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

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Strains of *Escherichia coli* containing the Nif$^+$ plasmid pRD1 were used to investigate the possibility that haem proteins are involved in the regulation by $O_2$ of *nif* expression. Strains lacking 5-aminolaevulinate synthase (HemA$^-$), and hence normally unable to synthesize haem proteins, showed an identical response to $O_2$ in the presence or absence of added aminolaevulinate (and hence of haem proteins). It was concluded that the regulatory protein NifL is not a haem protein.

## INTRODUCTION

Nitrogen fixation in the facultative anaerobe *Klebsiella pneumoniae* occurs only in anaerobic or microaerobic conditions (at less than 30 nM-$O_2$) (Hill *et al.*, 1984). At about 6 μM-$O_2$ 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 $O_2$ above 6 μM, expression from the *nifLA* promoter is also inhibited. The mechanism whereby $O_2$ regulates the expression of the *nif* genes is not known although the evidence suggests that in response to low concentrations of $O_2$, 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 $O_2$ 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 $O_2$. A more likely alternative involvement of haem protein in $O_2$ regulation is by way of a specific, $O_2$-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 $O_2$ 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 $O_2$ and the *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 μg $ml^{-1}$) and vitamin-free Casamino acids (200 μg $ml^{-1}$) (Difco). 5-Aminolaevulinic acid (5-ALA) (30 μM) (from Aldrich) was added as required. Strain JC5466 (pRD1) was grown in MM supplemented with tryptophan (20 μg $ml^{-1}$), carbenicillin (200 μg $ml^{-1}$) and kanamycin (30 μg $ml^{-1}$).

*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 μl 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 $Nif^+$ 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 μg $ml^{-1}$ 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 $N_2$ 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 $Nif^+$ 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 μM 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 μM). Aerobic cultures were shaken (50 oscillations $min^{-1}$) under air; anaerobic cultures were sparged for 20 min with sterile $N_2$ before capping with a Suba-seal. Growth was measured either as $OD_{540}$, 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 1. *Bacterial strains and plasmids*

All *E. coli* strains were derived from the K12 strain.

| Strain or plasmid | Genotype | Source/reference |
|---|---|---|
| A1002 | *mel ilv lacI metE* | NCIB 11825; Poole *et al.* (1979) |
| A1004a | *mel ilv lacI metE hemA* | Haddock (1973)<br>Haddock & Schairer (1973) |
| JC5466 | *trp his recA56 rpsE* | Dixon *et al.* (1977) |
| | Phenotype | |
| pRD1 | $Km^R Cb^R Tc^R His^+ Nif^+$ | Dixon *et al.* (1976) |

tryptone (Difco) (4 g $l^{-1}$) instead of Casamino acids and the trace element solution of Poole *et al.* (1979); 5-ALA (60 μM) 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 μl) and 1 M-NaOH (75 μl). 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 °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 °C. Whole bacteria and debris were removed by centrifugation for 20 min at 10000 *g* and the membrane particle fraction was obtained by subsequent centrifugation for 2 h at 140000 *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 °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 μm) 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 *o*-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 $C_2H_2$-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 μM) for the duration of the experiment. The control culture was maintained under anaerobic conditions. Samples (3–3·5 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 μCi (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 μl 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 μl 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 μl) 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 μl) (Pierce) was added to each well and the plates were incubated for 1 h at 50 °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. $C_2H_2$ 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 $2 \times 10^{-7}$. ND, Not determined. −, $<10$ pmol haem *b* (mg protein)$^{-1}$.

| | Growth without 5-ALA | | Growth with 5-ALA | |
|---|---|---|---|---|
| | A1002 (HemA$^+$) | A1004a (HemA$^-$) | A1002 (HemA$^+$) | A1004a (HemA$^-$) |
| Haem *b* [pmol haem *b* (mg protein)$^{-1}$] | 300 | − | ND | 1280 |
| NADH plus D(−)-lactate oxidase activity [nmol oxygen min$^{-1}$ (mg membrane protein)$^{-1}$] | 93 | 1 | ND | 35 |
| Acetylene reduction [nmol $C_2H_4$ min$^{-1}$ (mg protein)$^{-1}$] | 3·2–3·7 | 16–21 | 2·8–3·6 | 48–50 |

