Synthesis and Activity of Nitrogenase in Klebsiella pneumoniae Exposed to Low Concentrations of Oxygen

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Effects of very low concentrations of dissolved O₂ on nitrogenase activity in Klebsiella pneumoniae were studied in a stirred chamber system which enabled simultaneous measurements of steady-state O₂ concentrations, O₂ consumption and C₂H₂ reduction. A strain carrying a chromosomal nifH::lac fusion as well as the Nif⁺ plasmid pRD1, expressed nitrogenase activity with 80 nM-O₂, a concentration known to inhibit nifH::lac expression by about 50%. Thus nitrogenase activity in vivo was no more sensitive to O₂ than expression of nifH::lac. When compared with anaerobic treatments, dissolved O₂ near 30 nM apparently stimulated nitrogenase derepression and enhanced the activity of nitrogenase synthesized anaerobically. Thus, in this organism, N₂ fixation occurs in microaerobic as well as anaerobic conditions.

INTRODUCTION

Fixation of N₂ in the facultative anaerobe Klebsiella pneumoniae is principally associated with anaerobic metabolic processes, although enhancement by limiting O₂ has been obtained in chemostats (Hill, 1976). Excess O₂ rapidly inhibits both the activity and synthesis of nitrogenase (St John et al., 1974; Eady et al., 1978). Regulation of synthesis occurs, at least in part, at the level of transcription of nif DNA (Kaluza & Hennecke, 1981; M. Cannon, S. Hill, E. Kavanagh and F. Cannon, unpublished) by a mechanism that probably involves the nifL gene product as a negative controlling element (Hill et al., 1981; Merrick et al., 1982; Buchanan-Wollaston et al., 1981; Filser et al., 1983). Such studies have been facilitated by the construction of nif::lac gene fusions (Dixon et al., 1980), in which the Escherichia coli lac operon is fused into the various nif gene transcriptional units. Expression from the various nif promoters results in the O₂-stable enzyme, β-galactosidase, whose assay can be used to monitor expression of the various nif genes.

Recently Bergersen et al. (1982) employed a technique which exploits leghaemoglobin (Lb) to measure very low O₂ concentrations (Bergersen & Turner, 1979) to determine the influence of O₂ on nifH::lac expression (nifHDKY is the operon encoding the nitrogenase complex). At 100 nM-O₂, a concentration near the apparent Kₘ of the principal terminal oxidase (80 nM-O₂), derepression of nifH::lac was inhibited by 50% when compared with that in anaerobic treatments. The strain also carried the Nif⁺ plasmid pRD1, but our earlier experiments did not establish the relative O₂ sensitivity of nitrogenase activity and nifH::lac expression, because C₂H₂-reducing activity was not measured in situ. The experimental design has now been modified to show that active nitrogenase is produced at low concentrations of dissolved O₂. We also present further evidence that O₂ can stimulate derepression of nitrogenase (Bergersen et al., 1982) as well as activity (Hill, 1976).

METHODS

Media. The media were as described previously (Bergersen et al., 1982) unless otherwise stated. NFDM contained glucose (111 mM) as carbon source. Sucrose D medium was NFDM but with sucrose (59 mM) as carbon

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source, and ferric citrate (0.1-1 mm) in place of FeSO₄. Resuspension medium was the derepression medium of Bergersen et al. (1982) but with the sucrose concentration raised from 29 to 164 mm. These media contained various concentrations of (NH₄)₂SO₄ where indicated. For aerobic derepression in the apparatus described below, derepression medium contained 80-100 μM oxyleghaemoglobin (Lb) prepared as described by Appleby & Bergersen (1980).

**Bacteria and plasmids.** UNF767(pRD1) was constructed and tested for plasmid retention as described by Bergersen et al. (1982). There was no indication of plasmid loss during derepression experiments, therefore such tests were not performed after experiments with derepressed populations.

During tests for plasmid retention we observed some bacterial contamination. This was traced to some batches of stock solutions of Lb which were subsequently sterilized by membrane filtration. We consider that the level of N₂-fixing contamination was insufficient to have influenced the results.

**Cultures.** For derepression experiments, batch cultures under N₂ were grown in either NFDM or sucrose D, both with (NH₄)₂SO₄ (10 mm), harvested by centrifugation under Ar, and resuspended in 2 ml ice-cooled derepression medium under Ar as described previously (Bergersen et al., 1982). At zero-time, portions (0.2-0.6 ml) were then transferred by syringe to the reaction chambers described below. For experiments with derepressed populations, batch cultures were grown in sucrose D and contained, for overnight anaerobic growth under N₂, a small initial amount (1.5 mm) of (NH₄)₂SO₄. Four 40 ml portions from two 100 ml cultures were harvested by centrifugation under N₂, and resuspended in 2 ml ice-cooled resuspension medium under N₂. Portions (0.1-0.5 ml) were then transferred by syringe to the reaction chambers described below.

