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The major carotenoid in all known species of heliobacteria is the C_{30} carotenoid 4,4′-diaponeurosporene, not neurosporene

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Abstract The carotenoids of five species of heliobacteria (*Heliobacillus mobilis*, *Heliophilum fasciatum*, *Heliobacterium chlorum*, *Heliobacterium modesticaldum*, and *Heliobacterium gestii*) were examined by spectroscopic methods, and the C_{30} carotene 4,4′-diaponeurosporene was found to be the dominant pigment; heliobacteria were previously thought to contain the C_{40} carotenoid neurosporene. In addition, trace amounts of the C_{30} diapocarotenes diapolycopene, diapo-ζ-carotene, diapophytofluene, and diapophytoene were also found. Up to now, diapocarotenes have been found in only three species of chemoorganotrophic bacteria, but not in phototropic organisms. Furthermore, the esterifying alcohol of bacteriochlorophyll *g* from all known species of heliobacteria was determined to be farnesol (C_{15}) instead of the usual phytol (C_{20}). Heliobacteria may be unable to produce geranylgeranyol (C_{20}).

Key words Carotenoids · Diapocarotenes · 4,4′-Diaponeurosporene · Bacteriochlorophyll *g* · Heliobacteria · *Heliobacillus mobilis* · *Heliophilum fasciatum* · *Heliobacterium chlorum* · *Heliobacterium modesticaldum* · *Heliobacterium gestii*

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Abbreviation *BChl* Bacteriochlorophyll

Introduction

Heliobacteria are strictly anaerobic, anoxygenic, photosynthetic bacteria that contain bacterioclorophyll (BChl) *g* as their major chlorophyll pigment. Their photosynthetic system resembles that of photosystem I of green plants. Neither chlorosomes (typical of the green sulfur and the green filamentous bacteria) nor differentiated internal membranes (typical of the purple bacteria) are found in the heliobacteria (Madigan and Ormerod 1995).

The absorption spectrum of the first species of heliobacterium to be discovered (*Heliobacterium chlorum*) indicates that a neurosporene like carotenoid is present (Gest and Favinger 1983). This pigment was subsequently identified as neurosporene based on its absorption spectrum, which is identical to that of authentic neurosporene, and on a result of cochromatography of the two pigments on silica gel TLC (Van Dorssen et al. 1985). The same conclusion was obtained for the major carotenoid in *Heliobacillus mobilis* (Trost and Blankenship 1989). Following this, the major carotenoid in newly discovered heliobacteria such as *Heliobacterium modesticaldum* (Kimble et al. 1995), *Heliobacterium gestii*, and *Heliophilum fasciatum* (Ormerod et al. 1996) was assumed to be neurosporene based on absorption properties similar to those of *Hbt. chlorum* and *Hba. mobilis*.

In this study, the carotenoids of three genera (including five species) of heliobacteria were re-examined. In contrast to previous reports, the major carotenoid was found to be the C_{30} carotene 4,4′-diaponeurosporene. Minor amounts of other C_{30} diapocarotenes were also found.

Materials and methods

Five species of heliobacteria were used: *Hba. mobilis* (ATCC 43427^T; Beer-Romero and Gest 1987), *Hbt. chlorum* (ATCC 35205^T; Gest and Favinger 1983), *Hbt. modesticaldum* (ATCC 51547^T; Kimble et al. 1995), and *Hbt. gestii* and *Hph. fasciatum*

(ATCC 43375^T and ATCC 51790^T, respectively; Ormerod et al. 1996). They were grown phototrophically (anaerobic and light) as described in each reference.

Pigments were extracted twice from wet cells with a mixture of acetone and methanol (7:2, v/v), the two extracts were pooled, and the solvent was evaporated. The pigment extract was analyzed by HPLC equipped with a μBondapak C_{18} column (8 × 100 mm; Waters, USA) and eluted with methanol (2.0 ml/min) as previously described (Takaichi and Ishidsu 1992). For separation, the pigment extract was subjected to silica gel 60 (Merck, Germany) column chromatography, and carotenes were eluted with *n*-hexane while BChl *g* remained on the column. The carotene fraction was analyzed by HPLC equipped with a Novapak C_{18} column (8 × 100 mm; Waters) eluted with a mixture of acetonitrile, methanol, and tetrahydrofuran (58:35:7, by vol.; 2.0 ml/min). Finally, each carotene in the carotene fraction was purified from silica gel 60 HP-TLC (Merck, Germany) developed with a mixture of *n*-hexane and benzene (17:3, v/v).

