Effect of Carotenoid Overproduction on Oxygen Tolerance of Nitrogen Fixation in *Azospirillum brasilense* Sp7

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Carotenoid-overproducing mutants of *Azospirillum brasilense* Sp7, which contained about 100 times more carotenoids than the wild-type, were obtained after nitrosoguanidine mutagenesis. Growth studies with one of these mutants in oxygen-controlled batch and continuous cultures revealed a slightly improved oxygen tolerance of nitrogen fixation in the mutant as compared to the wild-type. The production of carotenoids was greatly enhanced by increasing the oxygen concentration under nitrogen-deficient conditions. Although nitrogen fixation was severely inhibited by increased oxygen concentrations, in both the mutant and the wild-type, the mutant showed significantly greater efficiency of nitrogen fixation at 12 µM dissolved oxygen, and it fixed five times more total nitrogen than the wild-type under these conditions. In conclusion, high levels of carotenoids slightly enhanced the oxygen tolerance of *Azospirillum brasilense* under conditions of oxygen stress, but did not extend the optimum pO₂ for nitrogen fixation to higher oxygen concentrations.

INTRODUCTION

Azospirillum spp. are Gram-negative soil bacteria, which live in the rhizosphere of a variety of plants all over the world (Krieg & Döbereiner, 1984; Patriquin *et al.*, 1983). Nitrogen fixation by *Azospirillum* is strictly dependent on low oxygen concentrations (Day & Döbereiner, 1976; Okon *et al.*, 1977). In a nitrogen-free semisolid medium, *Azospirillum* grows as a subsurface pellicle; a pronounced aerotactic response helps the organism to cope with the oxygen problem (Barak *et al.*, 1982).

Although oxygen is essential for aerobic life, it can pose a threat to cells if they are not adequately protected against toxic oxygen metabolites (Stouthamer *et al.*, 1979; Halliwell & Gutteridge, 1984; Krieg & Hoffman, 1986). Toxic forms of oxygen (superoxide anion, 'O₂' and hydrogen peroxide) appear spontaneously or during metabolic activities. In nonenzymic reactions the very reactive radical 'OH and singlet oxygen 'O₂ are formed. These may support autooxidation of unsaturated lipids, enzymes and redox-labile substrates.

Azospirillum brasilense has rather high respiratory activity, and it adapts to different oxygen concentrations (Bergersen & Turner, 1980; Okon *et al.*, 1983) and carries enzymes that detoxify superoxide and hydrogen peroxide (Nur *et al.*, 1982; Moore *et al.*, 1984). However, unlike *Azotobacter* spp., *Azospirillum* does not possess an efficient respiratory and conformational protection of nitrogenase (Robson & Postgate, 1980; Yates & Jones, 1974; Nelson & Knowles, 1978; Hochman *et al.*, 1987). Because the oxygen sensitivity of nitrogen fixation is a major

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limiting factor for growth of *Azospirillum* under nitrogen-deficient conditions, the improvement of its oxygen tolerance is of considerable interest. Nur *et al.* (1981) suggested a possible role of carotenoids in the protection of nitrogen fixation against oxygen damage in *Azospirillum*: after inhibition of carotenoid biosynthesis by diphenylamine, nitrogen fixation was reduced by oxygen stress. Many *Azospirillum* strains exhibit a faint pink colour from carotenoid pigments, and a few are coloured deep red or yellow by carotenoids (Tarrand *et al.*, 1978; Nur *et al.*, 1981). Carotenoids counteract photo-oxidative damage (Krinsky, 1979) and they actively quench singlet oxygen and have chain-breaking ability in radical-mediated autoxidation reactions (Burton & Ingold, 1984); they also control lipid peroxidation at low oxygen concentrations (Burton & Ingold, 1984).

The purpose of the present study was to determine the contribution of high levels of carotenoids to the oxygen tolerance of nitrogen fixation in *A. brasilense*. In a preliminary account, qualitative evidence was presented for the improvement of oxygen tolerance of nitrogen fixation in carotenoid-overproducing mutants of the low-pigment wild-type strain Sp7 (Hartmann *et al.*, 1983). Here we report on the quantitative contribution of carotenoids to the oxygen tolerance of nitrogen fixation in oxygen-controlled batch and continuous-culture experiments.