**Derepression experiments.** Synthesis of nitrogenase was monitored by assay of β-galactosidase produced from the nifH::lacDKY operon and by assay of activity of nitrogenase produced from the nif of pRD1. Anaerobic and O₂ treatments were run in parallel at 30 °C, by procedures similar to those in Bergersen et al. (1982). The following modifications allowed nitrogenase (C₂H₂-reducing) activity to be measured in situ.

For anaerobic treatments the gas composition in the capped 100 ml flasks, containing 16 ml of derepression media at 30 °C, was 20 % (%/v) C₂H₂ in Ar. In addition to withdrawing culture samples (1-1 ml) for β-galactosidase assays, samples (0.2 or 0.5 ml) of gas were removed in Ar-flushed syringes for gas analysis.

For O₂ treatments in flasks, the procedure was as above but the gas composition was 20 % C₂H₂ and either 0.5 % or 1.0 % (%/v) O₂ in Ar. For O₂ treatments, with Lb, the stirred flow chamber apparatus of Bergersen & Turner (1979), described and illustrated previously (Bergersen et al., 1982), was modified as follows.

(1) A cylindrical gas reservoir (31) fitted with a threaded piston (Turner & Gibson, 1980) was evacuated and then filled with 20 % O₂, or 40 % O₂, and 20 % C₂H₂ in Ar. The greater O₂ level was used in one experiment where a high O₂ concentration was maintained. The gas reservoir was then connected to the evacuated head-space of the medium reservoir which contained the degassed derepression medium plus Lb. The head-space was then brought to, and maintained at, atmospheric pressure with the aid of a manometer, by adjusting the piston of the cylinder.

(2) In addition to withdrawing culture samples (1-1 ml) from the reaction chamber for β-galactosidase assays, samples (4 ml) of the effluent flowing from the spectrophotometer flow cell were collected in a syringe for subsequent analysis of dissolved gases (Bergersen & Turner, 1979; Turner & Gibson, 1980). The concentration of free O₂ and the rate of O₂ consumption in the reaction chamber were calculated as described by Bergersen & Turner (1979) and Bergersen et al. (1982).

Previously the aggregation of strain UNF767(pRD1) in the stirred reaction chamber of O₂-treatments had been prevented by using the derepression medium in place of NFDM. However, although the same derepression medium was used in the present work, some loss of bacteria caused by aggregation and occlusion in the filter membrane occurred during the later stages of derepression. It was most pronounced when high O₂ concentrations with high flow rates were maintained but was not due to the presence of leghaemoglobin. The reason for this aggregation is unknown.

**Experiments with derepressed populations.** Anaerobic and O₂ treatments were run in parallel at 30 °C. The procedures were similar to those for derepression experiments except that resuspension medium was used in place of derepression medium, N₂ was used in place of Ar as diluent gas, and each treatment lasted for only 2 h. Thus several different O₂ concentrations could be tested on a particular population. Between each O₂ treatment the reaction chamber was washed out, refilled with medium and reconnected to the medium reservoir. A sample (0.1-0.5 ml) of ice-cooled bacterial suspension was injected into the reaction chamber and, at the same time, into a flask for the anaerobic treatment. Samples of effluent and of gas, from both O₂ and anaerobic treatments, were removed as described for derepression experiments.

**Treatment of samples of assays.** Samples of organisms were collected during derepression experiments and assayed for the amount of β-galactosidase present after chloroform-treatment (Bergersen et al., 1982).

Nitrogenase activity was assayed by the production of C₂H₄ from C₂H₂ measured by gas chromatography (Turner & Gibson, 1980). For anaerobic treatments the activity was calculated from the rate of accumulation of C₂H₂ in the gas phase above the culture. For O₂ treatments in the stirred chamber, dissolved gases were recovered from samples of effluent by decompression (Turner & Gibson, 1980). The rate of C₂H₄ production, in derepression experiments, was calculated from the concentration of C₂H₂ in the sample and the flux of derepression medium...
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through the reaction chamber; for experiments with derepressed populations the steady-state level of C₂H₄ production was determined (Bergersen & Turner, 1979).

Hydrogen was measured by gas chromatography (Turner & Gibson, 1980). Dry weights were estimated as described by Bergersen et al. (1982), and nitrogenase activity, O₂ consumption and H₂ evolution were expressed in terms of dry weight.

