A Haemoprotein Is Not Involved in the Control by Oxygen of Enteric Nitrogenase Synthesis

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INTRODUCTION

Nitrogen fixation in the facultative anaerobe *Klebsiella pneumoniae* occurs only in anaerobic or microaerobic conditions (at less than 30 nM-O₂) (Hill et al., 1984). At about 6 µM-O₂ nitrogenase is inactivated and transcription from all the nif operons except for nifLA (the regulatory operon) is inhibited (Dixon et al., 1980; Hill et al., 1981; Merrick et al., 1982; Cannon et al., 1985). At concentrations of O₂ above 6 µM, expression from the nifLA promoter is also inhibited. The mechanism whereby O₂ regulates the expression of the nif genes is not known although the evidence suggests that in response to low concentrations of O₂, the nifL product antagonizes the action of the nifA product, the nif-specific transcriptional activator (Hill et al., 1981; Merrick et al., 1982; Filser et al., 1983; Dixon et al., 1980; Buchanan-Wollaston & Cannon, 1984; Kennedy & Drummond, 1985). The mechanism by which the O₂ status of the bacterium is communicated to the nifL product remains a matter for speculation.

The sequence of nifL (Kim et al., 1986; Drummond & Wootton, 1987) revealed a -Cys-X-X-Cys- region that is homologous with redox-sensitive sites in disulphide reductases such as thioredoxin, and with metal-binding sites in redox centres of rubredoxins and cupredoxins. Drummond & Wootton (1987) also found a similarity between the amino acid sequences flanking the Cys-containing site and regions of cytochrome c in which the two cysteines are involved in covalent binding of haem; the amino acids responsible for the 5th and 6th ligands to the iron of the haem in cytochrome c (histidine and methionine) are, however, missing. Drummond & Wootton (1987) suggested that although the nifL product is not a cytochrome *sensu stricto*, a bound haem might be the basis of the redox sensitivity. This seems to us unlikely and also fails to explain how the nifL product might be sensitive to O₂. A more likely alternative involvement of haem protein in O₂ regulation is by way of a specific, O₂-binding haem protein which, by analogy with other such proteins, would require a histidine (or cysteine) 5th ligand to the iron in the haem. A second alternative involvement of a haem protein would be as an intermediate component of an electron transport chain which might interact with the nifL product. Such an interaction is consistent with the observation that expression from the nifH

Abbreviations: 5-ALA, 5-aminolaevulinic acid.
promoter (the nitrogenase structural operon) is inhibited by 50% at a dissolved O₂ concentration near the apparent \( K_m \) (100 nM) of the principal terminal oxidase in \( K. pneumoniae \) (Bergersen et al., 1982).

Clearly, one approach to limit the number of possible mechanisms to consider in explaining the role of O₂ and the \textit{nifL} product in regulation of nitrogenase synthesis would be to use an organism unable to synthesize haem proteins and to test whether or not its oxygen regulation is impaired. This paper reports the results of such experiments.

**METHODS**

**Bacterial strains and growth.** The bacterial strains used in this work are listed in Table 1; they were always grown and incubated at 30 °C.

Nutrient broth (NB) and nutrient agar (NA) were from Oxoid. Minimal medium (MM) and nitrogen-free medium (NFDM) containing glucose (1%, w/v) were described by Cannon (1980). For growth of A1002 and A1004a, MM and NFDM were supplemented with isoleucine, valine, methionine and cysteine (all at 40 \( \mu \)g ml\(^{-1} \)) and vitamin-free Casamino acids (200 \( \mu \)g ml\(^{-1} \)) (Difco). 5-Aminolaevulinic acid (5-ALA) (30 \( \mu \)M) (from Aldrich) was added as required. Strain JC5466 (pRD1) was grown in MM supplemented with tryptophan (20 \( \mu \)g ml\(^{-1} \)), carbenicillin (200 \( \mu \)g ml\(^{-1} \)) and kanamycin (30 \( \mu \)g ml\(^{-1} \)).

