The Behaviour of *Azotobacter chroococcum* in Oxygen- and Phosphate-limited Chemostat Culture

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SUMMARY

Oxygen-limited chemostat cultures of nitrogen-fixing *Azotobacter chroococcum* showed an inverse relation between biomass and dilution rate, accounted for largely by increased polysaccharide and polyhydroxybutyrate content. Abrupt increase in pO_2 led to immediate increase in CO_2 output followed later by increase in biomass and transition to N_2 limitation; viability on N-free and NH_4 -containing media remained at 80 to 100 % during O_2 stress. P-limited populations showed no respiratory response to O_2 stress, viability dropped rapidly on N-free medium though the populations were 100 % viable on NH_4 medium. These findings support the view that respiration in these bacteria has, in part, a protective function for nitrogenase.

INTRODUCTION

Continuous culture has been important in studies of azotobacters and has revealed unusual aspects of their physiology, notably O_2 -sensitivity when fixing N_2 , hypersensitivity in C-limited and P-limited conditions, N_2 -limited growth and 'switch-off' and 'switch-on' of nitrogenase in response to oxygen stress (see review by Hill, Drozd & Postgate, 1972). Nutritional status so far studied with *Azotobacter chroococcum* include NH_4^+ -, N_2 -, C-, P-, and S-limitations. O_2 -limitation has not so far been examined although Andreeva & Khmel (1970) recorded some properties of an O_2 -limited culture of *A. vinelandii* and Senior, Beech, Ritchie & Dawes (1972) used chemostat culture as part of their study of the involvement of oxygen limitation in poly- β -hydroxybutyrate synthesis by *A. beijerinckii*.

This paper reports a study of O_2 -limited *Azotobacter chroococcum* with particular reference to the relation of respiration to N_2 -fixation and extends previous studies on P-limited, N_2 -fixing cultures of *A. chroococcum*.

METHODS

Organisms and media. Stock cultures of *Azotobacter chroococcum* NCIB8003 were maintained and used as described by Dalton & Postgate (1969*a, b*) on 'mannitol B₆' medium. Where P-limitation was investigated, the phosphate concentration was 0.9 mg P/l, one half the concentration used by Dalton & Postgate (1969*b*).

Viabilities. Viability was assessed by slide culture (Postgate, Crumpton & Hunter, 1961) on mannitol B₆ medium solidified with agar. Viability was also assessed with solidified B₆ medium containing ammonium succinate 50 mg NH_4^+ -N/l (B₆-N medium).

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Fig. 1. The 5-litre receiver vessel. A, The flow line from the continuous culture vessel; B, the gas line to the CO_2 -measuring percolator apparatus; C, the air-release to the atmosphere.

Apparatus. The continuous culture apparatus (Baker, 1968) used had a working volume of 240 ml and was in all respects operated as described by Dalton & Postgate (1969*a, b*).

Analytical procedures. These were those used by Dalton & Postgate (1969*a, b*) except that the DNA content of the bacteria was not regularly measured. Early measurements showed that the DNA content was about 2 % of the bacterial dry wt under several conditions of culture. The contribution of DNA was thereafter disregarded in view of the fact that calculations of the N contributed by protein and RNA accounted for virtually all the total N in the cultures. One new analysis introduced was the measurement of the CO_2 -output of the cultures using a soil-percolator filled with glass beads in place of soil (Lees, 1949). A percolator needs a gas pressure slightly above one atmosphere if it is to operate properly. We therefore designed a simple effluent receiver vessel that trapped all CO_2 originating from the culture at the time of measurement and that excluded any CO_2 originating from the still-respiring effluent in the effluent vessel itself (Fig. 1). The percolator was initially filled with 110 ml 0.1 M- $\text{Ba}(\text{OH})_2$ plus phenolphthalein indicator. The air-line to the continuous culture vessel was then disconnected from the culture vessel and connected directly to the percolator for 5 min to ensure even distribution of the $\text{Ba}(\text{OH})_2$ in the percolator. Two 5 ml samples were then taken of the $\text{Ba}(\text{OH})_2$ and titrated against 0.1 M-HCl. The percolator, still connected in the same way, was run for 30 min and again two 5 ml samples were titrated; this gave the ' CO_2 -blank' of the air supply. The culture vessel was then reconnected to the air supply and the percolator to the gas issuing from the culture (Fig. 1). After 60 min a further two 5 ml samples of the $\text{Ba}(\text{OH})_2$ were titrated against 0.1 M-HCl. The difference between the second and third titrations, minus twice the blank shown by the difference between the first and second titrations, gave the CO_2 output of the culture. Test runs showed that, when a flask containing a known amount of KHCO_3

