

Studies of O₂ regulation of *nif* have been facilitated by the fusion of the *E. coli lacZ* gene into the various transcriptional units of the *nif* gene cluster because expression from the various *nif* promoters results in an O₂-stable product (β -galactosidase) (Dixon *et al.*, 1980). Such studies have revealed that a dissolved oxygen tension of 0.5% inhibited derepression of *nifHDK* but not of *nifLA* transcriptional units (Dixon *et al.*, 1980) and that the *nifL* gene product is involved as a negative effector in the O₂-regulatory mechanism specific to nitrogen fixation (Hill *et al.*, 1981). Although strains carrying *nif::lac* fusions provide an easy way of measuring expression from *nif* promoters, such strains are Nif⁻; therefore we have used a strain carrying the Nif⁺ plasmid pRD1, as well as a *nifH::lac* fusion.

In the present work we have used the technique of Bergersen & Turner (1979), which employs the O₂-binding protein leghaemoglobin to supply and monitor very low dissolved O₂ concentration, in order to determine the concentrations of O₂ that inhibit derepression of *nifHDK* and prevent nitrogenase activity *in vivo*.

METHODS

Media. Nutrient broth and nutrient agar were from Oxoid. NFD medium was that of Cannon *et al.* (1974). Where indicated, it included (NH₄)₂SO₄ (10 mM), histidine (20 µg ml⁻¹), kanamycin (Km, 20 µg ml⁻¹),

Table 1. *Bacteria and plasmid*

	Genotype or phenotype	Reference or source
<i>Klebsiella pneumoniae</i> UNF767	<i>hisD2 nifH2783 :: Mud(Aplac) lacA recA56</i>	Hill <i>et al.</i> (1981)
<i>Klebsiella pneumoniae</i> CK260	<i>hisD2 nifH2260 hsdR1 rpsL4</i>	Dixon <i>et al.</i> (1977)
<i>Escherichia coli</i> JC5466	<i>trp recA56 his rplE</i>	Dixon <i>et al.</i> (1976)
Plasmid pRD1	Km Tc Cb Gnd His Nif ShiA Tra IncP	Dixon <i>et al.</i> (1976)

streptomycin (Sm, 200 µg ml⁻¹) and Noble agar (1.5%, w/v; Difco). Derepression medium contained potassium phosphate (95 mM, pH 7.2), sucrose (29 mM), Na₂MoO₄·2H₂O (0.1 mM).

Bacteria and plasmid. These are shown in Table 1. The plasmid pRD1, which carries the *his-nif* region of the *K. pneumoniae* chromosome, was transferred from *E. coli* strain JC5466 to UNF767 by conjugation (Dixon *et al.*, 1976) on nutrient agar, followed by selection of transconjugants on NFDM plus NH₄⁺ and kanamycin. Several transconjugant colonies were transferred to and maintained on NFDM plus NH₄⁺ with minimal subculturing. In the course of the experiments, three different constructions were used, two prepared in Sussex and one in Canberra. In all respects they behaved similarly.

To ensure that subculturing and the experimental procedure did not produce an alteration or loss of pRD1 in the transconjugant strain, cultures were checked, after final sampling, for His⁺ Nif⁺ phenotype and for transfer of these characters to another His⁻ Nif⁻ mutant *K. pneumoniae* strain (CK260). Diluted culture samples were plated on NFDM + NH₄⁺ and, when grown, were replica plated either on to NFDM or on to lawns of 10⁷ cells of CK260 spread on the surfaces of 2 NFDM + Sm plates, one also containing NH₄⁺. Anaerobic growth was scored 3–4 d later. The former replica plating was performed after each experiment: for all data presented, the His⁺ Nif⁺ phenotype of UNF767(pRD1) was maintained. The latter replica plating ensured that *his* and *nif* were transferable on pRD1 to CK260: only CK260(pRD1) transconjugants can grow on both plates, since UNF767(pRD1) is Sm^s and CK260 is His⁺ and Nif⁻ but Sm^r. Transferability was tested periodically during these experiments, and once indicated the need to use a freshly constructed UNF767(pRD1) culture.

