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Unique Carotenoids in the Terrestrial Cyanobacterium *Nostoc commune* NIES-24: 2-Hydroxymyxol 2′-Fucoside, Nostoxanthin and Canthaxanthin

Shinichi Takaichi · Takashi Maoka · Mari Mochimaru

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Abstract Cyanobacteria produce some carotenoids. We identified the molecular structures, including the stereochemistry, of all the carotenoids in the terrestrial cyanobacterium, *Nostoc commune* NIES-24 (IAM M-13). The major carotenoid was β-carotene. Its hydroxyl derivatives were (3*R*)-β-cryptoxanthin, (3*R*,3′*R*)-zeaxanthin, (2*R*,3*R*,3′*R*)-caloxanthin, and (2*R*,3*R*,2′*R*,3′*R*)-nostoxanthin, and its keto derivatives were echinenone and canthaxanthin. The unique myxol glycosides were (3*R*,2′*S*)-myxol 2′-fucoside and (2*R*,3*R*,2′*S*)-2-hydroxymyxol 2′-fucoside. This is only the second species found to contain 2-hydroxymyxol. We propose possible carotenogenesis pathways based on our identification of the carotenoids: the hydroxyl pathway produced nostoxanthin via zeaxanthin from β-carotene, the keto pathway produced canthaxanthin from β-carotene, and the myxol pathway produced 2-hydroxymyxol 2′-fucoside via myxol 2′-fucoside. This cyanobacterium was found to contain many kinds of carotenoids and also displayed many carotenogenesis pathways, while other cyanobacteria lack some carotenoids and a part of carotenogenesis pathways compared with this cyanobacterium.

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S. Takaichi (✉)
Department of Biology, Nippon Medical School, Kosugi-cho, Nakahara, Kawasaki 211-0063, Japan
e-mail: takaichi@nms.ac.jp

T. Maoka
Research Institute for Production Development, Shimogamo-morimoto-cho, Sakyo, Kyoto 606-0805, Japan

M. Mochimaru
Department of Natural Sciences, Komazawa University, Komazawa, Setagaya, Tokyo 154-8525, Japan

Introduction

Nostoc, a genus of cyanobacteria, is one of the most widespread phototrophic bacteria. According to Rippka et al. [15], it is classified into subsection IV together with *Anabaena* and some other genera. They are filamentous and heterocystous cyanobacteria, commonly observed in both aquatic and terrestrial habitats.

The terrestrial cyanobacterium *Nostoc commune* (*N. commune*) is well known and highly drought tolerant. Because it sustains the capacity for cell growth for over 100 years in a dry state, it is considered an anhydrobiotic microorganism with oxygenic photosynthetic capabilities. Since *N. commune* does not differentiate into akinetes (spores), the mechanisms of its extreme desiccation tolerance most likely involve multiple processes including the cessation of photosynthesis and the accumulation of trehalose in response to desiccation [16]. Although some cyanobacteria have been investigated during the sequencing of their genome and the development of gene manipulation techniques, *N. commune* has not been well characterized in terms of its physiological or genetical attributes.

We have previously identified the molecular structures of the carotenoid and the glycoside moieties in some *Anabaena* and *Nostoc* species. *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) and *Nostoc punctiforme* (*N. punctiforme*) PCC 73102 contain β-carotene, echinenone, canthaxanthin, myxol 2′-fucoside, and 4-ketomyxol 2′-fucoside, but not 2′-rhamnoside [25]. *Anabaena variabilis* (*A. variabilis*) ATCC 29413 contains β-carotene,

echinenone, canthaxanthin, free myxol, and 4-hydroxymyxol but not myxol glycosides [26], while *A. variabilis* IAM M-3 (PCC 7118) contains β-carotene, echinenone, canthaxanthin, myxol 2′-fucoside, and 4-ketomyxol 2′-fucoside [25]. Based on functional analyses of their carotenogenesis genes, we have previously proposed the biosynthetic pathways for the carotenoids, their enzymes, and their genes, in these cyanobacteria [25, 26], and further we have summarized the biosynthetic pathways and their enzymes and genes, in cyanobacteria [22, 23].