Authentic neurosporene was obtained from cells of *Rhodospirillum rubrum* S1 cultured in a medium containing diphenylamine, which inhibits desaturation of phytoene. Other standard carotenes listed in Table 1 were obtained from the same *Rsp. rubrum* S1 or from carrot root (Takaichi et al. 1990).

Absorption spectra were recorded with a photodiode array detector (230–800 nm; MCPD-350; Otsuka Electronics, Japan) attached to the HPLC apparatus (Takaichi and Shimada 1992). The molecular masses were determined by field-desorption mass spectrometry using a double-focusing gas chromatograph/mass spectrometer equipped with a field-desorption apparatus (M-2500; Hitachi, Japan) (Takaichi 1993).

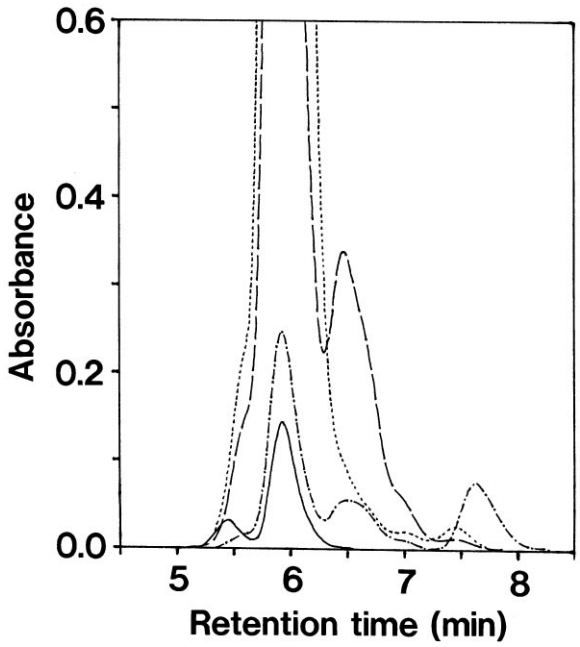

Fig. 1 Novapak C_{18} HPLC elution profile of the carotene fraction from *Heliobacillus mobilis*. Absorbance at 501 nm (———), 439 nm (----), 401 nm, (— —), and 349 nm (— - —) is shown. The observed absorbance at 439 nm was ca. 4.5

Results

Identification of photosynthetic pigments in *Hba. mobilis*

When pigments extracted from cells of *Hba. mobilis* were analyzed by μBondapak C_{18} HPLC, two major pigments were found. One component was identified as BChl *g* (containing a farnesol ester; Kobayashi et al. 1991) by its absorption spectrum (data not shown). The other component showed an absorption spectrum similar to that of neurosporene [7,8-dihydro-ψ,-ψ-carotene], but its retention time of 6.1 min was much shorter than that of authentic neurosporene (13.5 min). Next, the carotene fraction, which was separated by silica gel column chromatography, was analyzed by Novapak C_{18} HPLC. One major and several minor peaks were found (Fig. 1). The content of the major component was more than 95% of total carotenoids. For identification, each carotene was purified by silica gel TLC and finally by Novapak C_{18} HPLC.