Cultures. For derepression experiments, an overnight culture of UNF767(pRD1) was grown in NFDM + NH₄⁺ (10 ml) at 30 °C in air. This was used to inoculate 100 ml NFDM + NH₄⁺ in a Suba-Seal-capped Erlenmeyer flask (150 ml), and the culture was grown for 20 h at 30 °C under N₂ with shaking at 150 rev. min⁻¹. Two 40 ml portions of the culture were transferred by syringe to N₂-flushed, capped centrifuge tubes and the bacteria collected by centrifugation at 7500 g for 10 min. The pellets were kept under the supernatants at ice temperature, under N₂. Just before each experiment the supernatants were removed and the bacteria resuspended in 5 or 10 ml degassed, ice-cold derepression medium. Portions (1.0 or 1.5 ml) were then transferred by syringe to the experimental vessels described below.

Derepression experiments. Anaerobic and O₂ treatments were run in parallel. For anaerobic treatments the bacterial suspension was injected at zero time into 100 ml Suba-Seal-capped Erlenmeyer flasks containing 16 ml NFDM or derepression medium, degassed by evacuation and filled with 1 atm N₂. The flasks were shaken reciprocally at 150 excursions min⁻¹ at 30 °C in a water bath. Samples (2.0 ml) were withdrawn by argon-flushed syringes at intervals of approximately 1 h.

For O₂ treatments the apparatus of Bergersen & Turner (1979) was used (Fig. 1). The reaction chamber (approx. vol. 16.4 ml) equipped with a magnetic stirrer and water jacket (needed to maintain the chamber contents at 30 °C) was flushed with argon and filled completely with degassed NFDM or derepression medium. At zero time, 1.0 or 1.5 ml of bacterial suspension was injected into the chamber, and O₂ was then immediately supplied to the bacteria. This was achieved by pumping through the chamber air-saturated (240–267 µM-O₂ at 25 °C, according to the barometric pressure) derepression medium, containing about 100 µM-oxyleghaemoglobin (Appleby & Bergersen, 1980), at a pre-determined rate appropriate to maintain the desired free O₂ concentration in the effluent. The degree of oxygenation (Y) of the leghaemoglobin (Lb) and the equilibrium concentration of free dissolved O₂ in the effluent (assumed to be equal to that in the chamber) were calculated as before (Bergersen & Turner 1979). The rates of consumption of O₂ [nmol min⁻¹ (mg dry wt)⁻¹] in the chamber were calculated from the difference in concentrations of free O₂ and of oxyleghaemoglobin between the medium supplied and the effluent from the chamber (Bergersen & Turner 1979). Samples (2.0 ml) of bacteria were withdrawn from the chamber at intervals of about 1 h, using argon-flushed syringes connected through the port (i₂, Fig. 1) and closing tap (t). Removal of bacteria during sampling was taken into account when calculating rates of O₂ consumption in the chamber. In most experiments steady levels of oxygenation of leghaemoglobin in the effluent were achieved within 15–20 min of zero time and were maintained for up to 6 h by adjusting flow rates slightly as required, especially following sampling. Data from a typical experiment are given in Table 2.

Treatment of samples. Immediately following the collection of each sample, 0.5 ml was used for a nitrogenase assay. The balance was quickly chilled to 0–0.5 °C by delivery into a prechilled glass tube immersed in ice.

Fig. 1. A schematic diagram of the apparatus for steady O_2 supply: r, reservoir of derepression medium containing leghaemoglobin and stirred in air at 25 °C; g, reaction chamber with water jacket (for design details see Bergersen & Turner, 1979) and magnetic stirrer, m; f, spectrophotometer flow cell; t, a stopcock; i_2 , an injection port.

Table 2. Results of a typical experiment in the reaction chamber

Initially 8.79 mg (dry wt) of UNF767(pRD1) were present. The inflowing medium contained 101.5 μM -oxyleghaemoglobin and 245 μM free dissolved O_2 .