In another recent study, we have reported that the unicellular thermophilic cyanobacterium *Thermosynechococcus elongatus* (*T. elongatus*) strain BP-1 also contains two polyhydroxyl carotenoids, caloxanthin and nostoxanthin [8]. In *T. elongatus* strain BP-1, 2,2′-β-hydroxylase, CrtG, produced nostoxanthin from zeaxanthin via caloxanthin and 2-hydroxymyxol from myxol. This represented the first functional identification of this enzyme in cyanobacteria [8].

In this study, we identified the molecular structures, including the stereochemistry, of all the carotenoids in *N. commune* NIES-24. The major carotenoid was β-carotene, and its hydroxyl or keto derivatives. The myxol glycosides were myxol 2′-fucoside and 2-hydroxymyxol 2′-fucoside. This species is only the second one found to contain 2-hydroxymyxol. We proposed the carotenogenesis pathways based on the identification of the carotenoids, including the hydroxyl, keto, and myxol pathways. Thus, this bacterium was found to contain many kinds of carotenoids compared with other cyanobacteria, and also many carotenogenesis pathways.

Materials and Methods

Strain and Culturing Conditions

The cyanobacterium *N. commune* NIES-24 (IAM M-13) was grown in BG-11 medium with continuous shaking (110 rpm) at 26–28°C under continuous illumination from white fluorescent light (30–40 μE m^{-2} s^{-1}) for 2 weeks. The grown cells were then collected by centrifugation [25].

Purification and Identification of Pigments

The carotenoids were isolated and purified as follows (supplemental Fig. 1). We extracted the pigments with acetone/methanol (7:2, v/v) using an ultra sonicator, evaporated the solvent, dissolved them in a small volume of acetone, and loaded them on a column of DEAE-Toyopearl 650 M (Tosoh, Japan). A broad orange–yellow carotenoid band and then chlorophyll *a* (Chl *a*) were eluted with *n*-hexane/acetone (1:1, v/v), and a second polar orange carotenoid band was eluted with acetone. The first orange–yellow carotenoids were loaded on a column of silica gel 60 (Merck, Germany), and orange carotenoids were eluted with *n*-hexane/acetone (9:1, v/v), while the other orange carotenoids were eluted with *n*-hexane/acetone (7:3, v/v). Each fraction was further separated with KC-18 thin layer chromatography (TLC) (Whatman, USA) developed with methanol, and each band was collected. Each carotenoid was finally collected from the high performance liquid chromatography (HPLC) apparatus (described below) and eluted with methanol or methanol/water (9:1, v/v). The second polar orange carotenoids were purified with silica gel TLC (Merck) developed with dichloromethane/ethyl acetate/acetone/methanol (2:4:2:1, by vol), and then each carotenoid was collected from the HPLC apparatus eluted with methanol/water (9:1, v/v) [25].

Spectroscopic Analysis

The HPLC system was equipped with a μBondapak C_{18} column (8 × 100 mm, RCM type; Waters, USA) eluted with methanol/water (9:1, v/v) for the first 20 min and then with 100% methanol (2.0 ml min^{-1}) [8, 25].

We measured the absorption spectra of the pigments using an MCPD-3600 photodiode array detector (Otsuka Electronics, Japan) attached to the HPLC apparatus [21]. For quantitative analysis, the molar extinction coefficient in the HPLC eluent at the maximum wavelengths of each carotenoid was assumed to be the same, 140 mM^{-1} cm^{-1}, and that at 618 nm of Chl *a* was 16.8 mM^{-1} cm^{-1} [8]. The circular dichroism (CD) spectra of the carotenoids were measured using a J-820 spectropolarimeter (JASCO, Japan) in diethyl ether/2-pentane/ethanol (5:5:2, by vol) at room temperature. The relative molecular masses of the carotenoids were measured using an FD-MS; M-2500 double-focusing gas chromatograph-mass spectrometer (Hitachi, Japan) equipped with a field-desorption apparatus. The proton nuclear magnetic resonance (^{1}H-NMR) (500 MHz) spectra of the carotenoids, which were further purified using a column of DEAE-Toyopearl eluted with *n*-hexane/acetone (1:1, v/v), in $CDCl_3$ at 24°C were measured using the UNITY *INOVA*-500 system (Varian, USA).