**Bacterial matings.** The donor strain JC5466 (pRD1) and the recipient strains A1002 and A1004a were grown anaerobically for 18 h in MM supplemented as described, harvested and washed once in saline phosphate (containing 100 mM-NaCl and 50 mM-potassium phosphate buffer, pH 7.4). Matings were done by spotting 50 \( \mu \)l of the donor mixed with recipient on to the surface of a selective agar plate (MM supplemented with isoleucine, valine, methionine, carbenicillin, kanamycin and 5-ALA; concentrations as above). After 3 d, two transconjugants were purified on selective media and checked for maintenance of the Hem phenotype and for the presence of the \textit{Nif}\(^+\) phenotype by \( C_2H_2 \)-reduction assays.

**Inoculum cultures of strains A1004a(pRD1) and A1002(pRD1).** These were grown anaerobically in NFDM supplemented as described except that the Casamino acid concentration was increased to 500 \( \mu \)g ml\(^{-1} \) and 5-ALA was omitted; carbenicillin and kanamycin were included to maintain plasmid selection. Flasks (50 ml) containing 20 ml medium were inoculated with a single colony from a selective plate, sparged for 20 min with sterile N₂ and capped with a Suba-seal. Growth was for 18–40 h with shaking (50 oscillations min\(^{-1} \)). For A1004a(pRD1) this procedure gave acceptably low numbers of revertants and, because of the absence of 5-ALA, provided cultures lacking haem (see Scott & Poole, 1987).

**\( C_2H_2 \)-reduction assays.** A \textit{Nif}\(^+\) phenotype was confirmed for all transconjugants by growth and subsequent assay for \( C_2H_2 \) reduction after N-limited growth in supplemented-NFDM containing carbenicillin, kanamycin and 5-ALA (50 \( \mu \)M as required), as described by Cannon (1980). Quantitative measurements of \( C_2H_2 \) reduction were made under anaerobic conditions as described by Hill (1976).

**Growth curves.** Strain A1004a(pRD1) was grown in 20 ml of supplemented-NFDM in 100 ml Klett flasks anaerobically or aerobically in the presence or absence of 5-ALA (60 \( \mu \)M). Aerobic cultures were shaken (50 oscillations min\(^{-1} \)) under air; anaerobic cultures were sparged for 20 min with sterile N₂ and capped with a Suba-seal. Growth was measured either as OD\(_{420}\) or as optical density determined by using a Klett–Summerson photoelectric colorimeter. At the end of the experiment \( C_2H_2 \) (10%, v/v) was introduced into the gas phase and the specific \( C_2H_2 \)-reducing activity was determined.

**Detection of revertants.** Two methods of detecting Hem\(^*\) revertants were used. (1) Ability to grow aerobically on NA or MM in which succinate (50 mM) replaced glucose as sole carbon source. (2) Hem\(^*\) colonies grown anaerobically on a glucose MM plate in a GasPak anaerobic jar for 4 d were stained green within 30 s after flooding plates with the haem stain of Thomas et al. (1976); Hem\(^-\) colonies remained white.

**Haem content of bacteria.** Bacteria were grown in 1.5 l of supplemented-NFDM containing glucose (2%, w/v),

<table>
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<th>Table 1. Bacterial strains and plasmids</th>
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<td>All \textit{E. coli} strains were derived from the K12 strain.</td>
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<td>Strain or plasmid</td>
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tryptone (Difco) (4 g l\(^{-1}\)) instead of Casamino acids and the trace element solution of Poole et al. (1979); 5-ALA (60 \(\mu\)M) was added as required. Cultures were grown anaerobically for 24 h under a slow stream of \(N_2\). Glucose was still detectable at the time of harvesting. Cells were harvested by centrifugation, washed once in 20 mM-Tris/HCl, pH 8.0, containing EDTA (1 mM) and dithiothreitol (0.1 g l\(^{-1}\)) and resuspended in the same buffer to 20-50 mg protein ml\(^{-1}\). The cell suspension (0.75 ml) was treated with pyridine (175 \(\mu\)l) and 1 M-NaOH (75 \(\mu\)l). The dithionite-reduced minus persulphate-oxidized difference spectrum of the pyridine haemochrome was recorded using a Shimadzu UV-3000 spectrophotometer and haem determined with the extinction coefficient of Fuhrhop & Smith (1975).