Elapsed time (h)	Effluent medium		Bacteria in chamber (mg dry wt)	Medium flow (ml min ⁻¹)	O_2 consumption [nmol O_2 min ⁻¹ (mg dry wt) ⁻¹]
	Lb oxygenation (Y)	Free O_2 (μM)			
0.33	0.686	0.081	8.79	0.73	23.1
0.43	0.638	0.066		0.81	26.0
0.58	0.563	0.058		0.93	30.6
0.67	0.615	0.060		0.97	31.3
0.83	0.593	0.055		0.96	31.3
1.02	0.563	0.048	7.98	0.96	34.8
1.08	0.569	0.049		0.97	35.1
1.33	0.581	0.052		0.99	35.7
1.58	0.569	0.049		0.99	35.8
1.83	0.569	0.049		1.04	37.6
2.08	0.581	0.052	7.46	0.98	33.8
2.33	0.600	0.056		0.98	37.9
2.67	0.638	0.066		1.00	37.4
2.92	0.619	0.061		0.93	35.4
3.08	0.594	0.055		0.96	39.0
3.33	0.594	0.055	7.05	0.91	36.9
3.67	0.594	0.055		0.93	37.8
3.83	0.574	0.050		0.93	38.0
4.08	0.588	0.053		0.93	39.6
4.33	0.615	0.060		0.93	39.3
4.58	0.625	0.062	6.73	0.93	39.1
5.00	0.631	0.064		0.94	39.5
		$\bar{x} = 0.057$ (S.D. 0.007)			$\bar{x} = 34.6$ (S.D. 5.19)

Samples containing leghaemoglobin were centrifuged at 0 °C and the bacteria resuspended in the same volume (1.5 ml) of cold derepression medium and kept on ice. At the end of the experiment, the samples were assayed for β -galactosidase activity (0.5 ml) and the balance was used to determine absorbance at 600 nm and 700 nm.

Assays. Nitrogenase activity in samples from derepression experiments was assayed in capped small bottles (4.85 ml), filled with 5% C_2H_2 in argon. The samples of bacteria (0.5 ml) were injected and the bottle was shaken

for 30 min at 30 °C. The reactions were stopped by injecting 0.2 ml 10% (w/v) trichloroacetic acid. Production of C_2H_4 from C_2H_2 was measured by gas chromatography (Turner & Gibson 1980).

β -galactosidase activity was measured, using 0.5 ml portions of samples, by the method described by Miller (1972).

Leghaemoglobin concentrations were measured using the pyridine haemochrome method (Appleby & Bergersen, 1980).

Dry weights of bacteria present in samples were estimated using A_{700} values and a standard curve prepared previously for *K. pneumoniae* (Bergersen & Turner, 1980).

RESULTS AND DISCUSSION

Nutritional requirements during derepression of $nifH::lac(pRD1)$

Preliminary experiments utilized NFDM as the derepression medium (Dixon *et al.*, 1980); however, when high rates of O_2 supply were used in the chamber, the bacteria agglutinated into a brownish gel which adhered to the stirrer. This effect was minimized when sucrose was substituted for glucose and the $MgSO_4$, Na_2MoO_4 and $FeSO_4$, which were supplied in a stock solution, were omitted. However MoO_4^{2-} promotes derepression of β -galactosidase in another strain of *K. pneumoniae* containing a plasmid carrying $nifH::lac$ (Dixon *et al.*, 1980) and this was found to be the case with UNF767(pRD1) also (Fig. 2). Sucrose concentration also affected the rate of derepression (Fig. 2a). These results led us to use derepression medium containing 1% sucrose, 95 mM-potassium phosphate (as in NFDM) and 0.1 mM- Na_2MoO_4 ; in this medium derepression under anaerobic conditions was similar to that in NFDM, but agglutination of bacteria in the chamber was insignificant.

Time course of derepression of $nifH::lac$ with O_2

Low concentrations of O_2 (0.02–0.06 μM) invariably stimulated the early stages of derepression, compared with anaerobic treatments, but later on, organisms from anaerobic treatments always had higher activity; data for a typical experiment are shown in Fig. 3. With increased concentrations of O_2 , rates of derepression declined, reaching very low values at 2.6 μM - O_2 (Fig. 3). Results of nitrogenase assays were completely parallel to results of β -galactosidase assays (Fig. 4) but, as found previously (Dixon *et al.*, 1980), the appearance of β -galactosidase activity preceded the appearance of nitrogenase activity by about 1 h (Figs. 2, 4).