Results

Separation and Identification of Carotenoids

Figure 1 shows an elution profile of HPLC for the organic solvent-soluble pigments extracted from *N. commune* NIES-24. The peak-7 pigment (Fig. 1) was identified as Chl *a* by its absorption spectrum and the specific retention time on HPLC as compared with Chl *a* from *T. elongatus*

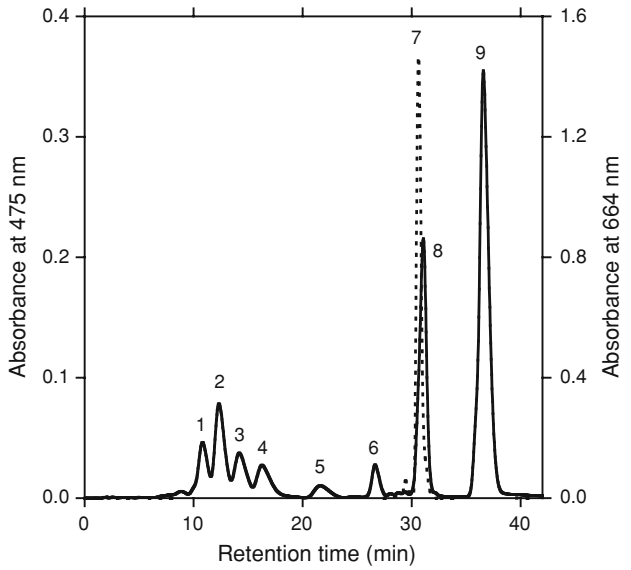

Fig. 1 HPLC elution profiles of pigments extracted from *N. commune* NIES-24. Elution was with methanol/water (9:1, v/v) for the first 20 min, and then 100% methanol (2.0 ml min^{-1}). Absorbance at 475 (*solid line*) and 664 (*dotted line*) nm are shown. Peaks are referred to in the text, table, and figures

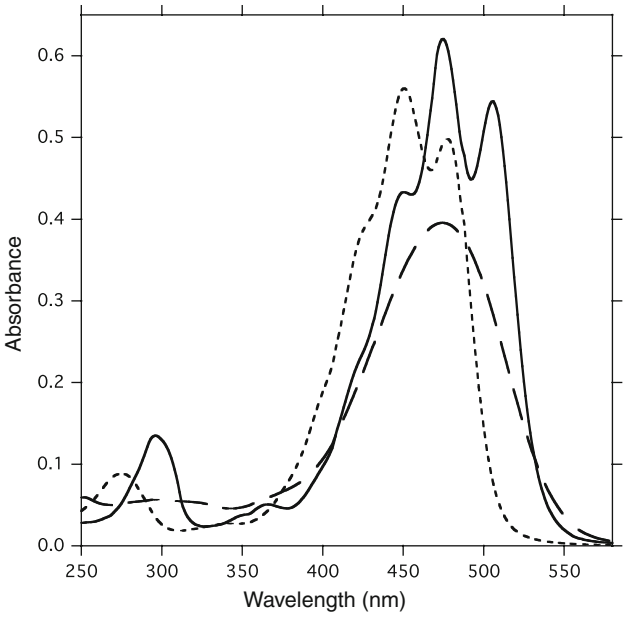

Fig. 2 Absorption spectra of 2-hydroxymyxol 2′-fucoside (1, *solid line*) and nostoxanthin (2, *dotted line*) in methanol/water (9:1, v/v), and canthaxanthin (6, *dashed line*) in methanol. Numbers in parentheses indicate the peak numbers in Fig. 1

strain BP-1 [8]. One minor peak eluted just before peak 7 had a Chl *a*-like spectrum.