RESULTS

Stimulation of derepression by O₂ in shaken flasks

During preliminary derepression experiments in flasks in the absence of Lb, levels of β-galactosidase were slightly higher under an initial pO₂ of either 0.5 or 1.0 kPa when compared with anaerobic treatments (Fig. 1a). Concomitant derepression of C₂H₂ reduction in flasks was not influenced by O₂ (Fig. 1b). For these and our earlier experiments (Bergersen et al., 1982) sucrose was used as the carbon source for anaerobic growth with NH₄⁺ and for derepression. Similar results were obtained in flasks when glucose replaced the sucrose (data not shown). In contrast, expression of nifH::lac and appearance of C₂H₂ reduction occurred significantly

Fig. 1. Influence of O₂ on sucrose-supported derepression of nifH::lac (a) and nitrogenase (b) with concomitant H₂ evolution (c). Derepression was in flasks under anaerobiosis (○, □) or with O₂ provided initially at either 0.5 kPa (●, ■) or 1.0 kPa (▲), and preceding anaerobic growth with NH₄⁺ was supported by either sucrose (circles and triangles) or glucose (squares).

Fig. 2. Derepression of nifH::lac (△, ▲) and nitrogenase (○, ●) with either (a) 17 nm-O₂, (b) 36 nm-O₂ or (c) 88 nm-O₂ (filled symbols) compared with anaerobic treatments (open symbols).
earlier with O$_2$ present when the removal of NH$_4^+$ was accompanied by a change in carbon source from glucose for growth to sucrose for derepression (Fig. 1a, b). Anaerobic expression of $nifH::lac$ and C$_2$H$_2$ reduction, during derepression supported by sucrose, was delayed when the carbon source for growth was glucose in place of sucrose (Fig. 1a, b).

*Klebsiella pneumoniae* possesses a hydrogenase, which dissipates reducing power generated during fermentation. Although it is inhibited by C$_2$H$_2$ (Smith *et al*., 1976), significant rates of H$_2$ evolution were detected under the derepressing conditions in flasks with 20% C$_2$H$_2$ (Fig. 1c) where H$_2$ evolution from nitrogenase would be negligible. The anaerobic rates of H$_2$ evolution were similar when growth and derepression were supported by either glucose (data not shown) or sucrose (Fig. 1c). However, when the shift to the derepressing condition was accompanied by the change in carbon source, the rate was significantly lower (Fig. 1c), suggesting that fermentation was restrained.

**Effects of controlled levels of O$_2$ on synthesis and activity of nitrogenase**

Synthesis and activity of nitrogenase and respiration were measured simultaneously during derepression in O$_2$ treatments with Lb in the stirred chamber system. Synthesis (units of β-galactosidase) and activity (C$_2$H$_2$-reduction) were detected earlier when O$_2$ was maintained at concentrations ranging from 8 to 36 nM than in anaerobic treatments (Fig. 2). The rate of derepression was also enhanced by these levels of O$_2$ and 2 h after the onset of expression levels of β-galactosidase with 30 nM O$_2$ were approximately four times greater than those in anaerobic treatments (Fig. 3). The rate of increase of nitrogenase activity was also stimulated by low concentrations of O$_2$ but not to the same extent (Fig. 3). At low O$_2$ concentrations, as expected, detection of β-galactosidase preceded detection of nitrogenase (Fig. 2a, b). At higher O$_2$ concentration (near 80 nM), as found by Bergersen *et al.* (1982), derepression was partially inhibited, but the time separating the appearance of the two activities was shorter (Fig. 2c). However with the latter O$_2$ concentrations, the bacteria tended to aggregate, so C$_2$H$_4$ production may represent nitrogenase activities under somewhat lower dissolved O$_2$ concentrations than that indicated by the spectrum of Lb.

The ability of cultures to reduce C$_2$H$_2$ during derepression with O$_2$ present may be a consequence of protective adaptation to the ambient aerobic conditions. Therefore populations derepressed under anaerobic conditions were tested for ability to reduce C$_2$H$_2$ at low O$_2$ concentrations with sucrose as carbon source. Three different derepressed populations of anaerobic activity 16 ± 9 nmol C$_2$H$_4$ min$^{-1}$ (mg dry wt)$^{-1}$ (which was unaffected by storage on ice) were used. For each steady-state O$_2$ concentration (maintained for 1–2 h), a fresh sample of

![Figure 3](image-url)
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Fig. 4. Influence of dissolved \(O_2\) concentration on nitrogenase activity in populations derepressed under anaerobic conditions. Nitrogenase activity (nmol \(C_2H_2\) mg\(^{-1}\) min\(^{-1}\)) is expressed as a percentage of that in anaerobic treatments. The different symbols represent three different experiments.