Consumption of O_2 and determination of apparent K_s

Steady-state rates of O_2 consumption were calculated as shown in Table 2. Data from experiments are illustrated in Table 3 and Fig. 5. The individual values (Table 3) were obtained with acceptable precision, being based on up to 35 estimations during each steady state. The estimate of the apparent K_s for O_2 uptake of 0.08 μM (Fig. 5) is very similar to that measured for another strain, *K. pneumoniae* strain 50231 (0.1 μM ; Bergersen & Turner, 1980). The experimental design in the present investigation did not permit the study of induction of this terminal oxidase of moderately high affinity. However, the data for strain 50231 (Bergersen & Turner, 1980) suggest that this principal terminal oxidase is constitutive, since similar values for apparent K_s were obtained from chemostat populations grown either anaerobically or at low concentrations of dissolved O_2 .

O_2 concentration and derepression

Hill *et al.* (1981) reported that derepression of β -galactosidase was inhibited by a dissolved oxygen concentration of 0.5% (6 μM - O_2) in strain UNF767 carrying pRD1. Our results show that even at an O_2 concentration of 2.6 μM , very little derepression occurs in this strain (Fig. 4). This concentration of 2.6 μM - O_2 is much lower than the dissolved oxygen tension of 10 mmHg (16 μM - O_2) which allowed some nitrogenase synthesis in another species of *Klebsiella* (Klucas, 1972). This suggests that there may be differences in the apparent

Fig. 2. Effects of composition of medium during *nifH::lac* derepression (data for anaerobic experiments). (a) NFDM, but with 1% sucrose instead of glucose (●); NFDM with 0.5% sucrose instead of glucose (○); 1% sucrose but with Mo, Fe and Mg at 1/10 concentration (Δ; see text for composition). (b) 1% sucrose in 95 mM-potassium phosphate (pH 7.2) with Fe, Mo and Mg as in NFDM (●), with 0.1 mM Na_2MoO_4 only (○), or with 0.8 mM $MgSO_4$ only (Δ). The continuous lines show β -galactosidase activity; the dashed lines show nitrogenase activity.

Fig. 3

Fig. 4

Fig. 3. Typical time course of derepression of *nifH::lac*. Anaerobic treatments were shaken under N_2 (○). Oxygen treatments: 0.027 μM - O_2 (●); 2.6 μM - O_2 (▼).

Fig. 4. Correspondence between derepression of *nifH::lac* (β -galactosidase activity: ●, ○) and pRD1 *nif* (nitrogenase activity: ▲, Δ). Anaerobic treatment (○, Δ) and 2.6 μM - O_2 (●, ▲).

sensitivities of the repression mechanism of nitrogenase synthesis in various *Klebsiella* species. Almost certainly there will be a gradient of O_2 concentration between that in the medium, the value measured, and that at the site of nitrogenase synthesis within the cell. Thus the different

Fig. 5. Estimation of apparent K_s of the principal terminal oxidase of UNF767(pRD1) (data from Table 3).

Table 3. *Respiration of UNF767(pRD1) during steady states*

The values in parentheses are standard deviations for means of 22–35 estimations in each experiment.

Expt. no.	[O ₂ free] (μM)	O ₂ consumption [nmol O ₂ min ⁻¹ (mg dry wt) ⁻¹]
3	0.0215 (0.0042)	21.5 (3.0)
1	0.027 (0.004)	18.7 (3.3)
4	0.057 (0.007)	34.6 (5.2)
5	0.193 (0.072)	42.8 (1.7)
2	2.59 (0.37)	106.6 (11.2)

Fig. 6. Relationship between concentration of O₂ and derepression of *nifH::lac* in UNF767(pRD1). Percentage derepression was calculated from each experiment as: $100 \times [\beta\text{-galactosidase (O}_2\text{)}] / [\beta\text{-galactosidase (anaerobic)}]$. The apparent K_s of the terminal oxidase of *K. pneumoniae* is indicated by arrows: (a) this work, (b) Bergersen & Turner (1980).

apparent sensitivities to repression may reflect the magnitude of the O₂ gradient in the different experimental systems used.

