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Sensitivity to Oxygen of Nitrogenase Activity in *Rhizobium* Strain ANU289 of the Non-legume *Parasponia* (Ulmaceae)

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Abstract

Nitrogenase activity can be detected in *Rhizobium* strain ANU289 of *Parasponia* in shaken liquid cultures. A combination of consistently low oxygen tension and appropriate cell density was found to be essential for rapid initiation and high specific rates (in the range of 50–60 nmoles of ethylene per milligram protein per hour). In the presence of succinate as carbon source and an oxygen concentration of 0·25% (v/v) in the gas phase, nitrogenase activity developed after incubation for 24 h. The requirement for an oxygen concentration in the range of 0·1 to 0·35% for derepression indicated a lack of any inherent tolerance to higher oxygen levels and thus suggests that plant-derived mechanisms are responsible for protection by oxygen in the nodule.

Introduction

The mechanism for protection by oxygen of the nitrogen-fixing *Rhizobium* cells in the *Parasponia* root nodule is still not understood. Early studies by Coventry *et al.* (1976) indicated a lack of haemoglobin. Recent work by Appleby *et al.* (1983), however, showed that by using an anaerobic isolation procedure, which prevents excessive tanning of nodule proteins, it is possible to demonstrate a haemoglobin within *Parasponia* nodules. Tjepkema and Cartica (1982) used sensitive oxygen microelectrodes and india ink infusion to demonstrate that the *Parasponia* nodule contains a boundary layer, which restricts free diffusion of oxygen into the infected zones.

We decided to use *in vitro* derepressed cultures of the *Parasponia* microsymbiont (*Rhizobium* strain ANU289) to test whether the bacterium itself has a tolerance to oxygen in terms of nitrogenase activity. This type of approach is based on the assumption that *in vitro* derepressed cultures represent the same state as the fully differentiated bacteroid. Data obtained by Sandeman (1983) with Siratro and *Parasponia* bacteroids indicated that at least in regards to oxygen regulation of nitrogenase activity, *in vitro* derepressed cultures and symbiotic bacteria were equivalent. The results shown here provide another line of evidence which suggest that (i) the *Parasponia* microsymbiont *Rhizobium* has no special adaptation for the expression of nitrogenase at elevated oxygen concentrations under the growth conditions used here, and (ii) plant mechanisms may be essential in the successful non-legume symbiosis as they are with legumes (Gresshoff *et al.* 1984*a*).

Strain ANU289 was earlier reported to show derepression of nitrogenase activity both in agar slope and stationary liquid culture (Mohapatra *et al.* 1983). Low specific activities and long lag period (4–7 days in stationary liquid cultures and 11–15 days on agar slopes) render these methods unsuitable for most physiological experiments (Mohapatra and Gresshoff 1983). Furthermore, the measurement of oxygen concentrations in the gas phase does not represent the dissolved oxygen in the agar or stationary liquid culture due to the

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formation of oxygen gradients either in the mucoid layer or the liquid 'cushion'. In the present experiments we used rapidly agitated liquid cultures designed to overcome these limitations. However, agitated liquid cultures are not as perfect as steady-state systems (chemostats), where oxygen concentration can be measured directly. In this paper, we demonstrate that nitrogenase activity can be detected in strain ANU289 in shaking liquid cultures with oxygen concentrations ranging from 0·11 to 0·35% in the gas phase. A comparison between strains 32H1 and ANU289 indicates that the latter is by no means special with regard to its oxygen requirement during derepression of nitrogenase activity and is equally susceptible to higher concentrations of oxygen.

Materials and Methods

Organism

Rhizobium strain ANU289 is a streptomycin-resistant (500 mg/l) derivative of strain CP283 and has been described previously (Cen *et al.* 1982; Mohapatra *et al.* 1983). Cowpea strain 32H1, a strain well characterized for its derepression kinetics (Keister and Evans 1976; Pankhurst 1981) was used in comparative experiments.

Media

Bacteria were grown in batch culture at 28°C. The basic medium contained (in mg/l): NaH_2PO_4 150, KCl 500, Na_2SO_4 250, $CaCl_2$ 150, $FeSO_4.7H_20$ 2·8, Na_2EDTA 3·7, nicotinic acid 0·1, pyridoxin-HCl 0·1, biotin 0·001, thiamine-HCl 0·001, inositol 10 and 1 ml of trace-element stock (Gamborg and Eveleigh 1968). In addition, the medium for growth (RGM30M, see Mohapatra *et al.* 1983) contained 10 mM each of mannitol and sodium glutamate as carbon and nitrogen source, whereas the medium used for derepression contained 50 mM sodium succinate and 3 mM glutamate and was buffered with 50 mM HEPES (*N*-(2-hydroxyethyl)-1-piperazine-*N'*-ethanesulfonic acid). The initial pH was adjusted to 6·8 with NaOH.