METHODS

Organisms and conditions of mutagenesis. Wild-type strains of *Azospirillum brasilense*, Sp7 (ATCC 29145), which has a low carotenoid content, and the naturally red-pigmented Cd (ATCC 29710), were obtained from the American Type Culture Collection. Mutagenesis was performed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) according to Adelberg *et al.* (1965). A culture was pregrown in tryptone broth to the exponential growth phase, centrifuged and resuspended in Tris/maleate buffer to a cell density of 4×10^7 cells ml⁻¹. The cell suspension was incubated with 100 µg MNNG ml⁻¹ for 30 min at 30 °C. These conditions yielded about 20% of surviving bacteria. After removing the mutagen by three washings, the mutagenized cultures were grown overnight in tryptone broth for segregation of the mutations. About 10² bacteria were plated on tryptone broth plates and incubated at 30 °C at ambient oxygen concentration to screen for colour mutants.

Media and culture conditions. Batch cultures were grown in tryptone broth and minimal medium according to Albrecht & Okon (1980). Growth in liquid, nitrogen-free minimal medium batch cultures was performed as described recently (Hartmann & Burris, 1987). Dissolved oxygen concentrations were controlled by an oxystat. Continuous cultures were grown in a nitrogen-free synthetic malate (5 g potassium DL-malate l⁻¹) medium according to Hurek *et al.* (1987). Precultures were grown at 35 °C in this medium plus (per litre) 0.5 g NH₄Cl and 0.1 g yeast extract.

Dissolved-oxygen-controlled chemostat cultures. A 2-litre Biostat V fermenter equipped with temperature controller, pH controller and oxygen controller (Braun) was used. Temperature and pH were maintained constant at 35 ± 0.2 °C and at 7.0 ± 0.05 respectively. The culture volume was 1250 ml and cultures were stirred at a constant rate of 400 r.p.m. Dissolved oxygen concentration was measured with an autoclavable Clark-type oxygen electrode (Ingold) and maintained constant at the desired levels with a relative accuracy of about 10%. Maintenance of the desired equivalent partial pressures and the expression of the oxygen tension as actual oxygen concentration (µM) were done according to Hurek *et al.* (1987). The culture vessel was inoculated with 2 ml of a mid-exponential-phase preculture. Before the nutrient pump was started, batch growth was allowed to proceed at 4 µM dissolved O₂. Changes of culture conditions were followed by passage of 4 to 6 culture volumes through the vessel before measurements were made of a new steady state.

Extraction and characterization of carotenoids. Carotenoid pigments were extracted from the cells with acetone/methanol (7:2, v/v) according to Nur *et al.* (1981). Carotenoid concentrations were calculated from the absorbance at 500 nm of the acetone/methanol extract, using an absorption coefficient of 2500 for a 1% (w/v) solution (Britton & Goodwin, 1971). To determine individual pigments, the extracts were evaporated, redissolved in a small volume of chloroform, and chromatographed on silica gel thin-layer plates with methanol/chloroform (7:93, v/v) as solvent. After extraction from the plates with methanol the visible spectra of the pigments were recorded with a Beckman spectrophotometer, model 25.

Analyses. Bacterial dry weight was determined as outlined by Kloss *et al.* (1983). Cellular protein was determined by the micro-Goa method (Bergersen, 1980), with bovine serum albumin as a reference standard. Total nitrogen was determined by Kjeldahl digestion by a procedure given by Bergersen (1980), and ammonia was measured enzymically by a test combination (no. 542946; Boehringer). Nitrogen-fixation activity was also measured by the acetylene reduction method (Burris, 1974). Ammonia in culture supernatants was determined by the phenol/hypochlorite method of Fawcett & Scott (1960).