Figure 6 shows the influence of dissolved O₂ concentration on β -galactosidase activities during the various experiments, expressed as a percentage activity of parallel anaerobic treatments, all measured 5 h after removal of NH₄⁺ and commencement of O₂ treatment. It is clear that 50% inhibition of derepression was obtained at an O₂ concentration close to the apparent K_s of the constitutive principal terminal oxidase. At this O₂ concentration sufficient O₂ may penetrate the cell to interfere with *nif* gene expression. Alternatively, due to the fairly high affinity of the principal constitutive terminal oxidase for O₂, the enzyme could act as a sensitive monitor for O₂ concentration. Inhibition of expression from the *nifH* promoter, a process probably mediated through the *nifL* gene product (Hill *et al.*, 1981), could thus be triggered by the oxidized form of the terminal oxidase or some other oxidized component of the electron transport pathway.

REFERENCES

- APPLEBY, C. A. & BERGERSEN, F. J. (1980). Preparation and experimental use of leghaemoglobin. In *Methods for Evaluating Biological Nitrogen Fixation*, pp. 315–335. Edited by F. J. Bergersen. Chichester: Wiley.
- BERGERSEN, F. J. & TURNER, G. L. (1979). Systems utilizing oxygenated leghaemoglobin and myoglobin as sources of free dissolved O₂ at low concentrations for experiments with bacteria. *Analytical Biochemistry* **96**, 165–174.
- BERGERSEN, F. J. & TURNER, G. L. (1980). Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and of N₂-fixing bacteria grown in continuous culture. *Journal of General Microbiology* **118**, 235–252.
- CANNON, F. C., DIXON, R. A., POSTGATE, J. R. & PRIMROSE, S. B. (1974). Chromosomal integration of *Klebsiella* nitrogen fixation genes in *Escherichia coli*. *Journal of General Microbiology* **80**, 227–239.
- DIXON, R. A., CANNON, F. & KONDOROSI, A. (1976). Construction of a P plasmid carrying nitrogen fixation genes from *Klebsiella pneumoniae*. *Nature, London* **260**, 268–271.
- DIXON, R., KENNEDY, C., KONDOROSI, A., KRISHNAPILLAI, V. & MERRICK, M. (1977). Complementation analysis of *Klebsiella* mutants defective in nitrogen fixation. *Molecular and General Genetics* **157**, 189–198.
- DIXON, R., EADY, R. R., ESPIN, G., HILL, S., IACCARINO, M., KAHN, D. & MERRICK, M. (1980). Analysis of regulation of *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions. *Nature, London* **286**, 128–132.
- EADY, R. R., ISSACK, R., KENNEDY, C., POSTGATE, J. R. & RATCLIFFE, H. D. (1978). Nitrogenase synthesis in *Klebsiella pneumoniae*: comparison of ammonium and oxygen regulation. *Journal of General Microbiology* **104**, 277–285.
- HILL, S. (1976). Influence of atmospheric oxygen concentration on acetylene reduction and efficiency of nitrogen fixation in intact *Klebsiella pneumoniae*. *Journal of General Microbiology* **93**, 335–345.
- HILL, S., KENNEDY, C., KAVANAGH, E., GOLDBERG, R. B. & HANAU, R. (1981). Nitrogen fixation gene (*nifL*) involved in oxygen regulation of nitrogenase synthesis in *Klebsiella pneumoniae*. *Nature, London* **290**, 424–426.
- KLUCAS, R. V. (1972). Nitrogen fixation by *Klebsiella* grown in the presence of oxygen. *Canadian Journal of Microbiology* **18**, 1845–1850.
- MILLER, J. H. (1972). Assay of β -galactosidase. In *Experiments in Molecular Genetics*, pp. 253–355. New York: Cold Spring Harbor Laboratory.
- TURNER, G. L. & GIBSON, A. H. (1980). Measurement of nitrogen fixation by indirect means. In *Methods for Evaluating Biological Nitrogen Fixation*, pp. 111–138. Edited by F. J. Bergersen. Chichester: Wiley.