Derepression of Nitrogenase Activity

Cells were grown in liquid medium (RGM30M) with shaking at 200 rpm at 28°C and were harvested in mid-exponential phase (3×10^9 cells/ml). Cells were washed twice (8000 *g*, 10 min) in derepression medium and resuspended to give a final density of about 1×10^9 cells/ml. Flat-bottomed Wolfes' bottles (400 ml) sealed with Subaseals (size 33, William Freedman Company) were evacuated and flushed four times with helium and finally filled with 96% (v/v) helium and 4% (v/v) acetylene (being optimal). Oxygen was injected at the described levels. The cultures (10 ml) were shaken at 140 rpm at 28°C. The oxygen concentration was monitored in the gas phase at intervals of approximately 8 h with a GOW-MAC 550 series gas chromatograph with thermal conductivity detector. Pure oxygen was added to the cultures to maintain the desired oxygen concentration. Samples removed from flasks were assayed for acetylene reduction using a Pye Unicam series 104 gas chromatograph with a Poropak R 80–100 column (at 55°C). Further details regarding the acetylene reduction, protein determination and microbiological procedures are outlined by Mohapatra *et al.* (1983).

Results and Discussion

The effects of various concentrations of oxygen on the initiation and rate of derepression of nitrogenase activity for *Rhizobium* strain ANU289 are shown in Fig. 1. The range of oxygen readings is given in the figure legend. Oxygen concentrations other than 0·25% (v/v) showed delayed initiation or lower specific rates or both. No acetylene reduction was found with oxygen concentrations at or higher than 0·5% (v/v) in the gas phase. These

oxygen levels are similar to those found optimal for continued nitrogenase activity in isolated bacteroids of strain ANU289 from Siratro and *Parasponia* nodules (Sandeman 1983; Gresshoff *et al.* 1984*b*). The lag period was dependent upon the level of oxygen used and varied from 12 to 48 h. The effects of a particular oxygen concentration were dependent on initial cell density. In the experiments described the initial cell density was $1 \cdot 1 \times 10^9$ cells/ml.

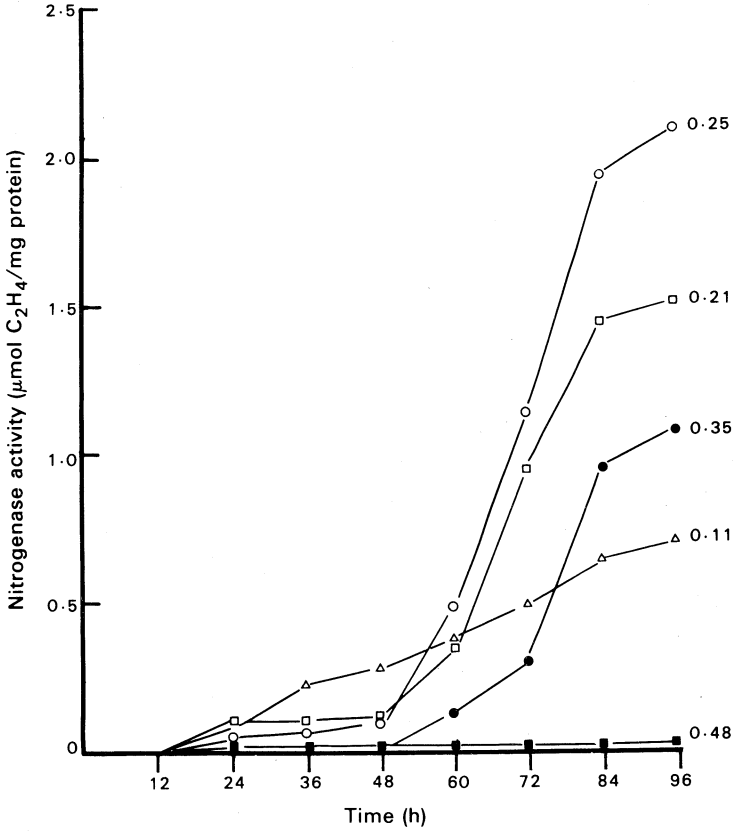

Fig. 1. Effect of various oxygen concentrations on the reduction of acetylene by *Rhizobium* strain ANU289 over a period of 96 h. Sodium succinate (50 mM) and sodium glutamate (3 mM) were added as carbon and nitrogen sources. The medium was buffered with 50 mM HEPES, pH 6·8. Oxygen concentrations in the gas phase are the average of readings taken at intervals of approximately 8 h. The ranges were 0·11% (0·08–0·15%), 0·21% (0·17–0·25%), 0·25% (0·20–0·30%), 0·35% (0·33–0·40%), 0·47% (0·43–0·50%). Protein was determined at the termination of the experiment, after washing to remove HEPES. Data are the averages of five flasks.

Derepression of nitrogenase activity in shaken liquid cultures provided an easy procedure for studying the oxygen requirement. The requirement for a consistently low oxygen tension was previously found to be mandatory for development of nitrogenase activity (Tjepkema and Evans 1975; Keister and Evans 1976). From our studies the oxygen requirement for strain ANU289 appeared to be very stringent, similar to that of other *Rhizobium* strains (Keister and Evans 1976) and considerably lower than that of other non-leguminous endophytes such as *Frankia* (Gauthier *et al.* 1981).