**Preparation of membrane particles and assay of oxidase activities.** Washed bacteria, grown as for measurement of haem content and stored at \(-20^\circ\text{C}\), were thawed, a few grains of DNAase were added and samples (20 ml) were disrupted by an ultrasonic disintegrator (MSE Soniprep 150) at full power with 10 cycles of 30 s separated by 30 s periods of cooling at \(0^\circ\text{C}\). Whole bacteria and debris were removed by centrifugation for 20 min at 10,000 g and the membrane particle fraction was obtained by subsequent centrifugation for 2 h at 140,000 g. The membranes were washed by resuspension and centrifugation, suspended in buffer (see above) (10-20 mg protein ml\(^{-1}\)) and stored in liquid nitrogen. \(O_2\) uptake by membrane particles was measured in a Rank oxygen electrode at 30 \(^\circ\text{C}\) in a reaction mixture (2 ml) containing sodium phosphate buffer (50 mM, pH 7.4), NADH (0.5 mM) and \(D(-)\)-lactate (20 mM). These substrates were chosen to give the greatest rate of \(O_2\) consumption by the membranes.

**Immunoblot analysis.** SDS-PAGE was done as described by Laemmli (1970) in 10% (w/v) polyacrylamide gels. Protein was transferred to nitrocellulose membrane (Schleicher and Schüll BA83, 0.2 \(\mu\)m) overnight at 5 V cm\(^{-1}\) (0.32 A) using the bicarbonate blotting system of Dunn (1986). Blots were developed with rabbit antiserum raised to *K. pneumoniae* nitrogenase (Rennie et al., 1978), and sheep anti-rabbit conjugated peroxidase (from Serotec). Immunologically reactive bands were stained as described by Towbin et al. (1979) except that diaminobenzidine dihydrochloride (Sigma) instead of \(\sigma\)-dianisidine was used.

**Pulse-labelling of derepressed cultures following exposure to \(O_2\).** Strain A1004a(pRD1) (10 ml) was inoculated into 100 ml of supplemented-NFDM containing carbenicillin and kanamycin. Cultures were sparged with a slow stream of 1% (v/v) \(CO_2\) in nitrogen for 18 h until the \(C_2H_4\)-reducing activity was more than 10 nmol \(C_2H_4\) produced min\(^{-1}\) (mg bacterial protein)\(^{-1}\) (early exponential phase). The number of Hem\(^+\) revertants was always less than 2 \(\times 10^{-6}\).

Samples (40 ml) were transferred in \(N_2\)-flushed syringes to two anaerobic reaction vessels supplied with \(N_2\) and mounted on magnetic stirrers. One vessel was equipped with a lead/silver oxygen electrode connected to an oxystat as described by Hill et al. (1981). Both vessels were isolated from the gas supply and \(C_2H_2\) (10%, v/v) was introduced into the gas phase. A small amount of air was introduced into the vessel with the oxygen electrode (oxystat culture) and the stirring speed automatically adjusted to maintain a low \(O_2\) concentration (1.4-2.6 \(\mu\)M) for the duration of the experiment. The control culture was maintained under anaerobic conditions. Samples (3-5 ml) of culture were removed from the reaction vessels as required and 2 ml of the sample was injected into an Ar-flushed conical polycarbonate tube containing 10 \(\mu\)l (370 kBq) \(^{14}\)C-labelled amino acids (Amersham) and capped with a Suba-seal. The tube was then incubated for 5 min with rapid shaking. Incorporation of labelled amino acids was stopped by addition of 100 \(\mu\)l of unlabelled Casamino acids (10 mg ml\(^{-1}\)). Subsequent estimation of total polypeptide synthesis, measured as incorporation of radioactivity into trichloroacetic-acid-precipitable material, and of nif polypeptide synthesis by autoradiography of SDS-PAGE gels were done as described by Eady et al. (1978) and Cannon (1980). Autoradiograms were scanned on a Chromoscan gel scanner. The nif gene products were identified by comparison with other pulse-labelling experiments in which the nif products were expressed from a multicopy plasmid during derepression (Cannon et al., 1985) and therefore accounted for 90-100% of total protein synthesis. In experiments where derepression had already occurred, the nif products accounted for only 20-30% of total protein synthesis.

