Carotenoid Composition and Function in Nitrogen-fixing Bacteria of the Genus *Azospirillum*

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Four different carotenoids were isolated and purified from membranes of the N_2 -fixing bacterium *Azospirillum brasilense* strain Cd, grown under aerobic conditions, and one from strain Cd-1. Carotenoid synthesis did not occur under microaerobic conditions. Cells of these strains formed aggregates (>0.1 mm in diameter) when subjected to aerobic conditions. Neither carotenoids nor cell aggregation were observed in *A. brasilense* strains Sp7, Sp81 and Sp51e. All of the differently pigmented *Azospirillum* strains tested contained similar amounts of soluble cytochrome *c*. In the presence of diphenylamine, which specifically inhibits carotenoid synthesis, the rate of acetylene reduction (N_2 fixation) in strain Cd decreased to 50% of the control. Carotenoid synthesis was inhibited in cells grown in the presence of NH_4Cl at concentrations higher than 10 mM. Carotenoid synthesis started in liquid cultures of strain Cd only after the concentration of NH_4Cl in the medium decreased, and N_2 fixation became evident at the same time. In comparison, strain Sp81 did not grow or fix nitrogen after NH_4Cl was depleted. Carotenoids appear to protect the nitrogenase of *A. brasilense* strains Cd and Cd-1 from oxidative damage.

INTRODUCTION

Colonies of different N_2 -fixing *Azospirillum* strains show pink, deep pink, red (Okon *et al.*, 1976; Eskew *et al.*, 1977; Tarrand *et al.*, 1978) or yellow (Nur *et al.*, 1980) pigmentation. This difference in pigmentation has been attributed to differences in the cytochrome content of cells (Tarrand *et al.*, 1978; Eskew *et al.*, 1977).

In liquid medium *Azospirillum* grows well under aerobic conditions in the presence of NH_4Cl . However, in semi-solid agar, both in the presence of NH_4^+ or while fixing N_2 , the organism forms a dense pellicle below the surface of the medium (Nur *et al.*, 1980) and behaves as a microaerophilic organism. These bacteria possess an oxygen-sensitive nitrogenase system (Okon *et al.*, 1977).

In this communication we describe carotenoid pigments produced by various strains of *Azospirillum* under aerobic, but not microaerobic, conditions and their possible role in growth and N_2 fixation.

METHODS

Organisms and growth conditions. The following *Azospirillum* strains were used: *A. brasilense* Sp7 (ATCC 29145); Sp51e and Sp81 from Brazil (Tarrand *et al.*, 1978); Cd (ATCC 29729); and Cd-1, an isolate from *Cynodon dactylon* from Rehovot (Nur *et al.*, 1980). These were grown at 30 °C on nutrient agar, potato dextrose agar or Trypticase soy agar, or in solid, semi-solid (0.05%, w/v, agar) or liquid synthetic malate medium (Okon *et al.*, 1977) either free of combined nitrogen or with 0.01 to 50 mM- NH_4Cl , KNO_3 or alanine.

For analytical purposes, the organisms were grown on a rotary shaker (New Brunswick) at 150 rev. min⁻¹ in 2 l Erlenmeyer flasks containing 1 l synthetic medium. Other experiments were carried out in 250 ml flasks with

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100 ml of liquid synthetic medium. Diphenylamine (10 to 100 μM), employed as an inhibitor of carotenoid synthesis (Liaaen-Jensen *et al.*, 1958), was added as a solution in 95% (v/v) ethanol; the final ethanol concentration never exceeded 0.10% (v/v) in the medium and this concentration did not affect growth.

Acetylene reduction was measured in 30 ml cultures grown in 110 ml bottles or in 5 ml cultures grown in 21 ml bottles; the synthetic medium was either semi-solid or liquid. A gas chromatograph with a flame ionization detector (Gow Mac, Madison, N.J., U.S.A.) was used to measure ethylene formed (Burris, 1974).

