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Identification of the Carotenoid Pigment Canthaxanthin from Photosynthetic *Bradyrhizobium* Strains

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Canthaxanthin (4,4′-diketo-β-carotene) is produced as the major carotenoid pigment by orange- and dark-pink-pigmented bacteriochlorophyll-containing *Bradyrhizobium* strains isolated from stem nodules of *Aeschynomene* species. These two new pigmentation groups differ from the well-studied strain BTAi1, which accumulates spirilloxanthin as the sole carotenoid.

Bacteria able to form nitrogen-fixing nodules on the stems of aquatic legumes of the genus *Aeschynomene* are unique among all other rhizobia because of their ability to produce photosynthetic pigments, including both bacteriochlorophyll *a* (Bchl *a*) and carotenoids (11, 13). These carotenoids are known to help in harvesting light energy and transferring it to Bchl and act as quenchers of oxygen radicals (1). We have isolated, from stem nodules of different tropical *Aeschynomene* species growing in Sénégal, a large number of photosynthetic *Bradyrhizobium* strains (13). In culture, most of these strains showed the same light pink coloration as strain BTAi1, the first Bchl-containing rhizobium described (4), whereas several strains produced orange or dark pink pigmentation. Carotenoid pigments have already been identified in most purple photosynthetic and marine bacteria (8, 10, 19) but not in symbiotic photosynthetic bradyrhizobia. The aim of this study was thus to identify the main carotenoid pigments of these orange and dark-pink *Bradyrhizobium* strains.

Photosynthetic *Bradyrhizobium* strains were subcultured as described previously (13). For purification of the pigments, cells were pelleted by centrifugation and extracted with cold acetone-methanol (7:2, vol/vol) (5). The absorption spectrum of the extract was then recorded between 300 and 800 nm, and the total cellular carotenoid content was determined (12). Carotenoids in the acetone-methanol extract were partitioned into an equal volume of hexane after addition of aqueous 10% NaCl. The hexane phase was reduced by vacuum distillation and applied to the top of an alumina N column. The retained carotenoids were eluted with diethyl ether, which was evaporated to dryness. The residue was reconstituted with either high-performance liquid chromatography (HPLC) solvent (see Fig. 2 and Table 2) or hexane for thin-layer chromatography (TLC) separation. TLC was performed on high-performance thin-layer chromatography silica gel plates (from Merck) with dichloromethane-ethyl acetate (95:5, vol/vol) as the eluent. Carotenoids were scraped off and extracted with methanol. The HPLC system consisted of a Philips PU4100 liquid chromatograph, fitted with a 20-μl loop, and an SP4190 integrator (Spectra-Physics, San Jose, Calif.). For carotenoid isomer identification and spirilloxanthin contents, a normal-phase system was used (see Table 2 for conditions). Fast-atom bombardment mass spectra of the pigments were recorded on an Autospec instrument (VG Analytical). The matrix was a mixture (1:1, vol/vol) of *m*-nitrobenzyl alcohol–glycerol, spiked with 1% trichloroacetic acid in water.

The three pigmentation groups. Photosynthetic *Bradyrhizobium* strains were placed into one of three groups on the basis of their culture pigmentation after growth under light-

TABLE 1. Representative photosynthetic stem-nodulating strains and their main pigment contents

Bacterial strain	Host plant	Strain color[a]	No. of carotenoids	Main carotenoid(s)	Cellular level of Bchl *a* (nmol/mg of dry cell wt)[b]	Carotenoid level (ng/mg of dry cell wt)[b]
BTAi1	*A. indica*[c]	LP	1	Spirilloxanthin	0.67	280
ORS292	*A. sensitiva*	LP	1	Spirilloxanthin	0.28	194
ORS294	*A. sensitiva*	LP	1	Spirilloxanthin	0.27	170
ORS278	*A. sensitiva*	O	7	Canthaxanthin, spirilloxanthin	0.13	1,594
ORS279	*A. sensitiva*	O	7	Canthaxanthin, spirilloxanthin	0.11	274
ORS282	*A. indica*	O	7	Canthaxanthin, spirilloxanthin	0.28	862
ORS296	*A. sensitiva*	DP	7	Canthaxanthin, spirilloxanthin	0.13	198
ORS344	*A. indica*	DP	7	Canthaxanthin, spirilloxanthin	0.06	184
ORS371	*A. indica*	DP	7	Canthaxanthin, spirilloxanthin	0.11	370

[a] LP, light pink; DP, dark pink; O, orange.
[b] Data are averages from three determinations. Bchl *a* concentrations were calculated with 76 $mM^{-1} \cdot cm^{-1}$ as the absorption coefficient (5).
[c] *Aeschynomene* is the genus.