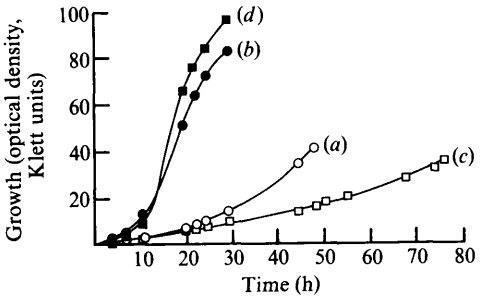


Fig. 1. Effect of 5-ALA (60 μM) on anaerobic (●, ○) and aerobic (■, □) growth in strain A1004a(pRD1). Cultures were grown in supplemented-NFDM as described in Methods either without (○, □) or with (●, ■) 5-ALA. The nitrogenase content, assessed by immunoblot analysis, in samples labelled (*a*), (*b*), (*c*) and (*d*) is shown in Fig. 2.

tested for $C_2H_2$-reducing activity in a standard assay procedure (Cannon, 1980), which has been used extensively to test expression of plasmid-borne *nif* in both *K. pneumoniae* and *E. coli* strains. The recipients did not reduce $C_2H_2$ (data not shown). Transconjugates of both the HemA$^-$ and wild-type strains reduced $C_2H_2$, but the activity in the mutant transconjugant was much greater than in that of the wild-type (Table 2).

Anaerobic growth, as well as $C_2H_2$ 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 $C_2H_2$-reducing activity (Table 2). However, the rate of anaerobic growth was also improved when haem biosynthesis was permitted (Fig. 1). When $C_2H_2$ reduction was measured at the end of this growth the difference in the specific activities was less marked [35 compared with 25 nmol $C_2H_4$ 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 HemA$^-$ E. coli(pRD1) transconjugate*

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. $C_2H_2$ 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 $O_2$ we could reliably monitor using a conventional $O_2$ electrode. When this $O_2$ 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 $C_2H_2$-reducing activity [14 nmol $C_2H_4$ 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 $O_2$ could not

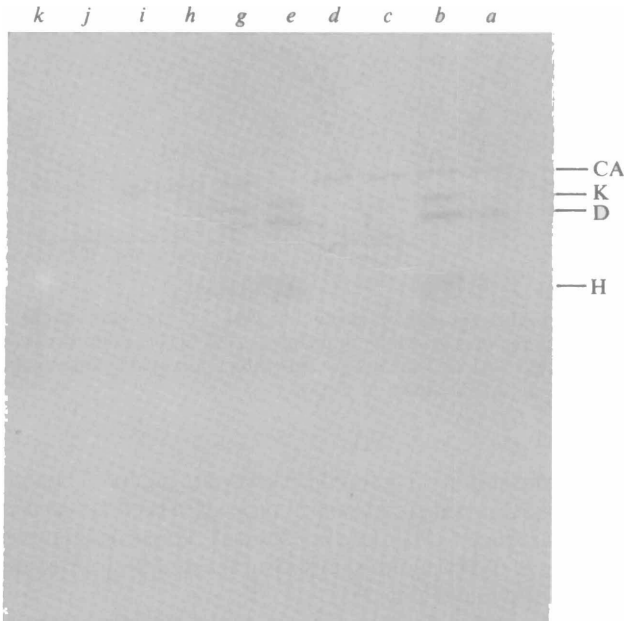


Fig. 2. Immunoblot analysis of nitrogenase levels in samples of A1004a(pRD1). Samples from growth depicted in Fig. 1 (samples *a*, *b*, *c* and *d*) and in Fig. 3 (samples *g*, *h*, *i*, *j* and *k*) were treated and analysed as described in Methods. Tracks contained 10 μg protein from anaerobic growth (*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).

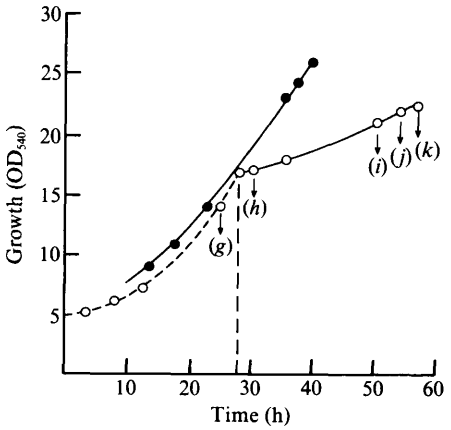


Fig. 3. Effect of $O_2$ on growth without 5-ALA of A1004a(pRD1). Two cultures were grown anaerobically in 200 ml of supplemented-NFDM sparged with 1% (v/v) $CO_2$ in $N_2$ (100 ml $min^{-1}$). During mid-exponential growth, indicated by the vertical dotted line, air was introduced into the gas supply of one culture (○) to maintain 2–6 μM dissolved $O_2$ for the subsequent 30 h of incubation. There was negligible $O_2$ 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).