Liquid cultures (10^9 cells ml^{-1}) were harvested after 48 h by centrifugation at 7000 g for 15 min at 4 °C, the pellet was washed twice with 0.06 M-potassium phosphate buffer, pH 7.0, and the cell paste was stored at -20 °C.

Pigment extraction and separation. Cells were resuspended in 0.06 M-potassium phosphate buffer, pH 7.0, disrupted in an ice bath for 10 min at 1.5 A with an MSE ultrasonic disintegrator, and centrifuged at 20000 g for 30 min. To recover a membrane fraction (Cole & Rittenberg, 1971) the supernatant was centrifuged at 140000 g for 2 h.

The carotenoid pigment was extracted (under dim light) by shaking 1 g (wet wt) cells or 0.1 g membranes in 100 ml acetone/methanol (7:2, v/v). The extract was centrifuged at 12000 g for 15 min, and the supernatant was dried in a vacuum evaporator. The deep red product obtained was partitioned between methanol and chloroform (95:5, v/v) in a separating funnel. The chloroform layer was washed several times with water to remove contaminating methanol, dried over anhydrous Na_2SO_4 and concentrated to 1% of its original volume under a stream of O_2 -free N_2 . The conventional extraction procedure (Kushawaha *et al.*, 1975) involving the use of alcoholic KOH was avoided because of the possible destruction of carbonyl (Sommer & Kofler, 1966) and/or phenolic isoprenoids (Kofler *et al.*, 1962).

Thin-layer chromatography (t.l.c.) was carried out on 20 $\times$ 20 cm silica gel H (0.75 cm thick) with the following solvents: acetone/benzene (35:65, v/v), acetone/chloroform (50:50, v/v), or methanol/chloroform (7:93, v/v). The separated fractions were eluted in methanol/chloroform (1:1, v/v) and their spectra were determined in a Cary 14 double-beam spectrophotometer.

Carotenoid concentrations were calculated from the absorbance of the acetone/methanol extracts at 500 nm, measured in a Varian 635 spectrophotometer, using an absorption coefficient of 2500 for a 1% (w/v) solution (Stern *et al.*, 1964).

Soluble cytochrome *c* was quantitatively determined in the 20000 g supernatant by measuring its reduced minus oxidized absorbance at 551 nm (Dailey, 1976). Protein was determined by the Folin phenol method on cells hydrolysed by boiling for 10 min in 1 M-NaOH or on the cell-free extracts.

Estimation of cell aggregates. Cell aggregates were defined as that fraction of the culture which did not pass through Whatman no. 1 filter paper. Their dry weight was measured by filtering 10 ml of a 48 h liquid culture and drying at 80 °C for 48 h.

Chemicals. Diphenylamine was obtained from Sigma; nutrient agar, potato dextrose agar and Trypticase soy agar from Difco; and silica gel H from Merck. All other chemicals were of analytical grade.

RESULTS

Effect of oxygen on pigment production in Azospirillum strains

Pink, red or yellow pigmentation was observed after 72 h in rough colonies of *Azospirillum* (strains Sp7, Sp81, Sp51e, Cd and Cd-1) grown under aerobic conditions on nitrogen-free malate agar and after 1 week in colonies grown on nutrient agar, potato dextrose agar or Trypticase soy agar. No pigments were formed by any of the strains in semi-solid medium (microaerobic conditions) with or without NH_4Cl , or in liquid medium (aerobic) with NH_4Cl at 10 mM or greater. In vigorously aerated liquid cultures with 5 mM- NH_4Cl , red and yellow pigments were formed in strains Cd and Cd-1, respectively, but they did not appear in strains Sp7, Sp81 or Sp51e. The pink coloration of the latter may be due to their cytochrome content. Strains Cd and Cd-1 also tended to form cell aggregates greater than 0.1 mm in diameter.

Agar plates containing nitrogen-free medium and inoculated with different *Azospirillum* strains were incubated for 96 h under 1% O_2 . The colonies formed were colourless and smooth. Following transfer to a normal atmosphere, the pigments were formed within 48 h and the colonies became rough.