Fig. 2 shows a comparison between strains ANU289 and 32H1 for nitrogenase derepression at oxygen concentrations of 0·2–0·25% (v/v) in the gas phase. The overall pattern of derepression kinetics was similar for both strains, but strain ANU289 displayed about a several-fold lower rate of activity. These results are in agreement with previous findings in agar and stationary cultures (Mohapatra *et al.* 1983). Such differences among *Rhizobium* strains are well documented in the literature (Mohapatra and Gresshoff 1983).

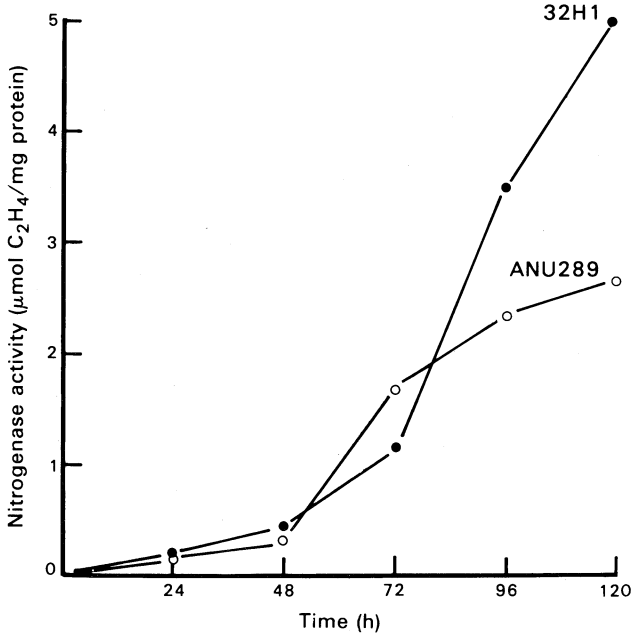

Fig. 2. Comparison of derepression kinetics between strains 32Hl and ANU289. The medium containing 50 mM sodium succinate and 3 mM sodium glutamate was buffered with 50 mM HEPES, pH 6·8. Experiments were done at an oxygen concentration of 0·25% (v/v) in the gas phase and with an initial cell concentration of $1·2 \times 10^9$ cells/ml. The nitrogenase activity was measured as μmoles of ethylene produced per milligram protein. Data are the averages of three flasks.

The other critical parameter governing derepression kinetics was cell density. Table 1 describes the effect of different cell densities on derepression of nitrogenase activity in strain ANU289. A cell density of 1×10^9 cells/ml showed maximal activity as well as highest specific rates. Higher or lower cell densities resulted in a decline of both. It was noted that flasks with different initial cell densities ended up with similar final cell numbers. Higher cell densities such as 2×10^9 cells/ml remained nearly stationary, whereas a lower density (such as $5·5 \times 10^8$ cells/ml) multiplied to reach nearly 2×10^9 cells/ml. The fact that an intermediate concentration was maximal, indicated that an appropriate combination of oxygen concentration and cell density permitting moderate growth was essential for optimal derepression. Both parameters (i.e. oxygen and cell concentration) are related, and indicate the multi-factored requirements involving carbon/nitrogen ratios as well as bacterial strain specificities for *in vitro* derepression of nitrogenase. For example, our data may be explained if oxygen concentration became limiting at high cell densities, while at higher oxygen concentrations the effect is probably on nitrogenase itself. It was assumed that if the rhizobia in *Parasponia* nodules were tolerant to higher oxygen levels, it would

be reflected during derepression of nitrogenase *in vitro*. As nitrogenase was derepressed only at a low oxygen tension, a respiratory protection by the bacterium could be ruled out. Thus, it is likely that mechanism for oxygen protection of nitrogenase in the *Parasponia*

Table 1. Effect of cell density on nitrogenase derepression in *Rhizobium* strain ANU289

Initial cell density (cells/ml)[A]	Final cell density (cells/ml)[A]	Specific activity ± s.d.[B] (μmol C_2H_4 per mg protein per hour)
$5{\cdot}5 \times 10^8$	2×10^9	42 ± 14[b]
$1{\cdot}1 \times 10^9$	2×10^9	68 ± 9[a]
$1{\cdot}7 \times 10^9$	2×10^9	26 ± 14[a]

[A] Cell numbers were determined at 72 h.
[B] Specific activities (determined over the period 60–72 h) having the same superscript letter differ significantly at $P<0{\cdot}05$ level; values with different letters differ at $P<0{\cdot}1$ level. Values are the average of five replicates.

nodule involves an anatomical barrier which restricts oxygen diffusion or that the haemoglobin (Appleby *et al.* 1983; Mohapatra 1983; Gresshoff *et al.* 1984*a*) may be functional or a combination of both.

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