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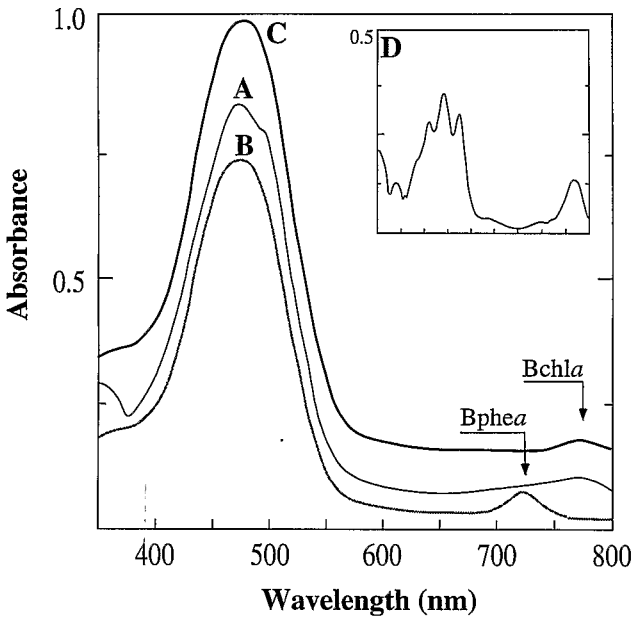

FIG. 1. Absorption spectra of an acetone-methanol extract from stem-nodulating photosynthetic rhizobia. (A and B) Extract from DP strain ORS371 before (A) and after (B) addition of one drop of 4 N HCl, resulting in the characteristic replacement of the band of Bchl *a* (770 nm) with the band of bacteriopheophytin *a* (Bphe *a*) at 745 nm. (C) Extract from O strain ORS278. (D) Extract from LP strain ORS292; spectrum identical to that of strain BTAi1 (5). Each extract corresponds to 50 ml of culture, except for strain ORS278, for which 25 ml was used.

dark cycles: the light-pink strains (LP) indistinguishable from strain BTAi1, the dark-pink strains (DP), and the orange strains (O) (Table 1). Strains of all three groups synthesized carotenoids (around 400 to 550 nm) and Bchl *a* with a main absorption band at 770 nm (Fig. 1). The DP and O groups (Fig. 1A and C, respectively) showed a single broad, high carotenoid peak (476 nm), different from the classical three-peaked spectrum (460, 490, and 525 nm) (Fig. 1D) of the BTAi1 group (5).

Spirilloxanthin is the only carotenoid produced by strain BTAi1 and LP strains. TLC analysis showed that strain BTAi1 and related strains produced only one pink compound (R_f, 0.85). Absorption maxima of this single compound suggest the presence of 13 conjugated double bonds (Table 2 [see spirilloxanthin, peak 5]). The proposed structure of spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetrahydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene) for this compound is consistent with mass spectra, UV-visible light data, and the partition coefficient (6) (Table 2). Natural spirilloxanthin purified from a *Rhodospirillum rubrum* strain (7) and carotenoid purified from BTAi1 cochromatographed as a single spot in silica gel TLC, thus confirming the structure of spirilloxanthin. In members of the families *Rhodospirillaceae* and *Chromatiaceae*, spirilloxanthin is commonly produced as the major carotenoid, most often under anaerobic conditions, and is known to be bound to the light-harvesting protein-associated complex (7–9, 19).