Fig. 2. The effect of dilution rate on O_2 -limited cultures of *Azotobacter chroococcum*.
○, Biomass; ●, CO_2 output.

was inserted in the air-line and suddenly acidified by tipping in excess H_2SO_4 , 98 % of the calculated CO_2 output was trapped by the $Ba(OH)_2$ within 10 min. The 'blank' values, recorded over an 8 month period, showed that the CO_2 content of the air supply was 0.03 %.

Experiments carried out under O_2 -limited and P-limited conditions were run for four days at each dilution rate and samples taken every day. All samples were analysed in duplicate. Each quoted analytical value is therefore the average of eight separate determinations. The number of replacement times used to establish a steady state before any samples were taken ranged from 7 to 21.

RESULTS

Continuous culture of O_2 -limited populations. A stirring rate was maintained so that, with air supplied to the cultures at 150 ml/min, the O_2 concentration within the culture approached the limit of detection (below $10 \mu M$) with B_6 medium. Evidence that the cultures were O_2 -limited under these conditions is given later. The dry weight of organisms fell steadily as the dilution rate (D) was increased, while the CO_2 output remained fairly constant (Fig. 2). No particular significance is attached to the dip in the CO_2 curve; it is probably due to slight changes in the stirring rate (the line voltage in Britain at the time varied markedly) and in any case represents no more than a rise of 14 % and a drop of 11 % from an average value of $16 \mu mol CO_2/ml$ culture/h.

The composition of the organisms changed with dilution rate (Table 1); we have no explanation for the abrupt rise in polysaccharide at $D = 0.22 h^{-1}$ but it was real. The lowest polysaccharide contents (6 to 8 %, w/w) approach the minimum values quoted by Dalton & Postgate (1969*b*) for chemostat populations of various nutritional status (4 to 5.4 %, w/w), which may represent the minimum requirement of this strain for structural

Fig. 3. The relation between replacement time and percentage of storage material (polysaccharide plus poly- β -hydroxybutyrate) in O_2 -limited chemostat cultures of *Azotobacter chroococcum*.

Table 1. The macromolecular composition of nitrogen-fixing *Azotobacter chroococcum* in continuous culture

Values as % dry weight of organisms; cultures at 30 °C in air except where stated.

Nutritional status of population	Dilution rate (h^{-1})	Protein	Poly-saccharide	PHB*	RNA
O_2 -limited	0.12	33	6	51	9
	0.16	44	9	34	11
	0.22	49	17	21	12
	0.30	58	6	25	9
N_2 -limited†	0.25	64	8	13	14
PO_4 -limited	0.15	68	12	6	14
PO_4 -limited, O_2 poisoned	0.15	66	12	7	14

* Poly- β -hydroxybutyrate.

† Growing with 0.4 atm O_2 , see text.

rather than storage polysaccharide. The percentage of 'storage material' (polysaccharide plus poly- β -hydroxybutyrate) in the organisms was linearly related to the replacement time (R) of the culture (Fig. 3). The residual mannitol in the culture rose from 3.5 mg/ml at $R = 11.8$ h to 8.8 mg/ml at $R = 6.7$ (the initial concentration was 10 mg/ml).

Proof that cultures were genuinely O_2 -limited was provided by experiments in which the stirring rate of the culture was kept constant while the inflowing air was supplemented by extra O_2 to give a final pO_2 of 0.4 atm. Experiments at several dilution rates all gave similar results; typical was an experiment at $D = 0.25$. An increase in CO_2 output was detectable within the first hour after the O_2 supply had been increased and after 24 h the CO_2 output had risen from 15 μ mol/ml culture/h to 25 μ mol/ml culture/h. The dry wt of the culture had risen from 0.50 to 0.73 mg/ml. The composition of the organisms changed slightly towards a lower percentage of 'storage material' (Table 1) while the residual mannitol in the culture dropped from 8.8 to 7.8 mg/ml. These values were maintained for the four days' duration of the experiment. The extra O_2 supplied almost certainly changed the cultures from O_2 -limitation to N_2 -limitation. The composition of the bacteria supplied with extra O_2 was virtually the same as the N_2 -limited bacteria described by Dalton &

Fig. 4. The dissolved O_2 concentration in chemostat cultures of *Azotobacter chroococcum*, grown in air in B_6 media and supplied with extra O_2 to 0.4 atm at start. ●, Initially O_2 -limited population; ×, PO_4 -limited population.

