Effect of Dissolved Oxygen Tension on Production of Carotenoids, Poly- β -hydroxybutyrate, Succinate Oxidase and Superoxide Dismutase by *Azospirillum brasilense* Cd Grown in Continuous Culture

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Azospirillum brasilense strain Cd was grown in a medium containing NH_4^+ in a chemostat at a range of constant dissolved oxygen tensions (d.o.t.) (0.007–0.18 atm). Poly- β -hydroxybutyrate (up to 12% of the cell dry weight) increased under oxygen limitation and moderate dilution rate ($D = 0.14 \text{ h}^{-1}$). The highest carotenoid content was observed at high d.o.t. and dilution rates up to 0.12 h^{-1} . The amount of protein varied with d.o.t. from 0.29 mg protein (mg dry wt) $^{-1}$ at 0.007 atm to 0.54 mg at 0.18 atm. The yield efficiency and respiration rate were highest at low d.o.t. and decreased significantly at a d.o.t. of 0.18 atm. Succinate dehydrogenase and malate dehydrogenase activities increased 2.5-fold at 0.10–0.18 atm O_2 , whereas succinate oxidase and NADH oxidase activities increased consistently with increasing d.o.t. *Azospirillum brasilense* showed a low specific activity for catalase; the specific activity of superoxide dismutase increased sharply above 0.16 atm O_2 .

INTRODUCTION

Although capable of aerobic growth, rhizosphere bacteria of the genus *Azospirillum* are capable of fixing molecular nitrogen only under low $p\text{O}_2$ (0.005 atm), probably because of the inactivation of nitrogenase by oxygen and its radicals (Okon *et al.*, 1976a; Day & Dobereiner, 1976; Nelson & Knowles, 1978).

In recent studies (Nur *et al.*, 1980; Okon *et al.*, 1980), it was found that although *A. brasilense* was capable of growing at faster rates at high $p\text{O}_2$ in the presence of NH_4Cl , if given the choice and in spite of the presence of combined nitrogen, *A. brasilense* actively sought microaerobic conditions, i.e. it developed as a pellicle below the surface in semi-solid medium or it entered capillaries containing only water or phosphate buffer, forming a band which moved towards a decreasing $p\text{O}_2$ gradient (aerotaxis to low $p\text{O}_2$). When grown on N_2 at low $p\text{O}_2$, *A. brasilense* strain Sp7 formed poly- β -hydroxybutyrate (PHB) which amounted to about 30% of the cell dry weight, whereas in cells growing under high $p\text{O}_2$ in the presence of NH_4Cl , PHB formed less than 1% of the cells weight (Okon *et al.*, 1976b). Nur *et al.* (1981) reported that red-pigmented *A. brasilense* strain Cd produced carotenoids, apparently involved in the protection of *A. brasilense* Cd nitrogenase from O_2 . Carotenoid synthesis started in liquid static cultures only after the concentration of NH_4Cl in the medium had decreased and N_2 -fixation became evident. Carotenoid synthesis did not occur under microaerobic conditions. When carotenoid synthesis was specifically inhibited in the presence of diphenylamine, the rate of acetylene reduction in strain Cd decreased by 50%.

Carbon-limited chemostat cultures of *A. brasilense* Sp7 have been studied on N-free medium at various dissolved oxygen concentrations (Nelson & Knowles, 1978). In this work we report the behaviour of *A. brasilense* Cd in the presence of NH_4Cl in a chemostat, under different dissolved O_2 concentrations and different dilution rates.

Abbreviations: d.o.t., dissolved oxygen tension(s); PHB, poly- β -hydroxybutyrate.

METHODS

Organism and growth conditions. Stock cultures of *Azospirillum brasilense* strain Cd, ATCC 29729 (Tarrand *et al.*, 1978), were maintained on N-free malate medium (Okon *et al.*, 1977). To propagate a continuous culture, a bench chemostat (New Brunswick, NBS model C30) of 1.5 l working capacity was used. Dissolved oxygen tensions (d.o.t.) were measured with an autoclavable galvanic-type electrode (NBS model M1016-0208). The oxygen probe was calibrated initially by passing pure nitrogen and then air through the stirred uninoculated growth vessel. D.o.t. values were kept constant during growth with a sterile mixture of nitrogen and air, the composition of which was varied automatically by an NBS oxygen controller (Model DO-80). The culture was stirred at a constant rate of 300 r.p.m. Growth temperature was maintained at 30 °C.