**Total bacterial protein and membrane protein.** This was measured by the bicinchoninic acid assay (Smith et al., 1985; Redinbaugh & Turley, 1986). Culture samples (0.5-5.0 ml) were harvested by filtration or centrifugation, washed once in saline phosphate and suspended in 200 \(\mu\)l 50 mM-Tris/HCl, pH 7.4. Various volumes of bacterial suspension (or membrane particles) were then diluted with an equal volume of 1% (w/v) SDS in 1.8% (w/v) EDTA, and boiled for 5 min. The boiled suspensions (10 \(\mu\)l) were placed in wells of a 96-well microtitre plate (Dynatech M129A) which was washed with Decon followed by acid between uses. BCA working reagent (200 \(\mu\)l) (Pierce) was added to each well and the plates were incubated for 1 h at 50 \(^\circ\text{C}\). Absorbance measurements were made on a MR700 microplate reader (Dynatech) with a 570 nm test filter and 450 nm reference. A standard curve was prepared with bovine serum albumin (0-2 mg ml\(^{-1}\)).

**RESULTS AND DISCUSSION**

**Anaerobic expression of nitrogenase in a HemA\(^+\) Escherichia coli(pRD1) transconjugate**

The Nif\(^+\) plasmid pRD1 was used to construct transconjugates of the HemA\(^-\) mutant *E. coli* A1004a and of the wild-type Hem\(^+\) *E. coli* A1002. The recipients and transconjugants were
Table 2. Effect of the presence of 5-ALA on the haem content and nitrogenase activity of wild-type E. coli and of a mutant lacking 5-ALA synthetase

Growth of bacteria and measurements of haem, oxidase activity and nitrogenase activity were as described in Methods. Cultures of strains without the Nif+ plasmid pRD1 were analysed for haem content (>10 pmol haem b (mg protein)⁻¹) and oxidase activity. C₂H₂ reduction was measured in strain A1004a(pRD1) after 18 h growth from a 4-8% inoculum, and in strain A1002(pRD1) after 36 h growth from an 8-16% inoculum. The optical density of the cultures of the latter strain were about half those of the cultures of strain A1004a(pRD1). The reversion frequency to Hem⁺ was less than 2 × 10⁻⁷. ND, Not determined. −, <10 pmol haem b (mg protein)⁻¹.

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<th>Growth with 5-ALA</th>
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<td>A1002 (HemA⁺)</td>
<td>A1004a (HemA⁻)</td>
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<tr>
<td>Haem b [pmol haem b (mg protein)⁻¹]</td>
<td>300</td>
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<td>NADH plus D(-)-lactate oxidase activity [nmol oxygen min⁻¹ (mg membrane protein)⁻¹]</td>
<td>93</td>
<td>1</td>
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<td>Acetylene reduction [nmol C₂H₂ min⁻¹ (mg protein)⁻¹]</td>
<td>3.2-3.7</td>
<td>16-21</td>
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tested for C2H2-reducing activity in a standard assay procedure (Cannon, 1980), which has been tested extensively to test expression of plasmid-borne nif in both K. pneumoniae and E. coli strains. The recipients did not reduce C2H2 (data not shown). Transconjugates of both the HemA− and wild-type strains reduced C2H2, but the activity in the mutant transconjugant was much greater than in that of the wild-type (Table 2).