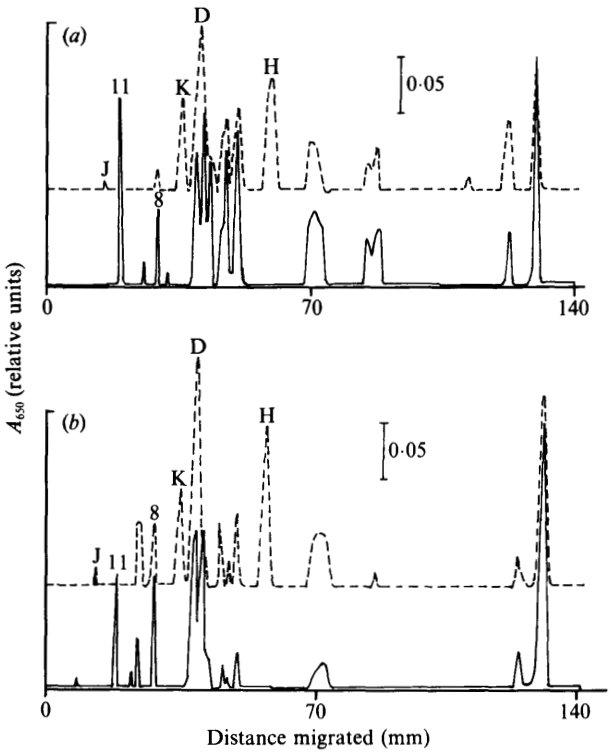


Fig. 4. Effect of $O_2$ on *nif* polypeptide synthesis in A1004a(pRD1). Cultures were supplemented ($Hem^+$) (*a*) or unsupplemented ($Hem^-$) (*b*) with 5-ALA, and exposed to either anaerobiosis (---) or $2 \pm 0{\cdot}6$ (SEM, $n = 16$) μM-$O_2$ (—) as described in Methods. Samples were removed at 10, 40, 70 and 100 min and pulse-labelled with $^{14}C$-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 $O_2$ has been used to differentiate mutants ($NifL^-$) that are defective in $O_2$ 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 $O_2$ concentration of 2 μM in an oxystat. When the culture was grown with added 5-ALA (to restore haem biosynthesis), this concentration of $O_2$ inhibited synthesis of the *nifH* and *nifK* polypeptides of nitrogenase within 10 min (*nifD* polypeptide was not sufficiently resolved to draw any conclusions) (Fig. 4*a*). 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. 4*b*). The synthesis of *nifJ* polypeptide (pyruvate:flavodoxin oxidoreductase), which is inhibited by $O_2$ (6 μM) in *K. pneumoniae* wild-type but not in $O_2$-constitutive $NifL^-$ mutants (Hill *et al.*, 1981; Cannon *et al.*, 1985) was inhibited by 2 μM-$O_2$ in A1004a(pRD1) both in the presence or absence of added 5-ALA (Fig. 4*a*, *b*). Thus the inability of strain A1004a(pRD1) to make haem does not alter the sensitivity to $O_2$ of the processes regulating the synthesis of nitrogenase and *nifJ* polypeptide.

During exposure to 2 μM-$O_2$, the synthesis of no other polypeptides besides *nifH*, *K* and *J* was completely inhibited, although the synthesis of two polypeptides was markedly enhanced (Fig. 4*a*, *b*). As expected, this level of $O_2$ completely inhibited $C_2H_2$-reducing activity (data not shown).

Our results demonstrate that $O_2$ 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 $O_2$ to $H_2O$; the redox status of the components of the chain is therefore 'locked' in the reduced state regardless of the presence or absence of $O_2$. Hence the direct involvement of a functional respiratory chain in the mechanism of $O_2$ regulation of *nif* expression is also not supported. Other possibilities for a mechanism of $O_2$ (redox) sensing could reside in alternative modes of reduction of $O_2$ 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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