The mineral growth medium containing potassium phosphate buffer (pH 6.8) (Okon *et al.*, 1977) was supplemented with 2 g malic acid, 0.8 g NaOH and 0.5 g NH_4Cl per litre. The pH of the medium was kept at pH 6.8 throughout the experiment by an NBS pH controller (model pH-22).

Inocula were prepared by growing cultures at 30 °C on a rotary shaker in 250 ml Erlenmeyer flasks containing 100 ml malate medium supplemented with 0.5 g NH_4Cl l⁻¹. Bacterial growth was measured either turbidimetrically with a Junior II Coleman spectrophotometer or by determining bacterial dry weight. Bacterial suspensions (20 ml) were centrifuged at 7000 g for 10 min at 4 °C. The pellet was resuspended in 5 ml distilled water, and dried at 80 °C for 24 h. The relation between bacterial dry weight and A_{420} was constant at all dry weight values: dry weight (mg ml⁻¹) = $1.180 A_{420}$ (S.D. $\pm$ 0.05).

Analytical procedures. Total dissolved carbon in the culture medium was determined by the dichromate method (Johnson, 1949). Cell extracts were obtained by sonication (5 min at 4 °C) using an MSE ultrasonic disintegrator or by overnight extraction with 0.5 M-NaOH (for determination of protein). The protein content of extracts was determined by a modification of Lowry's procedure (Markwell *et al.*, 1979) using bovine serum albumin (Sigma) as a standard.

Bacterial carotenoids were extracted and determined as described previously (Nur *et al.*, 1981), using an absorption coefficient of 2500 at 500 nm for a 1% (w/v) solution (Stern *et al.*, 1964).

The poly- β -hydroxybutyrate content of *A. brasilense* was determined by the modified method of Senior *et al.* (1972). Each point was determined in six replicates (S.E. < 10%).

Enzyme activities. All assays were done at 30 °C. Oxygen consumption was followed polarographically, using a biological oxygen monitor (model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). The potential respiration rate of bacteria growing in the chemostat was determined as follows. Samples taken from the growth vessel were rapidly diluted in 0.1 M-potassium phosphate buffer (pH 7.2). The respiratory activity of the buffer-suspended cells was measured immediately at 30 °C, both in the absence and in the presence of sodium malate (final concentration 0.2 M). Activity was expressed as $\mu\text{l O}_2$ consumed h⁻¹ (mg dry wt)⁻¹.

Crude extracts of *A. brasilense* Cd grown at various d.o.t. values were obtained by sonicating buffer-washed cells for 5 min at 4 °C. The extract was centrifuged at 17000 g for 20 min at 4 °C, and the supernatant was diluted to 2–3 mg protein ml⁻¹.

The rate of oxygen uptake by extracts was estimated by a modification of the method of Stone & Wilson (1952). The reaction mixture contained 1 ml 0.2 M-potassium phosphate buffer, (pH 7.0), containing 0.02 M-MgSO₄, 1 ml extract, and succinate and/or NADH at final concentrations of 40 mM, and 12.2 mM, respectively.

Malate dehydrogenase and succinate dehydrogenase were assayed by procedure of Courtright & Henning (1970).

Superoxide dismutase was assayed as described by Buchanan & Leeds (1976). One unit of enzyme was defined as the amount of enzyme giving 50% inhibition of the rate of reduction of cytochrome c. Catalase was estimated by the conventional polarographic oxygen electrode method, to determine the production of oxygen from 10 $\mu\text{mol H}_2\text{O}_2$ in 1 min, after correction with the appropriate controls. Increasing the quantity of H_2O_2 or the time of incubation resulted in a decrease of the enzyme activity due to its inactivation by the substrate.

RESULTS

Effect of dissolved oxygen tension on growth parameters

The growth of *A. brasilense* Cd in the presence of NH_4Cl at a constant dilution rate (generation time 8.66 h) was followed under various d.o.t. conditions ranging from air saturation (d.o.t. = 0.21 atm) to oxygen-limiting conditions (Fig. 1). Cell mass (dry weight) increased rapidly from 0.525 mg ml⁻¹ at 0.015 atm, reaching a steady state that was maintained over a wide range of d.o.t. (0.015–0.18 atm). The cell mass of air-saturated cultures was significantly lower (Fig. 1a). At low d.o.t. (0.007 atm), the protein content of the cells was low, but it increased at d.o.t. = 0.03 atm and remained constant at d.o.t. values up to 0.125 atm. At higher d.o.t. values protein content increased in spite of a decrease in cell mass (Fig. 1a).