Anaerobic growth, as well as C2H2 reduction, of the wild-type transconjugate A1002(pRD1) was poor compared with that of the HemA− mutant A1004a(pRD1). This was attributed to an apparent greater sensitivity to the added kanamycin which was needed to retain pRD1. This unexpected difference in phenotype of the wild-type and HemA− mutant suggests that these strains are not isogenic. Because the addition of 5-ALA restored haem biosynthesis and membrane oxidase activities (Haddock, 1973; Haddock & Schairer, 1973) to the HemA− mutant (Table 2), the behaviour of cultures of this strain with 5-ALA was considered to be equivalent to that of a wild-type (Hem+) phenotype.

Supplementing cultures of the HemA− transconjugate with 5-ALA resulted in a 2–3-fold increase of C2H2-reducing activity (Table 2). However, the rate of anaerobic growth was also improved when haem biosynthesis was permitted (Fig. 1). When C2H2 reduction was measured at the end of this growth the difference in the specific activities was less marked [35 compared with 25 nmol C2H4 produced min−1 (mg protein)−1]. The presence of a significant amount of nitrogenase was confirmed by an immunoblotting procedure (Fig. 2). Since growth was less affected by the addition of 5-ALA if a richer medium such as nutrient broth was used (data not shown), haem may play a role during anaerobic growth under N-limitation, but this possibility was not pursued further. Nevertheless, these results show that under anaerobic conditions haem is required neither for nitrogenase synthesis nor for electron transport to the enzyme in E. coli.

Oxygen inhibition of nitrogenase synthesis in the wild-type K. pneumoniae

As expected, aerobic growth of the HemA− transconjugate was extremely poor unless 5-ALA was added (Fig. 1). The slow growth was probably due to the lack of hydroperoxidases and of respiratory haemoproteins. C2H2 was not reduced by these cultures. In neither the presence nor absence of added 5-ALA were the polypeptides of nitrogenase detected by the immunoblotting procedure at the end of aerobic growth (Fig. 2). The concentration of fixed N in the medium was that allowing anaerobic derepression of nitrogenase (as for Fig. 1).

Oxygen at 2 μM inhibits nitrogenase synthesis in wild-type K. pneumoniae (Hill et al., 1981; Bergersen et al., 1982), and was the lowest concentration of O2 we could reliably monitor using a conventional O2 electrode. When this O2 concentration was introduced into an anaerobic culture of A1004a(pRD1), growing without 5-ALA (Fig. 3), the levels of nitrogenase polypeptides rapidly decreased (Fig. 2) and the C2H2-reducing activity [14 nmol C2H4 produced min−1 (mg protein)−1 in sample g, Fig. 3] dropped to zero (in sample h, Fig. 3). Since the nitrogenase polypeptides were unstable under this aerobic condition, the effect of O2 could not
Fig. 2. Immunoblot analysis of nitrogenase levels in samples of A1004a(pRD1). Samples from growth depicted in Fig. 1 (samples a, b, c and d) and in Fig. 3 (samples g, h, i, j and k) were treated and analysed as described in Methods. Tracks contained 10 μg protein from anaerobic growth (a), anaerobic growth with 5-ALA (b), aerobic growth (c), aerobic growth with 5-ALA (d), anaerobic growth (g) and anaerobic growth followed by 2.5 h (h), 22.5 h (i), 27.5 h (j) and 30 h (k) treatment with air. Track e contained purified K. pneumoniae nitrogenase (0.4 μg). Bands labelled K, D and H indicate the polypeptides of nitrogenase and CA indicates the Gram-negative common antigenic polypeptide (Dr D. Nunn, personal communication).