The major carotene present showed absorption maxima at 416, 439, and 468 nm, and its spectral fine structure of %III/II [the ratio of the peak height of the longest wavelength absorption band from the minimum between the two peaks to that of the middle absorption band from the minimum; see Takaichi and Shimada (1992)] was 95 in the eluent (Table 1). A low value of $\%D_B/D_{II}$ [the ratio of the *cis*-peak height to the middle-peak height; see Takaichi and Shimada (1992)] indicated the high purity of this carotenoid. The absorption maxima and the value of %III/II indicate that this pigment is an acyclic carotenoid with nine conjugated double bonds and that its absorption spectrum is similar to that of neurosporene (Takaichi and Shimada 1992). The mol. mass of this pigment was *m/z* = 402 (Table 1). These results indicate that this carotenoid is a C_{30} acyclic carotene with nine conjugated double bonds and one nonconjugated double bond. Consequently, this major carotene can be identified as 4,4′-diaponeurosporene [7,8-dihydro-4,4′-diapocarotene] (Fig. 2; Straub 1987).

A minor carotenoid species detected from absorbance at 501 nm and eluted at 5.4 min, just before diaponeurosporene (Fig. 1), is similarly identified as 4,4′-diapolycopene [4,4′-diapocarotene] (Fig. 2) based on the absorption spectrum and the mol. mass (Table 1). An additional minor component was detected from absorbance at 401 nm and eluted just after diaponeurosporene. The absorption spectrum of this pigment was the same as that of ζ-carotene [7,8,7′,8′-tetrahydro-ψ,ψ-carotene] and was distinct from asymmetrical ζ-carotene [7,8,11,12-tetrahydro-ψ,ψ-carotene]. Therefore, this pigment is identified as 4,4′-diapo-ζ-carotene [7,8,7′,8′-tetrahydro-4,4′-diapocarotene]. Further, one minor carotenoid detected from absorbance at 349 nm is 4,4′-diapophytofluene [7,8,11,12,7′,8′-hexahydro-4,4′-diapocarotene], and a next minor component eluting at 10 min (not shown in Fig. 1) is 4,4′-diapophytoene [7,8,11,12,7′,8′,11′,12′-octahydro-4,4′-diapocarotene].

Identification of photosynthetic pigments in *Hph. fasciatum* and other *Heliobacterium* species

The carotenoids from *Hph. fasciatum* and three species of *Heliobacterium* (*Hbt. chlorum*, *Hbt. modesticaldum*, and *Hbt. gestii*) were similarly analyzed. Novapak C_{18} HPLC

Table 1 Absorption spectra in the eluent and retention times by Novapak C_{18} HPLC, and mol. masses of diapocarotenes from heliobacteria and standard carotenes. (*Parentheses* absorption shoulder) [*nd* not determined, *Hba. Heliobacillus, Hph. Heliophilum, Hbt. Heliobacterium*]

Carotenoid	λ_{max} (nm)					%III/II[a]	%D_B/D_{II}[a]	mol. mass (Da)	R_t (min)
Diaponeurosporene									
Hba. mobilis	267	331	416	439	468	95	8	402	5.9
Hbh. fasciatum	266	330	416	439	468	94	7	402	6.0
Hbt. chlorum	267	330	416	439	468	90	8	402	5.8
Hbt. modesticaldum	267	329	416	439	468	90	8	402	6.0
Hbt. gestii	267	330	416	440	468	96	6	402	5.9
Neurosporene	269	333	418	442	470	91	7	538	13.4
Diapolycopene									
Hba. mobilis	294	361	445	470	501	67	11	400	5.4
Hbh. fasciatum	293	359	446	471	502	85	8	400	5.4
Hbt. modesticaldum	294	364	444	470	501	75	8	*nd*	5.5
Hbt. gestii	293	361	444	471	501	85	9	*nd*	5.5
Lycopene	296	364	447	473	504	70	8	536	11.4
Diapo-ζ-carotene									
Hba. mobilis	234	295	380	401	426	94	7	404	6.5
Hbt. modesticaldum	*nd*	*nd*	377	402	426	*nd*	*nd*	*nd*	6.6
Hbt. gestii	236	295	381	401	425	99	7	*nd*	6.5
ζ-Carotene	237	294	381	401	426	100	4	540	16.3
Asymmetrical ζ-carotene	238	298	377	397	421	89	8	540	16.8
Diapophytofluene									
Hba. mobilis	*nd*	255	333	349	368	83	6	406	7.6
Hbh. fasciatum	*nd*	*nd*	336	349	366	*nd*	*nd*	*nd*	7.7
Hbt. gestii	*nd*	247	334	349	367	*nd*	*nd*	*nd*	7.6
Phytofluene	*nd*	255	334	349	368	71	8	542	20.5
Diapophytoene									
Hba. mobilis	*nd*	*nd*	(277)	287	(296)	*nd*	*nd*	408	10.0
Hbh. fasciatum	*nd*	*nd*	(273)	280	(289)	*nd*	*nd*	*nd*	9.5
Hbt. chlorum	*nd*	*nd*	*nd*	287	*nd*	*nd*	*nd*	*nd*	9.4
Hbt. modesticaldum	*nd*	*nd*	*nd*	288	*nd*	*nd*	*nd*	*nd*	9.9
Phytoene	*nd*	*nd*	(278)	288	298	*nd*	*nd*	544	26.9