Fig. 1. Growth of *A. brasilense* Cd in continuous culture with $0.5 \text{ g NH}_4\text{Cl l}^{-1}$, at different dissolved oxygen tensions (0.007 to 0.21 atm). □, Bacterial dry weight; ■, protein content; ○, malate in culture supernatant; ●, Y_m [g cells (mol malate utilized)⁻¹]; △, Q_{O_2} [$\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$]; ▲, carotenoid content. The dilution rate was 0.08 h^{-1} .

There was a direct relationship between the carotenoid content of the cells and the d.o.t. of the growth medium. However, under air-saturated conditions there was a small decrease in carotenoid content (Fig. 1b). Q_{O_2} values [$\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$], malate yield coefficients Y_m [g organism formed (mol malate utilized)⁻¹] and residual malate concentration were also measured (Fig. 1a, b). The Q_{O_2} and Y_m values decreased with increasing d.o.t., reaching a plateau at d.o.t. = 0.03 atm . In air-saturated cultures, Q_{O_2} and Y_m values were markedly lower (Fig. 1b). There was a direct relationship between Q_{O_2} and Y_m throughout all O_2 concentrations (Fig. 1b). It should be noted that these results differ from those reported by Senior *et al.* (1972) and Ward *et al.* (1977), who observed in *Azotobacter beijerinckii* an inverse relation between Q_{O_2} and yield, due probably to the uncoupling of the electron transport system at high O_2 concentration.

The highest residual malate concentration was observed at d.o.t. = 0.007 atm , but at all other d.o.t. values the residual concentration was very low (Fig. 1a), which indicated that oxygen limitation occurred only at 0.007 atm at the dilution rate used.

Effect of d.o.t. on cell mass and growth rate

The maximum biomass obtained under steady-state conditions in the chemostat was 0.84 mg ml^{-1} . Limiting O_2 concentration, either by lowering d.o.t. in the growth chamber or by increasing the dilution rate, led to a decrease in cell concentration.

Generally, bacterial biomass in carbon-limited *Azospirillum* cultures remained constant at all the dilution rates tested. However, when oxygen limitation was imposed an initial decrease in bacterial concentration was followed by readjustment to the new environment resulting in a new steady state (Fig. 2).

Effect of d.o.t. and dilution rate on carotenoids and PHB

The maximum specific carotenoid content, $0.57 \mu\text{g} (\text{mg protein})^{-1}$, was observed at d.o.t. = 0.18 atm and at dilution rates up to 0.12 h^{-1} ; at higher dilution rates it decreased rapidly to $0.2 \mu\text{g} (\text{mg protein})^{-1}$ (Fig. 3), suggesting that carotenoid synthesis occurs mainly at low growth

Fig. 2. Variation of steady-state biomass with growth rate in a malate-limited chemostat culture at three different dissolved oxygen concentrations: $\blacktriangle$, 0.18 atm; $\circ$, 0.015 atm; $\bullet$, 0.007 atm. For experimental details see text.

Fig. 3. Variation in carotenoid and PHB content with dilution rate. $\blacktriangle$, carotenoid content at 0.18 atm; $\circ$, PHB content at 0.015 atm; $\bullet$, PHB content 0.007 atm (PHB was not detected at 0.18 atm). For experimental details see text.

rates and when oxygen is not a limiting factor. Maximum PHB content (12% of the biomass) was observed under microaerobic conditions and at moderate growth rates (Fig. 3).

Effect of d.o.t. on enzyme activities

NADH oxidase and succinate oxidase activities increased in proportion to the d.o.t. of the medium (Fig. 4a). In contrast, succinate and malate dehydrogenase activities increased markedly only above 0.16 atm (Fig. 4b). Superoxide dismutase activity increased slightly as d.o.t. was increased to intermediate levels. A sharp increase in superoxide dismutase activity, reaching a maximum of 72 units (mg dry wt)⁻¹, was obtained at d.o.t. above 0.16 atm (Fig. 5). Catalase activities were generally low, and decreased with increasing d.o.t. (Fig. 5).

DISCUSSION

In this work we present further evidence on the capability of *Azospirillum brasilense* Cd, growing under steady state conditions in the chemostat, to adapt readily to different d.o.t. values and dilution rates.

