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 O_2 on nitrogenase activity in *Klebsiella pneumoniae* were studied in a stirred chamber system which enabled simultaneous measurements of steady-state O_2 concentrations, O_2 consumption and C_2H_2 reduction. A strain carrying a chromosomal *nifH::lac* fusion as well as the Nif^+ plasmid pRD1, expressed nitrogenase activity with 80 nM- O_2 , a concentration known to inhibit *nifH::lac* expression by about 50%. Thus nitrogenase activity *in vivo* was no more sensitive to O_2 than expression of *nifH::lac*. When compared with anaerobic treatments, dissolved O_2 near 30 nM apparently stimulated nitrogenase derepression and enhanced the activity of nitrogenase synthesized anaerobically. Thus, in this organism, N_2 fixation occurs in microaerobic as well as anaerobic conditions.

INTRODUCTION

Fixation of N_2 in the facultative anaerobe *Klebsiella pneumoniae* is principally associated with anaerobic metabolic processes, although enhancement by limiting O_2 has been obtained in chemostats (Hill, 1976). Excess O_2 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at the level of transcription of *nif* DNA (Kaluza & Hennecke, 1981; M. Cannon, S. Hill, E. Kavanagh and F. Cannon, unpublished) by a mechanism that probably involves the *nifL* gene product as a negative controlling element (Hill *et al.*, 1981; Merrick *et al.*, 1982;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 O_2 -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 O_2 concentrations (Bergersen & Turner, 1979) to determine the influence of O_2 on *nifH::lac* expression (*nifHDKY* is the operon encoding the nitrogenase complex). At 100 nM- O_2 , a concentration near the apparent K_s of the principal terminal oxidase (80 nM- O_2), 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 O_2 sensitivity of nitrogenase activity and *nifH::lac* expression, because C_2H_2 -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 O_2 . We also present further evidence that O_2 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 (0.11 mM) in place of FeSO_4 . 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 $(\text{NH}_4)_2\text{SO}_4$ 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 N_2 -fixing contamination was insufficient to have influenced the results.

Cultures. For derepression experiments, batch cultures under N_2 were grown in either NFDM or sucrose D, both with $(\text{NH}_4)_2\text{SO}_4$ (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 N_2 , a small initial amount (1.5 mM) of $(\text{NH}_4)_2\text{SO}_4$. Four 40 ml portions from two 100 ml cultures were harvested by centrifugation under N_2 , and resuspended in 2 ml ice-cooled resuspension medium under N_2 . 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 O_2 treatments were run in parallel at 30 °C, by procedures similar to those in Bergersen *et al.* (1982). The following modifications allowed nitrogenase (C_2H_2 -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/v) C_2H_2 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 O_2 treatments in flasks, the procedure was as above but the gas composition was 20% C_2H_2 and either 0.5% or 1.0% (v/v) O_2 in Ar. For O_2 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 (3 l) fitted with a threaded piston (Turner & Gibson, 1980) was evacuated and then filled with 20% O_2 , or 40% O_2 , and 20% C_2H_2 in Ar. The greater O_2 level was used in one experiment where a high O_2 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 O_2 and the rate of O_2 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 O_2 -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 O_2 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 O_2 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, N_2 was used in place of Ar as diluent gas, and each treatment lasted for only 2 h. Thus several different O_2 concentrations could be tested on a particular population. Between each O_2 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 O_2 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 C_2H_4 from C_2H_2 measured by gas chromatography (Turner & Gibson, 1980). For anaerobic treatments the activity was calculated from the rate of accumulation of C_2H_4 in the gas phase above the culture. For O_2 treatments in the stirred chamber, dissolved gases were recovered from samples of effluent by decompression (Turner & Gibson, 1980). The rate of C_2H_4 production, in derepression experiments, was calculated from the concentration of C_2H_4 in the sample and the flux of derepression medium

through the reaction chamber; for experiments with derepressed populations the steady-state level of C_2H_4 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, O_2 consumption and H_2 evolution were expressed in terms of dry weight.

RESULTS

Stimulation of derepression by O_2 in shaken flasks

During preliminary derepression experiments in flasks in the absence of Lb, levels of β -galactosidase were slightly higher under an initial pO_2 of either 0.5 or 1.0 kPa when compared with anaerobic treatments (Fig. 1*a*). Concomitant derepression of C_2H_2 reduction in flasks was not influenced by O_2 (Fig. 1*b*). For these and our earlier experiments (Bergersen *et al.*, 1982) sucrose was used as the carbon source for anaerobic growth with NH_4^+ 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 C_2H_2 reduction occurred significantly

Fig. 1

Fig. 2

Fig. 1. Influence of O_2 on sucrose-supported derepression of *nifH::lac* (a) and nitrogenase (b) with concomitant H_2 evolution (c). Derepression was in flasks under anaerobiosis ($\circ$, $\square$) or with O_2 provided initially at either 0.5 kPa ($\bullet$, $\blacksquare$) or 1.0 kPa ($\blacktriangle$), and preceding anaerobic growth with NH_4^+ was supported by either sucrose (circles and triangles) or glucose (squares).