Table 1. Carotenoid content and nitrogen fixation activity under oxygen stress conditions of *A. brasilense* Sp7 and carotenoid-overproducing mutants

Strain	Carotenoid content* [$\mu\text{g (mg protein)}^{-1}$]	Nitrogenase activity† ($\text{nmol C}_2\text{H}_4 \text{ h}^{-1} \text{ per } 10^8 \text{ cells}$)
<i>A. brasilense</i> Sp7	0.01	0
Red-coloured mutant C1	0.97	134
Red-coloured mutant C2	0.78	81
Red-coloured mutant C3	1.04	124

* The carotenoid content of cells from cultures grown aerobically to stationary phase in malate/ammonium medium was estimated using the absorbance at 500 nm of the acetone/methanol extracts. Under these conditions the cell density was similar in wild-type and mutant cultures.

† Liquid nitrogen-free medium (2 ml) was inoculated with 2×10^6 cells ml^{-1} , using washed cells of an exponential-phase tryptone broth culture, and incubated under stagnant conditions at 30 °C for 16 h before acetylene was injected to assay nitrogenase activity. Under these conditions of oxygen stress, the wild-type reached a cell density of $5 \times 10^6 \text{ ml}^{-1}$ and the mutants of $1.0\text{--}1.25 \times 10^7 \text{ ml}^{-1}$. The experiment was repeated three times and values are means of four replicates.

RESULTS

Isolation and characterization of the mutants

After mutagenesis of *A. brasilense* Sp7 with MNNG, red-coloured mutants were obtained with a frequency of 10^{-1} to 10^{-3} . Red-pigmented colonies appeared after 3–5 d incubation at 30 °C under air. Mutant colonies with different degrees of red pigmentation occurred, but only the deep red mutants are listed in Table 1. In contrast to the wild-type, the mutants with a high level of carotenoid overproduction grew and fixed nitrogen under oxygen stress conditions, achieved according to Nur *et al.* (1981) (Table 1). Mutants with low or medium levels of carotenoids showed no consistent nitrogen fixation activity under oxygen stress (not shown). When the inoculum size was increased from 2×10^6 to 5×10^6 cells ml^{-1} the wild-type also could grow and reduce acetylene. To prove that the pigment-overproducing mutants were derived from Sp7, a streptomycin- and rifampicin-resistant mutant of Sp7 was mutagenized. All coloured mutants revealed the double resistance.

The pigments of the mutant C1 were characterized further. When chromatographed on silica gel thin-layer plates (solvent: methanol/chloroform, 7:93, v/v) the extracted pigments had R_F values of 0.25, 0.40 and 0.45, similar to the main pigments of *A. brasilense* Cd (Nur *et al.*, 1981). The pigments isolated from these bands gave carotenoid-like visible spectra; Fig. 1 compares the spectra of the pigment obtained from the spot of R_F 0.45 from mutant C1 (derived from the wild-type Sp7) with that of the wild-type Cd. A fiftyfold concentrated extract of Sp7 revealed a red pigment which cochromatographed on thin-layer plates with the main carotenoid pigment of the mutant.

Effect of oxygen stress on *A. brasilense* Sp7 and the mutant C1 in oxygen-controlled ammonium-limited batch cultures

To demonstrate improved oxygen tolerance due to carotenoid overproduction, the wild-type Sp7 and its carotenoid-overproducing mutant C1 were grown under nitrogen-fixing conditions at 4% air saturation (about $10 \mu\text{M}$ dissolved O_2); under these conditions derepression of nitrogen fixation was almost completely inhibited in *A. brasilense* Sp7 (A. Hartmann, unpublished results). During the rapid growth phase, when the initial low amount of ammonium was utilized, no difference in growth was observed between the strains (Fig. 2). Under nitrogen-deficient conditions (with continuing oxygen stress), however, the cells clumped and cellular protein decreased (Fig. 2). After a considerable period of adaptation, the mutant C1 recovered and resumed slow growth, giving a growth yield which exceeded that attainable from the limiting amount of ammonium. This oxygen-tolerant growth was preceded by a dramatic increase of carotenoids, which was evidently triggered by oxygen stress under conditions of nitrogen limitation. In the wild-type no measurable carotenoids were synthesized, and growth under

Fig. 1

Fig. 2

Fig. 1. Visible spectra of acetone/methanol extracts of wild-type *A. brasilense* Sp7 (A), its red-coloured mutant C1 (B) and the naturally red-coloured wild-type *A. brasilense* Cd (C). Cells from 2 d old, stationary cultures were extracted and the spectra of the main red pigments were recorded as described in Methods.