As shown by the higher Y_s (Y_m) values obtained under low pO₂ and low dilution rates, conditions generally encountered in the rhizosphere, *A. brasilense* Cd utilized its energy and carbon sources more efficiently. Similarly, high N₂ fixation efficiencies [i.e. mg N fixed (g

Fig. 4. Effect of variation in d.o.t., in a chemostat culture of *A. brasilense* Cd, on the activities of NADH oxidase [$\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$, ○], succinate oxidase [$\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$, △], malate dehydrogenase [$\text{nmol NADH min}^{-1} (\text{mg dry wt})^{-1}$, ●] and succinate dehydrogenase [$\text{nmol succinate min}^{-1} (\text{mg dry wt})^{-1}$, ▲]. Details as for Fig. 1.

Fig. 5. Effect of different dissolved oxygen tensions on superoxide dismutase (○) and catalase (△) activities in a chemostat culture of *A. brasilense* Cd. Details as in Fig. 1. Standard deviation bars are shown.

carbon substrate utilized) $^{-1}$] have been observed at microaerobic conditions under limited malate supply (Day & Döbereiner, 1976; Okon *et al.*, 1976a). In addition, under these conditions, *A. brasilense* produced PHB in larger quantities, 12% in strain Cd (Fig. 4) and 30% in strain Sp7 in batch cultures (Okon *et al.*, 1976b). PHB may serve as further energy and carbon source when carbon is limiting; high hydroxybutyrate dehydrogenase activities were observed under low pO_2 (Okon *et al.*, 1976b), and this corroborates the relationship between oxygen limitation and PHB accumulation observed in other N_2 -fixing organisms grown on NH_4 or N_2 (Ward *et al.*, 1977).

At intermediate d.o.t. values *A. brasilense* Cd produced red carotenoids, which are apparently capable of protecting the cell against oxidative damage, because of their ability to quench singlet oxygen and possibly oxygen radicals (Krinsky, 1979). In addition, carotenoids may act as a rigid

insert reinforcing the membrane bilayer (Rottem & Markowitz, 1979) thus reducing O₂ diffusion into the cytoplasm. In this work, a clear relationship was observed between carotenoid content in *A. brasilense* Cd and oxygen concentration. The same relationship was obtained under N₂-fixing conditions in batch cultures (Nur *et al.*, 1981).

Under high d.o.t. and/or high dilution rates (conditions difficult, but not impossible to find in the rhizosphere) production of PHB was markedly decreased (Fig. 4, and Okon *et al.*, 1976*b*). At high oxygen concentrations, carotenoid content may be reduced either by inhibition of synthesis or as a result of destruction by oxygen radicals. When exposed to air, purified *A. brasilense* Cd carotenoids were rapidly destroyed (Nur *et al.*, 1981).

Under d.o.t. and high dilution rates PHB and carotenoid production were inhibited. In contrast, the protein content of the cells, and the specific activities of succinic dehydrogenase and succinic oxidase, increased under high d.o.t. Succinic dehydrogenase is an FAD membrane-bound enzyme found as a complex with cytochrome *b* in *E. coli*, a *Bacillus* species (Hendler & Burgess, 1974) and *A. brasilense* (Y. Okon & R. H. Burris, unpublished results). This system may protect the bacterium by scavenging oxygen in a way similar to the protective oxidase system, reported recently by Bergersen & Turner (1980) in *Rhizobium* bacteroids and in *Azospirillum brasilense* Sp7.

Superoxide dismutase activity of *A. brasilense* Cd was induced under increased d.o.t., but catalase activity was relatively weak, especially at high d.o.t. values, when its function is probably most needed. Stouthamer *et al.* (1979) suggested that under atmospheric oxygen pressure, the respiratory system of microaerophilic bacteria was organized in such a way that large amounts of toxic oxygen metabolites are formed during respiration, and that the activity of enzymes of decomposition of O₂⁻ and H₂O₂ was insufficient to prevent damage to the cell. This may apply also to *A. brasilense*. However, in this bacterium the damage did not affect the respiratory enzymes (see Fig. 3*a, b*) but affected the intact cell respiration and yield efficiency (see Fig. 1*b*). Although *A. brasilense* Cd contains protecting agents (such as carotenoids) against oxygen and its radicals it still actively seeks, by aerotaxis (Okon *et al.*, 1980), an environment where oxygen is limiting (microaerobic environment); this behaviour is probably less costly in energy terms than the synthesis of carotenoids or respiration enzymes. Therefore *A. brasilense* differs significantly from *Azotobacter beijerinckii* which possesses high catalase activity and is capable of fixing N₂ under high d.o.t.