The first orange–yellow carotenoids eluted from the DEAE-Toyopearl column (supplemental Fig. 1) were loaded on the column of silica gel 60, and orange carotenoids, which corresponded to peaks 6, 8, and 9 on HPLC (Fig. 1), were eluted with *n*-hexane/acetone (9:1, v/v); the next orange carotenoids, which corresponded to peaks 2, 4, and 5, were eluted with *n*-hexane/acetone (7:3, v/v). Each fraction was further purified with KC-18 TLC developed with methanol, and then each band was collected. Finally, each carotenoid was collected from the HPLC apparatus eluted with methanol for peaks 6, 8, and 9 or with methanol/water (9:1, v/v) for peaks 2, 4, and 5.

The absorption maxima of the peak-9 carotenoid in methanol were 273, 342, 426 (shoulder), 449, and 475 nm, and the spectral fine structure of %III/II was 16; this is the ratio of the peak heights of the longest and the middle wavelength absorption bands from the trough between the two peaks [21]. It had a relative molecular mass of 536, and hence this peak-9 carotenoid was identified as β-carotene (Fig. 3). The peak-6 (Fig. 2, dashed line) and -8 carotenoids showed broad absorption spectra in methanol, and their absorption maxima were around 475 and 460 nm, respectively. They had relative molecular masses of 564 and 550, respectively, and hence these peak-6 and -8 carotenoids were identified as canthaxanthin (Fig. 3) and echinenone, respectively. A minor carotenoid in this fraction was identified as β-cryptoxanthin based on the compatibility of its absorption spectrum with that of β-carotene, and its relative molecular mass of 552. Further, it eluted at around 31 min on HPLC and was hidden under Chl *a* (see Fig. 1). The specific retention times on HPLC of these carotenoids were also compatible with those from *N. punctiforme* PCC 73102 [25].

The absorption maxima of the peak-2 carotenoid in methanol/water (9:1, v/v) were 275, 344, 428 (shoulder), 451, and 477 nm (Fig. 2, dotted line), and the spectral fine structure of %III/II was 38, which were compatible with those of β-carotene. Its CD spectrum in diethyl ether/2-pentane/ethanol (5:5:2, by vol) was compatible with that of (2*R*,3*R*,2′*R*,3′*R*)-nostoxanthin from *T. elongatus* strain BP-1 [8]. It had a relative molecular mass of 600, and its ^{1}H-NMR spectrum (Table 1) was compatible with that of nostoxanthin from *T. elongatus* strain BP-1 [8]. Hence, this peak-2 carotenoid was identified as (2*R*,3*R*,2′*R*,3′*R*)-nostoxanthin (Fig. 3). The absorption spectra of the peak-4 and -5 carotenoids were compatible with that of nostoxanthin (Fig. 2, dotted line). The CD spectrum of the peak-4 carotenoid was compatible with that of the peak-2 carotenoid. [Note: (2*R*,3*R*,2′*R*,3′*R*)-nostoxanthin, (2*R*,3*R*,3′*R*)-caloxanthin, (3*R*,3′*R*)-zeaxanthin, and (3*R*)-β-cryptoxanthin have the same CD spectra [2].] The peak-4 and -5 carotenoids had relative molecular masses of 584 and 568, respectively, and were thus identified as (2*R*,3*R*,3′*R*)-caloxanthin and (3*R*,3′*R*)-zeaxanthin (Fig. 3), respectively. The specific retention times on HPLC of these carotenoids were also compatible with those from *T. elongatus* strain BP-1 [8].