The ice-cooled suspension was used, and in parallel the activity was measured anaerobically. Nitrogenase activity was highest (twice the anaerobic rate), at about 30 nm\(O_2\) (Fig. 4). At concentrations above 80 nm\(O_2\), which is the apparent \(K_c\) of the principal terminal oxidase (Bergersen et al., 1982), activity was partially inhibited, but was still detected with 550 nm\(O_2\) (2.6 nmol \(C_2H_2\) min\(^{-1}\) (mg dry wt\(^{-1}\)). The reason for the apparent decrease in activity compared with the anaerobic level (Fig. 4), when the \(O_2\) concentration was maintained below 20 nm, is unclear. With anaerobically derepressed bacteria there was no aggregation in the stirred chamber. Over all experiments rates of \(C_2H_2\) reduction were proportional to \(O_2\) consumption up to 80 nmol \(O_2\) min\(^{-1}\) (mg dry wt\(^{-1}\)), 2.5 nmol \(O_2\) being consumed per mol \(C_2H_2\) reduced above the anaerobic rate (cf. Bergersen, 1978).

DISCUSSION

In our earlier work (Bergersen et al., 1982) samples were removed at timed intervals from the stirred chamber containing the derepressing culture of UNF767(pRD1), maintained at a low \(O_2\) concentration with Lb, and nitrogenase activity in the samples was measured anaerobically. This assay may have misrepresented the activity in the chamber because inhibition of nitrogenase by \(O_2\) is partially reversible (Hill, 1976) and relief from \(O_2\) repression of \(nifHDK\) mRNA and polypeptide synthesis is very rapid (M. Cannon, S. Hill, E. Kavanagh and F. Cannon, unpublished). The processes regulating \(nifH::lac\) expression may be more tolerant of \(O_2\) than one or more subsequent steps needed for nitrogenase activity. The present experiments were therefore designed to measure nitrogenase activity during derepression in the presence of \(O_2\) and to establish whether \(nifH::lac\) expression could be enhanced by low concentrations of \(O_2\). Nitrogenase activity was no more sensitive to \(O_2\) than expression of \(nifH::lac\). This also implies that none of the synthetic processes, following transcription from the \(nifH\) promoter (measured as expression of \(nifH::lac\)) and resulting in the appearance of \(C_2H_2\) reduction, are markedly inhibited by microaerobic conditions. Besides the translation of the \(nif\) mRNA, such processes involve the subsequent assembly and processing of the polypeptides to yield the two \(O_2\)-sensitive metal-containing nitrogenase components, and the provision of sources of reducing power and ATP for activity.

Jensen & Kennedy (1982) showed that anaerobic nitrogenase synthesis is selectively curtailed when the ATP level \textit{in vivo} is low. Thus the slower derepression after the change in carbon source may reflect the time taken for relief from catabolic repression for sucrose utilization. The provision of some \(O_2\) during derepression following the change in carbon source, may
temporarily upgrade the energy status and thereby allow earlier and more rapid derepression of nitrogenase. Such an upgrading might be brought about by a stimulation of invertase induction thus lifting the restraint on fermentation. Conditions of low O$_2$ supply giving rise to early nitrogenase derepression in flasks, rather than stimulating, partially inhibited H$_2$ evolution (Fig. 1c); however, the presence of O$_2$ may suppress fermentative processes and aerobic catabolic processes supported by endogenous substrates might be responsible for the upgrading. Steady-state rates of O$_2$ uptake [10–65 nmol min$^{-1}$ (mg dry wt)$^{-1}$] in the stirred chamber system maintained at O$_2$ concentrations ranging from 8 to 36 nM, were similar to those observed by Bergersen et al. (1982), suggesting that respiratory activity was not affected by the change in carbon source. An enhancement of catabolic activity by O$_2$ could lead to a general effect on macromolecular synthesis rather than to a more specific effect on energy status regulating carbon source. An enhancement of catabolic activity by O$_2$ was apparently excluded during the measurements, so her results do not preclude the possibility that, as a result of aerobic respiratory activity, a sufficient proton motive force is generated across the membrane to yield additional supplies of either reducing power or ATP for nitrogenase activity.

The activity of nitrogenase, fully derepressed during anaerobic growth, was also clearly enhanced with free, dissolved O$_2$ at 30 nM. This, together with the correlation between enhancement of nitrogenase activity and O$_2$ consumption, indicates involvement of increased energetic efficiency of aerobic versus anaerobic pathways of metabolism supporting nitrogenase. The observations are consistent with evidence that nitrogenase activity in $K$. pneumoniae can be supported, in part, by aerobic processes (Hill, 1976). This hypothesis has been challenged by Kashket (1981), who was unable to detect an electrical component ($\Delta \psi$) of the proton motive force in anaerobic N$_2$-fixing batch cultures of $K$. pneumoniae strain M5a1. However, O$_2$ was apparently excluded during the measurements, so her results do not preclude the possibility that, as a result of aerobic respiratory activity, a sufficient proton motive force is generated across the membrane to yield additional supplies of either reducing power or ATP for nitrogenase activity.

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**REFERENCES**