[a] Takaichi and Shimada (1992)

elution profiles of their carotene fractions were similar to the profile of *Hba. mobilis* (data not shown). The carotenes in these fractions are identified as indicated in Table 1 based on their retention times on HPLC, their absorption spectra, and their molecular masses. The major carotenoid component in all organisms was also diaponeurosporene, and its content was again more than 95% of total carotenoids. Trace amounts of other diapocarotenes were also found.

Absorption spectra of BChl from these heliobacteria were the same as the spectrum of BChl *g* from *Hba. mobilis*, and their retention times by μBondapak C_{18} HPLC were almost identical to the retention time of *Hba. mobilis* (data not shown). Consequently, the esterifying alcohol of BChl *g* from all species of heliobacteria is the C_{15} alcohol, farnesol.

Discussion

In this study, the carotenoids from all known species of heliobacteria (three genera including five species) were reidentified using spectroscopic methods, and the major carotenoid in each was found to be the C_{30} acyclic carotene 4,4′-diaponeurosporene. Diapolycopene, diapo-ζ-carotene, diapophytofluene, and diapophytoene were also found as minor components. In addition, one minor carotene eluting at 3.8 min by Novapak C_{18} HPLC was found in all species of heliobacteria, and two additional carotenoids were also found in trace amounts in cells of *Hph. fasciatum*. Identification of these carotenoids is in progress.

Previously, the major carotenoid in all species of heliobacteria was reported to be neurosporene by absorption spectra and the R_f value on silica gel TLC (Gest and Favinger 1983; Van Dorssen et al. 1985; Trost and Blankenship 1989). This was understandable since the absorption spectra of both diaponeurosporene and neurosporene were almost the same in the HPLC eluent (Table 1) and were identical in methanol (unpublished results). Moreover, on silica gel TLC it is difficult to distinguish these carotenes judging from the developing solvent used (Van Dorssen et al. 1985). However, by both μ Bondapak C_{18} and Novapak C_{18} HPLC, diaponeurosporene