Quantitative analysis of soluble cytochrome c

Soluble cytochrome *c* of *Azospirillum* has an absorption spectrum with α , β and γ peaks at 551, 523 and 420 nm, respectively. Eskew *et al.* (1977) suggested that cytochrome *c* levels

Table 1. R_F values of the carotenoids isolated from *A. brasilense* strain Cd and chromatographed on silica gel H

Carotenoid (see Fig. 1)	Colour	Solvent system (v/v)		
		Methanol/chloroform (7:93)	Acetone/benzene (35:65)	Acetone/chloroform (50:50)
R ₁	Red	0.43	0.67	0.94
R ₂	Red	0.39	0.58	0.88
R ₃	Red	0.25	0.55	0.73
R ₄	Yellow-orange	0.19	0.50	0.61

may influence the colour of colonies of the different *Azospirillum* strains. In strains Cd, Cd-1 and Sp7 the contents of cytochrome *c* were similar [0.017 to 0.021 ± 0.003 reduced minus oxidized absorbance units (mg protein)⁻¹] although the levels of coloration were different.

Characterization and localization of pigments

When *A. brasilense* strain Cd was grown aerobically in liquid malate medium with 5 mM-NH₄Cl, the organism formed aggregates that became red after 48 h. Following extraction of the pigments, four different spots were detected on t.l.c. plates (Table 1). The R_F values of the spots obtained by using different solvent mixtures present evidence of the polarity of the carotenoids. All fractions showed absorption spectra in chloroform typical of carotenoids (Fig. 1). Fractions R₁, R₂ and R₃ were red, whereas R₄ was pale yellow. The carotenoid pigment from *A. brasilense* Cd-1 extracted by the same method was yellow and showed absorption maxima at 462 and 437 nm and a shoulder at 485 to 495 nm (not shown). No carotenoids were recovered from *A. brasilense* Sp7, Sp81 or Sp51e cultures grown aerobically on liquid or solid media even when their colonies were pale or intense pink.

Carotenoids with the same spectra as those extracted from whole cells were extracted from membrane preparations of strains Cd and Cd-1 but were not detected in membranes of strains Sp7, Sp81 or Sp51e. No carotenoids were detected in other fractions of the disrupted cells.

Effect of ammonium concentration on formation of carotenoids and aggregates by *A. brasilense* strain Cd

Cultures were grown under vigorous aeration with NH₄Cl (0 to 50 mM), harvested after 48 h, and the total protein and carotenoid contents and formation of cell aggregates were determined (Fig. 2). The protein content of the cultures reached a maximum when the organism was grown in 12 mM-NH₄Cl. Carotenoid production and aggregate formation were highest in cultures grown with 5 mM-NH₄Cl and were not observed in cultures supplied with NH₄Cl above 10 mM. Similar protein (approximately 100 µg ml⁻¹) and carotenoid (approximately 0.1 µg ml⁻¹) concentrations were produced after 48 h in cultures with other nitrogen sources such as 5 mM-KNO₃ or alanine.

Effect of diphenylamine on carotenoid formation in *A. brasilense* strain Cd

Addition of diphenylamine (final concentration 10 to 100 µM) to cultures growing under aerobic conditions in liquid malate medium with 5 mM-NH₄Cl inhibited carotenoid production in proportion to diphenylamine concentration, but only affected the total protein content of the cells slightly, even at the highest concentration tested (Fig. 3).

In liquid medium free of combined nitrogen, cultures of *A. brasilense*, either static or shaken, did not grow or reduce acetylene, apparently because without a small amount of agar to minimize mixing of O₂ into the medium, the small inoculum could not reduce the dissolved O₂ to a level permitting N₂ fixation and thus growth.

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Absorption spectra of the purified carotenoids (R_1 , R_2 , R_3 and R_4) from *A. brasilense* strain Cd.

Fig. 2. Effect of NH_4Cl concentration on carotenoid content (O), protein synthesis (▲) and on the formation of cell aggregates (Δ) in *A. brasilense* strain Cd after 48 h growth under aerobic conditions in a shaker.