Canthaxanthin is the major carotenoid pigment produced by DP and O strains. Strains belonging to the DP and O groups exhibited identical TLC profiles and contained seven carotenoids, two of which (R_f, 0.48 and 0.85) were most colored. Characterization by HPLC showed that the major pigment isolated from these strains was canthaxanthin (4,4′-diketo-β-carotene) (R_f of 0.48 in TLC, peak 2 in HPLC; Table 2 and Fig. 2, respectively). Reduction of synthetic canthaxanthin and of the purified compound with $NaBH_4$ led to the formation of the same carotenoid (λ_{max}, 452 and 478 nm) of the predicted reaction product isozeaxanthine (4,4′-dihydroxy-β-carotene) (2, 6). Coelution of spirilloxanthin purified from strain BTAi1 showed that peak 5 corresponds to the structure

TABLE 2. Spectroscopic and polar properties of carotenoids purified from the photosynthetic rhizobial strain ORS278 (orange strain) and their relative abundances in the cell

Compound[a]	Peak[b]	Functional group(s)	Spectroscopic properties (λ_{max} [nm] in methanol)[c]	R_f[d]	Partition coefficient[e]	% of total pigment[f]
Compound 1	1	1 C=O	358, 384, (445), 473, (504)	0.65	85:15	4
Compound 1 (reduced)		1 OH	369, 444, 468, 494			
trans-Canthaxanthin	2	2 C=O	476	0.48	50:50	68
trans-Canthaxanthin (authentic)		2 C=O	476	0.48	48:52	
trans-Canthaxanthin (reduced)		2 OH	(428), 452, 478		22:78	
9-*cis*-Canthaxanthin[g]	3	2 C=O	365 ff 465	0.37	44:56	9.8
13-*cis*-Canthaxanthin[g]	4	2 C=O	365, 463	0.26	43:56	7.8
Spirilloxanthin	5	2 OCH_3	364, 384, 461, 488, 521	0.85	90:10	5.8
Spirilloxanthin (authentic)		2 OCH_3	364, 384, 462, 488, 521	0.85	90:10	
Echinenone	6	1 C=O	463	0.90	100:0	3.6
Echinenone (authentic)		1 C=O	463	0.90	100:0	
Echinenone (reduced)		1 OH	(425), 452, 476		85:15	
Unidentified	7			0.93		1

[a] Compound 1 (see text), canthaxanthin (4,4′-diketo-β-carotene), canthaxanthin reduced (or isozeaxanthin, 4,4′-dihydroxy-β-carotene), spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene), echinenone (4-keto-β-carotene), echinenone reduced (or isocryptoxanthin, 4-hydroxy-β-carotene). Isozeaxanthin and isocryptoxanthin were prepared from canthaxanthin and echinenone, respectively, after reduction with $NaBH_4$ (2).

[b] See Fig. 2.

[c] Wavelengths in parentheses indicate a shoulder rather than a distinct peak. ff, absorbance very weak.

[d] TLC solvent was dichloromethane-ethyl acetate (95:5, vol/vol).

[e] See reference 16. Values are very similar to the collected values of Foppen (6).

[f] The percentage of each pigment was determined by reverse-phase HPLC.

[g] Identification was made by using a normal-phase HPLC system: column, 5 μm Lichrosorb Si60 (100 by 4.5 mm, Merck); eluent, dichloromethane-ethyl acetate (95:5, vol/vol); flow rate, 0.8 ml/min; detection, 470 nm. Peaks were compared to geometric canthaxanthin isomers prepared as previously described (14).