Fig. 3. Effect of O₂ on growth without 5-ALA of A1004a(pRD1). Two cultures were grown anaerobically in 200 ml of supplemented-NFDM sparged with 1% (v/v) CO₂ in N₂ (100 ml min⁻¹). During mid-exponential growth, indicated by the vertical dotted line, air was introduced into the gas supply of one culture (O) to maintain 2-6 μM dissolved O₂ for the subsequent 30 h of incubation. There was negligible O₂ uptake. Samples (10 ml) taken before (g) and after (h, i, j and k) the introduction of air were assayed for nitrogenase content by immunoblot analysis (Fig. 2).
Fig. 4. Effect of O₂ on nif polypeptide synthesis in A1004a(pRD1). Cultures were supplemented (Hem⁺) (a) or unsupplemented (Hem⁻) (b) with 5-ALA, and exposed to either anaerobiosis (---) or 2 ± 0.6 (SEM, n = 16) μM-O₂ (—) as described in Methods. Samples were removed at 10, 40, 70 and 100 min and pulse-labelled with 14C-labelled amino acids for the measurement of the rate of total polypeptide synthesis and of nif polypeptide synthesis (see Methods). The microdensitometer traces shown are of autoradiographs of SDS-PAGE of extract prepared from the samples removed after an exposure of 40 min. J, D, K and H show the position of the nif polypeptides and 8 and 11 show two unidentified polypeptides.

be assessed reliably by using the accumulation of antigenic material as a measure of synthesis.

In K. pneumoniae the rate of nitrogenase polypeptides synthesis, measured by a pulse-labelling technique, in cultures exposed to a constant concentration (6 μM) of dissolved O₂ has been used to differentiate mutants (NiFL⁻) that are defective in O₂ regulation of nitrogenase, from the wild-type (Hill et al., 1981; Cannon et al., 1985). Therefore rates of nitrogenase polypeptide synthesis were measured in A1004a(pRD1), by a pulse-labelling technique, in cultures exposed to anaerobiosis and to a dissolved O₂ concentration of 2 μM in an oxystat. When the culture was grown with added 5-ALA (to restore haem biosynthesis), this concentration of O₂ inhibited synthesis of the nifH and nifK polypeptides of nitrogenase within 10 min (nifD polypeptide was not sufficiently resolved to draw any conclusions) (Fig. 4a). On the other hand, it did not significantly affect total protein synthesis (data not shown). When 5-ALA was omitted (to prevent haem biosynthesis), similar results were obtained (Fig. 4b). The synthesis of nifJ polypeptide (pyruvate:flavodoxin oxidoreductase), which is inhibited by O₂ (6 μM) in K. pneumoniae wild-type but not in O₂-constitutive NiFL⁻ mutants (Hill et al., 1981; Cannon et al., 1985) was inhibited by 2 μM-O₂ in A1004a(pRD1) both in the presence or absence of added 5-ALA (Fig. 4a, b). Thus the inability of strain A1004a(pRD1) to make haem does not alter the sensitivity to O₂ of the processes regulating the synthesis of nitrogenase and nifJ polypeptide.
During exposure to 2 μM-O₂, the synthesis of no other polypeptides besides nifH, K and J was completely inhibited, although the synthesis of two polypeptides was markedly enhanced (Fig. 4a, b). As expected, this level of O₂ completely inhibited C₂H₂-reducing activity (data not shown).

Our results demonstrate that O₂ regulation of nif expression mediated by the nifL product is not lost when cells are deprived of haem. It seems unlikely that traces of haem, undetected by us, satisfied the presumptive requirement of the nifL product, but not of that for the respiratory components, particularly since the nifL product is made in relatively high amounts (Cannon et al., 1985). Hence the suggestion of Drummond & Wootton (1987) that the nifL polypeptide might contain a bound haem as the basis of its ‘redox sensitivity’ is not supported. The lack of haem proteins renders the constitutively expressed respiratory chain unable to reduce O₂ to H₂O; the redox status of the components of the chain is therefore ‘locked’ in the reduced state regardless of the presence or absence of O₂. Hence the direct involvement of a functional respiratory chain in the mechanism of O₂ regulation of nif expression is also not supported. Other possibilities for a mechanism of O₂ (redox) sensing could reside in alternative modes of reduction of O₂ or its dismutation. Whether there is a role for nitrogenase itself, or for a component of the electron transport chain to nitrogenase, as a signal for the prevention of nif expression mediated by the nifA product remains to be seen, but possible candidates for the transducing protein (the nifL product or a nifAL protein complex) have not yet been excluded.

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