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REFERENCES

- BERGERSEN, F. J. & TURNER, G. L. (1980). Properties of terminal oxidase systems of bacteroids from roots of soybean and cowpea and of N₂-fixing bacteria grown in continuous culture. *Journal of General Microbiology* **118**, 235–252.
- BUCHANAN, A. G. & LEES, H. (1976). The effects of pH and temperature on the assays of superoxide dismutase. *Canadian Journal of Microbiology* **22**, 1643–1646.
- COURTRIGHT, J. B. & HENNING, U. (1970). Malate dehydrogenase mutants in *Escherichia coli* K-12. *Journal of Bacteriology* **102**, 722–728.
- DAY, J. M. & DÖBEREINER, J. (1976). Physiological aspects of N₂-fixation by a *Spirillum* from *Digitaria* roots. *Soil Biology and Biochemistry* **8**, 45–50.
- JOHNSON, M. J. (1949). A rapid micromethod of estimation of non-volatile organic matter. *Journal of Biological Chemistry* **181**, 707–711.
- HENDLER, R. W. & BURGESS, A. H. (1974). Fractionation of electron-transport chain of *Escherichia coli*. *Biochimica et biophysica acta* **357**, 215–230.
- KRINSKY, N. I. (1979). Carotenoid protection against oxidation. *Pure and Applied Chemistry* **51**, 649–660.
- MARKWELL, A. M., SUZANNE, K., HASS, M., BIEBER, L. L. & TOLBERT, N. E. (1979). Modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* **87**, 206–210.
- NELSON, L. M. & KNOWLES, R. (1978). Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. *Canadian Journal of Microbiology* **24**, 1395–1403.
- NUR, I., OKON, Y. & HENIS, Y. (1980). Comparative studies of nitrogen-fixing bacteria associated with grasses in Israel with *Azospirillum brasilense*. *Canadian Journal of Microbiology* **26**, 714–718.
- NUR, I., STEINITZ, Y. L., OKON, Y. & HENIS, Y. (1981). Carotenoid composition and function in nitrogen-fixing bacteria of the genus *Azospirillum*. *Journal of General Microbiology* **122**, 27–32.
- OKON, Y., ALBRECHT, S. L. & BURRIS, R. H. (1976*a*).

- Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. *Journal of Bacteriology* **127**, 1248–1254.
- OKON, Y., ALBRECHT, S. L. & BURRIS, R. H. (1976*b*). Carbon and ammonia metabolism of *Spirillum lipoferum*. *Journal of Bacteriology* **128**, 592–597.
- OKON, Y., ÇAKMAKCI, L., NUR, I. & CHET, I. (1980). Aerotaxis and chemotaxis of *Azospirillum brasilense*. *Microbial Ecology* **6**, 277–280.
- OKON, Y., HOUGHINS, J. P., ALBRECHT, S. L. & BURRIS, R. H. (1977). Growth of *Spirillum lipoferum* at constant partial pressures of oxygen and the properties of its nitrogenase in cell-free extracts. *Journal of General Microbiology* **98**, 87–93.
- ROTTM, S. & MARKOWITZ, O. (1979). Carotenoids act as reinforcers of *Acholeplasma laidlawii* lipid bilayer. *Journal of Bacteriology* **140**, 944–948.
- SENIOR, P. J., BEECH, G. A., RITCHIE, G. A. F. & DAWES, E. A. (1972). The role of oxygen limitation in the formation of poly- β -hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. *Biochemical Journal* **128**, 1193–1201.
- STERN, A. I., SCHIFF, J. A. & EPSTEIN, H. T. (1964). Studies of chloroplast development in *Euglena*. V. Pigment biosynthesis, photosynthetic oxygen evolution and carbon dioxide fixation during chloroplast development. *Plant Physiology* **39**, 200–226.
- STONE, R. W. & WILSON, P. W. (1952). Respiratory activity of cell-free extracts from *Azotobacter*. *Journal of Bacteriology* **63**, 605–617.
- STOUTHAMER, A. H., DE VRIES, W. & NIEKUS, H. G. D. (1979). Microaerophily. *Antonie van Leeuwenhoek* **45**, 5–12.
- TARRAND, J. J., KRIEG, N. R. & DÖBEREINER, J. (1978). A taxonomic study of the *Spirillum lipoferum* group, with description of a new genus *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Canadian Journal of Microbiology* **24**, 967–980.
- WARD, A. C., ROWLEY, B. I. & DAWES, E. A. (1977). Effect of oxygen and nitrogen limitation on poly- β -hydroxybutyrate biosynthesis in ammonium-grown *Azotobacter beijerinckii*. *Journal of General Microbiology* **102**, 61–68.