Fig. 2. Growth (as cellular protein; ●, ○) and carotenoid production (■, □), and ammonium concentration in the medium (▲, △), in ammonium-limited batch cultures of *A. brasilense* Sp7 wild-type (open symbols) and its carotenoid-overproducing mutant C1 (filled symbols) at 10 μM dissolved O_2 concentration (about 4% air saturation).

Table 2. Effect of oxygen on growth and nitrogen fixation of *A. brasilense* Sp7 and its mutant C1 grown on N_2 in a chemostat at a dilution rate of 0.08 h^{-1}

For each steady state, three subsamples were taken with intervals of one volume change. Analyses were done in duplicate. The experiments were done at least twice with very similar results.

Strain	Dissolved O_2 concn (μM)	Carotenoids [μg (mg cellular protein) $^{-1}$]	Malate in supernatant ($\mu\text{g ml}^{-1}$)	Cell dry weight ($\mu\text{g ml}^{-1}$)	Protein* (% of dry weight)	Total nitrogen fixed* ($\mu\text{g ml}^{-1}$)	Nitrogen-fixation efficiency [$\mu\text{g N}$ (mg malate) $^{-1}$]
Sp7	1.7	≤ 0.01	≤ 0.2	177	45 ± 4	12.5 ± 1.0	12.5
	7.6	≤ 0.01	≤ 0.2	213	27 ± 2	8.0 ± 0.6	8.0
	13.2	≤ 0.01	902	4.4	14 ± 1	0.08 ± 0.006	0.8
	16.0	≤ 0.01	959	4.0	8 ± 1	0.04 ± 0.004	0.9
Sp7C1	1.5	0.25	≤ 0.2	183	48 ± 4	13.2 ± 1.1	13.2
	8.9	0.54	≤ 0.2	206	26 ± 3	7.8 ± 0.6	7.8
	12.7	1.29	850	23	14 ± 1	0.43 ± 0.03	2.9
	17.0	3.36	912	9	4 ± 1	0.25 ± 0.02	2.8

* These results are means $\pm$ SEM.

oxygen stress was almost completely abolished. During the slow oxygen-tolerant growth of the mutant C1, only low acetylene reduction activities were measured (not shown). To evaluate the quantitative influence of high levels of carotenoids on nitrogen fixation activity during oxygen stress, N_2 -dependent continuous culture experiments and measurements of nitrogen fixation by direct means were performed.

Effect of oxygen on N_2 -dependent growth of *A. brasilense* Sp7 and its carotenoid-overproducing mutant C1 in continuous culture

The growth of both strains in the absence of combined nitrogen was followed at a constant dilution rate (generation time, 8.7 h) under various dissolved oxygen concentrations from about $2\text{ }\mu\text{M-O}_2$ to $17\text{ }\mu\text{M-O}_2$ (Table 2). Between 2 and $8\text{ }\mu\text{M-O}_2$ no malate ($\leq 0.2\text{ }\mu\text{g ml}^{-1}$) was found in the supernatants, indicating that these steady-state cultures were malate limited. At these

relatively low oxygen concentrations no differences were found between the strains in cell dry weight, malate in the supernatant, percentage protein of dry bacteria, and total nitrogen fixed (Table 2). Increasing the dissolved oxygen concentration above $8\ \mu\text{M-O}_2$ decreased nitrogen fixation, percentage protein of dry cells, efficiency of nitrogen fixation and bacterial dry weight of both strains (Table 2). In addition, residual malate in the supernatant was increased to a concentration that suggested that malate was no longer limiting. With these high oxygen concentrations, when malate was not limiting, the strains differed in their response to O_2 (Table 2). Cell dry weight and efficiency of nitrogen-fixation of the carotenoid-overproducing mutant C1 were significantly higher and the residual malate concentration was significantly lower than for the wild-type strain Sp7. This specific response of the mutant was accompanied by a marked increase in carotenoid synthesis. In contrast, Sp7 did not produce carotenoids in significant amounts (Table 2).