Postgate (1969*b*). Fig. 4 shows that, 10 min after the extra O_2 was supplied to the culture, the dissolved O_2 had risen from 7 μM to 80 μM but after 25 min it had stabilized at 20 μM : oxygen consumption had evidently become rapidly adjusted to the increased oxygen supply.

Behaviour of phosphate-limited cultures. The response of P-limited cultures to raised pO_2 was quite different. When exposed to 0.4 atm O_2 for 4 h, the composition of the organisms did not change (Table 1). That growth virtually ceased was shown by the fact that the turbidity, dry wt, and concentrations of protein, RNA, poly- β -hydroxybutyrate and polysaccharide all fell in accordance with washout as calculated from the dilution rate. Unlike the O_2 -limited cultures on medium B_6 , the P-limited cultures were quite incapable of restoring the O_2 concentration within the culture to normal levels (Fig. 4); moreover, the CO_2 output of the cultures dropped steadily as exposure to excess O_2 continued. There was a small increase in residual mannitol concentration during the 4 h.

Viability studies. O_2 -limited cultures had a viability of $75 \pm 5\%$ on solidified B_6 medium but 100% (no non-dividing cells were seen) on solidified B_6 -N medium. This suggests a slight physiological damage due to O_2 deprivation. The viability of P-limited cultures before the cultures were challenged by excess O_2 was about 100% on both B_6 and B_6 -N media, but, after 4 h of exposure to excess O_2 , the viability on B_6 medium was virtually zero while that on B_6 -N medium remained at 100%, although the colonies were very small. These findings are consistent with Dalton & Postgate's report (1969*a*) on the effect of O_2 on P-limited cultures but those authors did not test viability on a solidified medium containing NH_4^+ .

DISCUSSION

It seems that, under O_2 -limited, N_2 -fixing conditions, *Azotobacter chroococcum* always synthesizes its essential protein and RNA at any dilution rate, but the percentage of 'storage material' (polysaccharide and poly- β -hydroxybutyrate) increases as the dilution rate is decreased, i.e. as the residence time of the bacteria in the culture vessel is increased (Fig. 3). This observation is consistent with the demonstration of Senior *et al.* (1972) that O_2 -limited *A. beijerinckii* synthesized much more poly- β -hydroxybutyrate than C- or N_2 -limited populations. They suggested that the polymer served as an 'electron sink' as well as a storage polymer. When supplied with extra O_2 and thus returned to a N_2 -limited state, *A. chroococcum* reverted to a composition almost exactly like that given by Dalton & Postgate (1969a) for N_2 -limited *A. chroococcum* under the same culture conditions.

While O_2 -limited cultures subjected to excess O_2 were easily able to increase their respiration so as to keep the level of dissolved O_2 low, P-limited cultures were not. The composition of P-limited cultures did not change when excess O_2 was supplied (Table 1) but the bacteria simply ceased to grow and CO_2 evolution fell. This suggests that the terminal-respiration system ceased to function properly, possibly because excess O_2 drove all the transferable nucleotide P into ATP so that a normal ADP/ATP ratio could not be maintained. This would be in harmony with the results of Yates (1970) who presented evidence for continual respiratory control by the ADP/ATP ratio in *Azotobacter chroococcum*.

The viability tests showed that the 'lethal' effect of O_2 stress on P-limited *Azotobacter chroococcum* reported by Dalton & Postgate (1969a) is not real. The organisms remain viable after O_2 stress if tested on a medium containing NH_4^+ . It follows that O_2 stress specifically affects nitrogenase or something closely associated with it. This is direct evidence for the view that respiration protects nitrogenase in N_2 -fixing organisms as suggested by Postgate and colleagues (see Hill *et al.* 1972).

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