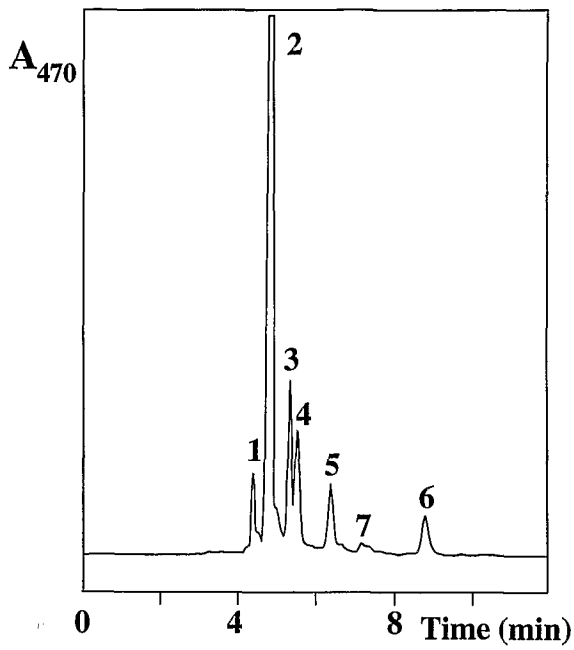

FIG. 2. Reverse-phase liquid chromatography of an extract of strain ORS278. Column: 5 μm Hypersil C_{18} (250 by 4.6 mm, Alltech). Eluent: acetonitrile-methanol-dichloromethane (40:50:10, vol/vol/vol). Flow rate: 0.8 ml/min. Detection: 470 nm. Peak identities: peak 1, see Table 2; peak 2, all-*trans*-canthaxanthin; peaks 3 and 4, *cis*-canthaxanthin isomers; peak 5, spirilloxanthin; peak 6, echinenone; peak 7, unidentified.

of spirilloxanthin. Peaks 3 and 4 (Fig. 2 and Table 2) exhibited symmetrical spectra and had the same partition coefficient as that of canthaxanthin but a λ_{max} 12 nm lower. On the basis of the presence of a characteristic "*cis*-peak" at 365 nm for both compounds, the structure of *cis*-canthaxanthin was assigned to peaks 3 and 4. To determine the structure of these isomers, the strain ORS278 cell extract was injected on a normal-phase system (Table 2). Comparison of the chromatogram with that of canthaxanthin stereomutation mixtures (14) permitted assignment of the tentative structures of 9-*cis*- and 13-*cis*-canthaxanthin to peaks 3 and 4, respectively. Other carotenoids were detected as minor compounds. Peak 6 was found to be the common mono-keto precursor of canthaxanthin synthesis, echinenone. Compound 1 was found to contain 12 conjugated double bonds and one carbonyl group (Table 2). Fast-atom bombardment mass spectrometric analysis revealed an even-numbered mass of 888 for this compound. It is thought to be a monocyclic keto-carotenoid, one part of which is substituted with a group of high mass, which was not identified.

The orange strain ORS278 showed the highest concentration of canthaxanthin, which represents 85% (all *trans* plus *cis*) of the total carotenoid complement, as in the other representative DP and O strains (Tables 1 and 2). Analysis by HPLC of total canthaxanthin yielded a value of 1.34 mg/g of dry cell weight (0.78 mg/liter) for strain ORS278 and 0.26 mg/g of dry cell weight (0.16 mg/liter) for strain ORS371. The amount of spirilloxanthin was 0.146 mg/g of dry cell weight (0.085 mg/liter) for strain ORS278 and 0.085 mg/g of dry cell weight (0.017 mg/liter) for strain ORS371. Strains ORS278 and ORS371 showed different ratios of canthaxanthin (Cx) versus spirilloxanthin (Sp) content (Cx/Cx + Sp), 90% and 75%, respectively, which could explain the difference in pigmentation between O and DP strains.

Canthaxanthin is a carotenoid pigment of great economic value, but its levels in strain ORS278 remain insufficient to make this organism a realistic candidate for the production of "natural" canthaxanthin (15). Canthaxanthin had not been previously identified in either photosynthetic bradyrhizobia or the closely related photosynthetic non-sulfur bacteria (20). In other photosynthetic organisms, canthaxanthin had been found only in cyanobacteria and in green algae as a minor pigment (3, 9). In other aerobic photosynthetic bacteria, the major forms of carotenoids in the cells are not bound to the Bchl-protein complex (17, 18, 21). Similarly, in orange and dark-pink *Bradyrhizobium* strains, canthaxanthin might not function as a light-harvesting pigment. It could have a role in photoprotection against damage caused by a combination of light and oxygen for better adaptation to the aerobic conditions of stem nodulation.

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