Fig. 2. Derepression of *nifH::lac* (Δ , $\blacktriangle$) and nitrogenase ($\circ$, $\bullet$) with either (a) 17 nM- O_2 , (b) 36 nM- O_2 or (c) 88 nM- O_2 (filled symbols) compared with anaerobic treatments (open symbols).

Fig. 3. Influence of O₂ concentration on the rate of increase of β -galactosidase (○) and of nitrogenase (●) activities during derepression. The activities measured shortly after their appearance and then after a further 2 h were used to estimate the rate of increase. The vertical bars indicate the standard deviations for anaerobic values.

earlier with O₂ present when the removal of NH₄⁺ was accompanied by a change in carbon source from glucose for growth to sucrose for derepression (Fig. 1*a, b*). Anaerobic expression of *nifH*:*lac* and C₂H₂ reduction, during derepression supported by sucrose, was delayed when the carbon source for growth was glucose in place of sucrose (Fig. 1*a, b*).

Klebsiella pneumoniae possesses a hydrogenase, which dissipates reducing power generated during fermentation. Although it is inhibited by C₂H₂ (Smith *et al.*, 1976), significant rates of H₂ evolution were detected under the derepressing conditions in flasks with 20% C₂H₂ (Fig. 1*c*) where H₂ evolution from nitrogenase would be negligible. The anaerobic rates of H₂ evolution were similar when growth and derepression were supported by either glucose (data not shown) or sucrose (Fig. 1*c*). However, when the shift to the derepressing condition was accompanied by the change in carbon source, the rate was significantly lower (Fig. 1*c*), suggesting that fermentation was restrained.

Effects of controlled levels of O₂ on synthesis and activity of nitrogenase

Synthesis and activity of nitrogenase and respiration were measured simultaneously during derepression in O₂ treatments with Lb in the stirred chamber system. Synthesis (units of β -galactosidase) and activity (C₂H₂-reduction) were detected earlier when O₂ 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 O₂ and 2 h after the onset of expression levels of β -galactosidase with 30 nM-O₂ 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 O₂ but not to the same extent (Fig. 3). At low O₂ concentrations, as expected, detection of β -galactosidase preceded detection of nitrogenase (Fig. 2*a, b*). At higher O₂ 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. 2*c*). However with the latter O₂ concentrations, the bacteria tended to aggregate, so C₂H₄ production may represent nitrogenase activities under somewhat lower dissolved O₂ concentrations than that indicated by the spectrum of Lb.

The ability of cultures to reduce C₂H₂ during derepression with O₂ 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 C₂H₂ at low O₂ concentrations with sucrose as carbon source. Three different derepressed populations of anaerobic activity 16 ± 9 nmol C₂H₄ min⁻¹ (mg dry wt)⁻¹ (which was unaffected by storage on ice) were used. For each steady-state O₂ concentration (maintained for 1–2 h), a fresh sample of

Fig. 4. Influence of dissolved O_2 concentration on nitrogenase activity in populations derepressed under anaerobic conditions. Nitrogenase activity ($\text{nmol C}_2\text{H}_4 \text{ mg}^{-1} \text{ min}^{-1}$) 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_s of the principal terminal oxidase (Bergersen *et al.*, 1982), activity was partially inhibited, but was still detected with 550 nM- O_2 ($2.6 \text{ nmol C}_2\text{H}_4 \text{ min}^{-1} (\text{mg dry wt})^{-1}$). 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 $O_2 \text{ min}^{-1} (\text{mg dry wt})^{-1}$, 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 *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 O₂ supply giving rise to early nitrogenase derepression in flasks, rather than stimulating, partially inhibited H₂ evolution (Fig. 1c); however, the presence of O₂ may suppress fermentative processes and aerobic catabolic processes supported by endogenous substrates might be responsible for the upgrading. Steady-state rates of O₂ uptake [10–65 nmol min⁻¹ (mg dry wt)⁻¹] in the stirred chamber system maintained at O₂ 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 O₂ could lead to a general effect on macromolecular synthesis rather than to a more specific effect on energy status regulating *nif* gene expression. Further work should reveal whether the apparent stimulation by O₂ of nitrogenase derepression can be correlated with elevated levels of ATP.

The activity of nitrogenase, fully derepressed during anaerobic growth, was also clearly enhanced with free, dissolved O₂ at 30 nM. This, together with the correlation between enhancement of nitrogenase activity and O₂ 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 protonmotive force in anaerobic N₂-fixing batch cultures of *K. pneumoniae* strain M5a1. However, O₂ was apparently excluded during the measurements, so her results do not preclude the possibility that, as a result of aerobic respiratory activity, a sufficient protonmotive force is generated across the membrane to yield additional supplies of either reducing power or ATP for nitrogenase activity.

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