Fig. 3. Effect of increasing concentrations of diphenylamine in the growth medium of *A. brasilense* strain Cd on protein (▲) and carotenoid (O) content, measured after 48 h growth under aerobic conditions in the presence of 5 mM- NH_4Cl .

Strains Cd and Sp81 grown in static culture in liquid malate medium with 0.5 mM- NH_4Cl and with or without 25 μM -diphenylamine produced similar biomass during the first 48 h by growth at the expense of NH_4Cl ; growth then slowed down (Fig. 4c). Cultures of strain Cd grew near the surface but strain Sp81 developed only at the bottom of the culture. After 48 h, acetylene reduction was detected in cultures of strain Cd in the absence of diphenylamine (Fig. 4b), protein content continued to increase (Fig. 4c) and *de novo* synthesis of carotenoids was observed (Fig. 4a). Cultures of strain Cd with diphenylamine showed lower acetylene reduction (about 50%) (Fig. 4b), lower growth rates (Fig. 4c) and carotenoids were not formed (Fig. 4a). However, cell aggregates were observed whether or not diphenylamine was present. Cultures of strain Sp81 (with or without diphenylamine), that reached a similar biomass to Cd during the first 48 h incubation, subsequently showed very low acetylene

Fig. 4. Carotenoid synthesis (a), acetylene reduction (b) and protein synthesis (c) in static liquid cultures (0.5 mM-NH₄Cl) of *A. brasilense* strains Cd (△, ▲) and Sp81 (○, ●) in the presence (△, ○) or absence (▲, ●) of diphenylamine (25 µM).

reduction rates (Fig. 4b), and during the next 3 d incubation there was no significant increase in their protein content (Fig. 4c).

DISCUSSION

The four different red or yellow pigments isolated and purified from the membrane fraction of *Azospirillum brasilense* strain Cd were identified as carotenoids according to their solubility in organic solvents and their optical absorption spectra. In comparison with the published absorption spectra of purified carotenoids from *Halobacterium cutirubrum* (Kushawaha *et al.*, 1975) and bacterial carotenoids studied by Arpin *et al.* (1972), the carotenoids of *A. brasilense* resemble those of the bacterioruberin group.

In producing carotenoids only under aerobic conditions *A. brasilense* resembles *Rhodospseudomonas capsulata* (Weaver *et al.*, 1975) and the mycelium of *Fusarium aqueductum* (Rau, 1969) and *Neurospora crassa* (Theimer & Rau, 1970). Carotenoids have been shown to act as protecting agents against oxidative damage in micro-organisms, owing to their ability to quench singlet oxygen and possibly oxygen radicals (Krinsky, 1979).

The decreased rates of N₂ fixation following inhibition of carotenoid synthesis by diphenylamine, the production of carotenoids by *A. brasilense* strain Cd only under aerobic conditions, and the known role of carotenoids in protecting cells from oxidative damage (Krinsky, 1979), suggest that the carotenoids of *A. brasilense* may play a role in protection of the sensitive nitrogenase system from O₂. In agreement with this suggestion, we have shown that in *A. brasilense* strain Sp81, which lacks carotenoids, growth and N₂ fixation were inhibited under aerobic conditions in medium free of combined nitrogen. Additionally, in the presence of NH₄Cl, when there is no need to protect the repressed nitrogenase system, carotenogenesis was repressed in *A. brasilense* strains Cd and Cd-1. Moreover, decreasing the NH₄Cl concentration in the medium to a low level led to the derepression of nitrogenase with concomitant carotenoid synthesis.

In addition to carotenogenesis, cell aggregates were formed under aerobic conditions; these seem to provide a microaerophilic environment for the N_2 -fixing system. Such aggregates were formed neither under microaerobic conditions nor under aerobic conditions in the presence of high concentrations (above 10 mM) of NH_4Cl .

Finally, cytochrome *c* was present at similar concentrations in the highly pigmented strains and in the pale and colourless strains. Therefore cytochrome *c* content cannot explain the pigmentation of *A. brasilense* strain Cd as proposed previously (Eskew *et al.*, 1977).

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