DISCUSSION

The purpose of this investigation was to evaluate the contribution of carotenoids to the oxygen tolerance of the extremely oxygen-sensitive nitrogen fixation of *A. brasilense*, by comparing the pale wild-type Sp7 with carotenoid-overproducing red mutants. We demonstrated a significant contribution of a high carotenoid content to the protection of nitrogen-limited *A. brasilense* against oxygen damage. It is quite possible that the carotenoids supported the recovery process from the oxygen shock, eventually enabling the bacteria to start nitrogen fixation. Under various conditions of mild oxygen stress (Table 1, Fig. 2) the carotenoid-overproducing mutants grew better than the wild-type. The efficiency of nitrogen fixation and the total amount of nitrogen fixed was significantly higher in the mutant C1 than in the wild-type under oxygen stress (Table 2). However, the presence of high levels of carotenoids did not alter the general pattern of growth inhibition in N_2 -fixing chemostat cultures at increased oxygen concentrations. Apparently carotenoids make no major contribution to the oxygen tolerance of the nitrogen-fixation system.

Carotenoid overproduction was also found in some mutants of *A. lipoferum* SpRG20a after Tn5 transposon mutagenesis (Abdel-Salam & Klingmüller, 1987). In some ordinary light pink strains of *A. brasilense*, deep red mutants occurred spontaneously (Tarrand *et al.*, 1978). Possibly the carotenoid-overproducing mutants are altered in a regulatory gene (or site) analogous to the *carS* gene in *Phycomyces* (Cerdeira-Olmedo & Torres-Martinez, 1979), whose mutation caused overproduction of carotenoids. The ability to synthesize carotenoids may be quite common in *Azospirillum* as a cryptic property left from a common ancestor. *Azospirillum* spp. are taxonomically closely related to the photosynthetic bacterium *Rhodospirillum rubrum* (Woese *et al.*, 1982; Reinhold *et al.*, 1987), which contains carotenoids that function in its photosynthetic apparatus and protect against photo-oxidation damage. Interestingly, carotenoid biosynthesis in *A. brasilense* was triggered under oxygen stress conditions (Table 2). Under nitrogen-limiting conditions (Fig. 2), the organism may also experience oxygen stress because of the concomitant decline in respiration.

Differences in oxygen tolerance of *Azospirillum* have been demonstrated at the species level (Hartmann *et al.*, 1985; Fu & Knowles, 1986) and the strain level (Hurek *et al.*, 1987), but there was no obvious correlation with pigmentation. Bothe *et al.* (1983) failed to demonstrate a contribution of carotenoids to oxygen protection, when they compared differently pigmented wild-type strains (*A. brasilense* Sp7 and SpCd). Differences in oxygen tolerance due to other protective activities may have overshadowed the small effect of carotenoids.

In a number of nitrogen-fixing bacteria, including *Azospirillum* (Yates & Jones, 1974; Hochmann & Burris, 1981; Goldberg *et al.*, 1987; Hartmann & Burris, 1987), the rapid and partially reversible inhibition of nitrogen fixation by oxygen may result from a shortage of electrons, because the limited supply has been diverted for respiration or production of poly- β -hydroxybutyrate, which accumulates under oxygen stress in *Azospirillum* (Hurek *et al.*, 1987). Therefore the inhibition of nitrogen fixation by increased oxygen concentrations may be caused primarily by a mechanism which does not involve the direct toxic effects of oxygen or oxygen

metabolites. An increased production of reducing equivalents, enabling cells to respire at enhanced rates and to fix nitrogen simultaneously, may be the key to improved oxygen tolerance in *Azospirillum*, as is the case in *Azotobacter* (Ramos & Robson, 1985). In concert with other protective mechanisms, carotenoids play only a limited role, but they may be of some ecological advantage to the poorly oxygen protected nitrogen-fixing *A. brasilense* in certain oxygen stress situations.

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