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 N₂-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 (N₂ fixation) in strain Cd decreased to 50% of the control. Carotenoid synthesis was inhibited in cells grown in the presence of NH₄Cl at concentrations higher than 10 mM. Carotenoid synthesis started in liquid cultures of strain Cd only after the concentration of NH₄Cl in the medium decreased, and N₂ fixation became evident at the same time. In comparison, strain Sp81 did not grow or fix nitrogen after NH₄Cl was depleted. Carotenoids appear to protect the nitrogenase of A. brasilense strains Cd and Cd-1 from oxidative damage.

INTRODUCTION

Colonies of different N₂-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 NH₄Cl. However, in semi-solid agar, both in the presence of NH₄⁺ or while fixing N₂, 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 N₂ 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 isolates 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-NH₄Cl, KNO₃ 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 (10⁶ 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 Na₂SO₄ and concentrated to 1 ml.

Estimation of cell aggregates. Cell aggregates were defined as that fraction of the culture which did not pass through a membrane fraction (Cole & Rittenberg, 1971) suggested that cytochrome c levels

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 from Difco; and silica gel H from Merck. All other chemicals were of analytical grade.

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
Carotenoids of *Azospirillum*

Table 1. *R*<sub>f</sub> values of the carotenoids isolated from *A. brasilense* strain Cd and chromatographed on silica gel H

<table>
<thead>
<tr>
<th>Carotenoid (see Fig. 1)</th>
<th>Colour</th>
<th>Solvent system (v/v)</th>
<th>Carotenoid Methanol/chloroform</th>
<th>Acetone/benzene</th>
<th>Acetone/chloroform</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Methanol/chloroform (7:93)</td>
<td>Acetone/benzene (35:65)</td>
<td>Acetone/chloroform (50:50)</td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Red</td>
<td>0.43</td>
<td>0.67</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Red</td>
<td>0.39</td>
<td>0.58</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Red</td>
<td>0.25</td>
<td>0.55</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>R&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Yellow-orange</td>
<td>0.19</td>
<td>0.50</td>
<td>0.61</td>
<td></td>
</tr>
</tbody>
</table>

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)<sup>-1</sup>] 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-NH<sub>4</sub>Cl, 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*<sub>f</sub> 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 R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> were red, whereas R<sub>4</sub> 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 NH<sub>4</sub>Cl (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-NH<sub>4</sub>Cl. Carotenoid production and aggregate formation were highest in cultures grown with 5 mM-NH<sub>4</sub>Cl and were not observed in cultures supplied with NH<sub>4</sub>Cl above 10 mM. Similar protein (approximately 100 μg ml<sup>-1</sup>) and carotenoid (approximately 0.1 μg ml<sup>-1</sup>) concentrations were produced after 48 h in cultures with other nitrogen sources such as 5 mM-KNO<sub>3</sub> 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-NH<sub>4</sub>Cl 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 O<sub>2</sub> into the medium, the small inoculum could not reduce the dissolved O<sub>2</sub> to a level permitting N<sub>2</sub> fixation and thus growth.
Fig. 1. Absorption spectra of the purified carotenoids (R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub>) from A. brasilense strain Cd.

Fig. 2. Effect of NH<sub>4</sub>Cl concentration on carotenoid content (O), protein synthesis (▲) and on the formation of cell aggregates (▲) 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<sub>4</sub>Cl.

Strains Cd and Sp81 grown in static culture in liquid malate medium with 0·5 mM NH<sub>4</sub>Cl and with or without 25 μM diphenylamine produced similar biomass during the first 48 h by growth at the expense of NH<sub>4</sub>Cl; 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
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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 Rhodopseudomonas 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 N₂-fixing system. Such aggregates were formed neither under microaerobic conditions nor under aerobic conditions in the presence of high concentrations (above 10 mM) of NH₄Cl.

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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**REFERENCES**