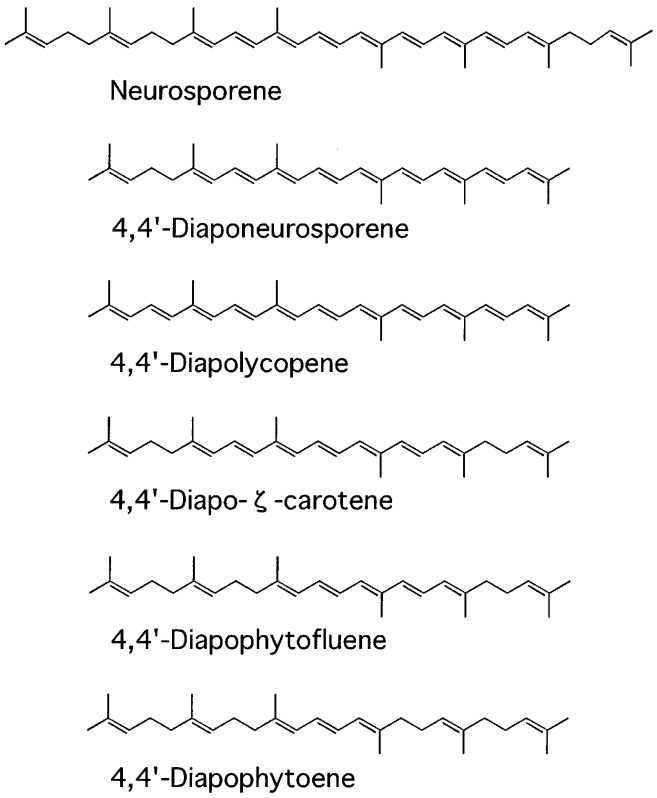

Fig. 2 Structure of neurosporene, 4,4′-diaponeurosporene, and minor diapocarotenes of heliobacteria

and neurosporene are clearly resolvable and, thus, the identification of the major carotenoids of heliobacteria has had to wait until examination by these methods.

The presence of 4,4′-diapocarotene derivatives has been reported only for three species of nonphototrophic bacteria: *Staphylococcus aureus*, *Streptococcus faecium*, and *Pseudomonas rhodos* (present name, *Methylobacterium rhodinum*) (Taylor 1984; Straub 1987). These organisms produce diaponeurosporene and/or diapolycopene, and their final products are carotenoid glycosides or carotenoic acid glycosides. Recently, two genes encoding proteins in the early steps of diapocarotene biosynthesis have been cloned and characterized from *S. aureus* (Wieland et al. 1994). Dehydrosqualene synthase (CrtM) converts two molecules of farnesyl diphosphate (C_{15}) into 4,4′-diapophytoene in a way similar to the one in which phytoene synthase (CrtB) combines two C_{20} units in the production of C_{40} carotenoids. Diapophytoene is then successively desaturated by a diapophytoene desaturase (CrtN) to form 4,4′-diaponeurosporene, analogous to the activity of phytoene desaturase (CrtI). In heliobacteria, the major final product is diaponeurosporene. Thus, heliobacteria may well have genes homologous to those encoding CrtM and CrtN in *S. aureus*.

The esterifying alcohol of BChl *g* of *Hba. mobilis* and *Hbt. chlorum* has been reported to be farnesol (C_{15}) instead of the usual phytol (C_{20}) of BChls *a* and *b* (Michalski et al. 1987; Kobayashi et al. 1991); this study shows that this is true of other species of heliobacteria as well. Both phytoene and the esterifying alcohol of BChls *a* and *b* are produced from geranylgeraniol (C_{20}), which is a precursor of phytol. It is thus possible that heliobacteria are unable to produce geranylgeraniol; if this is true, it would be a unique situation among anoxygenic phototrophs (green sulfur bacteria contain farnesol as the esterifying alcohol on BChls *c*, *d*, or *e* but contain phytol on the small amount of BChl *a* present in the cytoplasmic membrane).

All carotenoids found so far in phototrophic organisms are C_{40} carotenoids and their derivatives. All phototrophs necessarily have some kind of xanthophyll in the pigment-protein complexes, and some phototrophic organisms also have carotenes in the complexes. Exceptionally, some strains of photosynthetic bacteria (e.g. *Rsp. rubrum* S1 and *Rhodobacter sphaeroides* 2.4.1) have more than 90% content of one kind of xanthophyll (Schmidt 1978). By contrast, all species of heliobacteria contain a single C_{30} carotene that comprises more than 95% of the total carotenoids, but they contain no xanthophylls. In these respects, heliobacteria are, therefore, unique phototrophic organisms.

Since the number of conjugated double bonds in diaponeurosporene is the same as that in neurosporene (Fig. 2), its spectroscopic and physicochemical properties are similar to those of neurosporene. However, since diaponeurosporene is smaller than the usual C_{40} carotenoids, the interactions of this molecule with the pigment-protein complexes in the photosynthetic membrane may differ from those of other anoxygenic phototrophs. In this connection, neurosporene (now identified as diaponeurosporene) has been reported to be firmly bound to the reaction center-core complex of heliobacteria and is not easily removed by detergents (Trost and Blankenship 1989; Van de Meent et al. 1990). Thus, this pigment may play a unique role in heliobacterial photosynthesis, but such conclusions await functional studies.

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