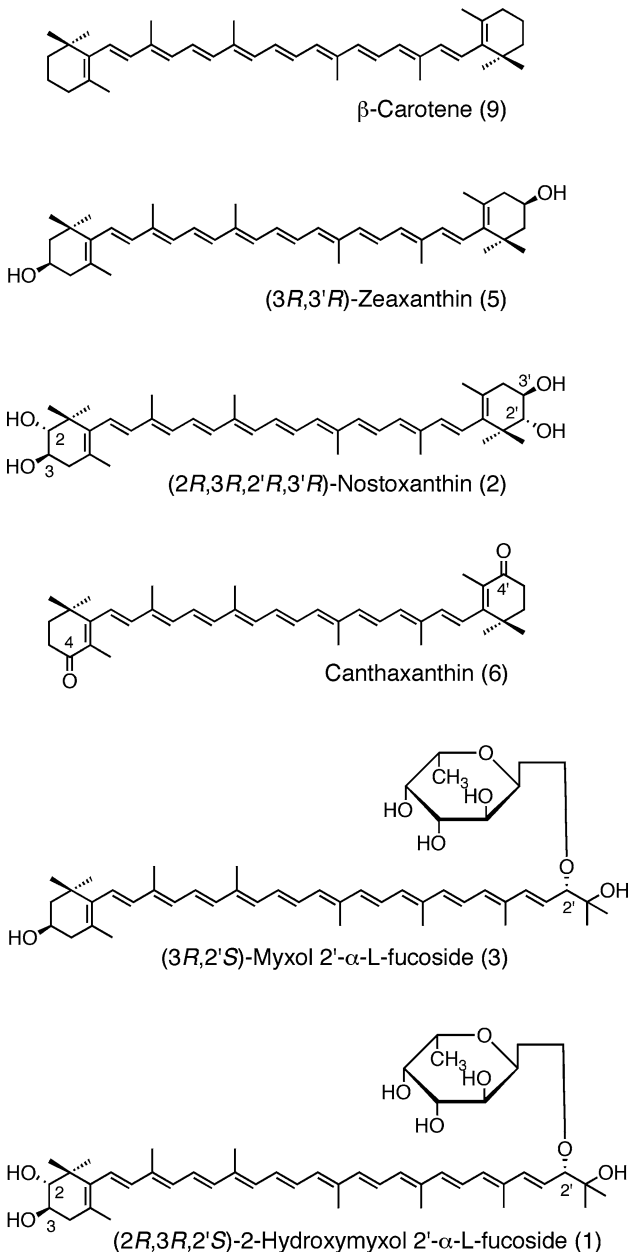

Fig. 3 Structures of selected carotenoids. Numbers in parentheses indicate the peak numbers in Fig. 1

Separation and Identification of Polar Carotenoids

The second polar orange carotenoids eluted from the DEAE-Toyopearl column (supplemental Fig. 1) were purified with silica gel TLC (Merck) developed with dichloromethane/ethyl acetate/acetone/methanol (2:4:2:1, by vol). We collected one broad polar orange band, separated it via HPLC, and it was eluted with methanol/water (9:1, v/v). The products corresponded to the peak-1 and -3 carotenoids on HPLC (Fig. 1). Based on their characteristics on the silica gel TLC and C_{18}-HPLC, they were considered to be carotenoid glycosides [24].

The absorption maxima of the purified peak-1 carotenoid in methanol/water (9:1, v/v) were 296, 366, 451, 475, and 506 nm (Fig. 2, solid line), and the spectral fine structure of %III/II was 56. Based on these results, the carotenoid moiety was identified as a derivative of γ-carotene with 12 conjugated double bonds [21]. Its CD spectrum was compatible with that of (2*R*,3*R*,2′*S*)-2-hydroxymyxol 2′-fucoside from *T. elongatus* strain BP-1 [8]. The relative molecular mass was 746. The ^{1}H-NMR spectrum (Table 1) was compatible with that of 2-hydroxymyxol 2′-α-L-fucoside from *T. elongatus* strain BP-1 [8]. Hence, the structure of the peak-1 carotenoid was identified as 2-hydroxymyxol 2′-fucoside, (2*R*,3*R*,2′*S*)-2-hydroxymyxol 2′-α-L-fucoside (Fig. 3). The IUPAC–IUBMB semi-systematic name is (2*R*,3*R*,2′*S*)-2′-(α-L-fucopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-carotene-2,3,1′-triol.

The absorption spectrum of the purified peak-3 carotenoid was compatible with that of the peak-1 carotenoid (Fig. 2, solid line). Its CD spectrum was compatible with that of the peak-1 carotenoid. The relative molecular mass was 730. The ^{1}H-NMR spectrum (Table 1) was compatible with that of myxol 2′-α-L-fucoside from *T. elongatus* strain BP-1 [8] and *Anabaena* sp. PCC 7120 [25]. Thus, the structure of the peak-3 carotenoid was identified as myxol 2′-fucoside, (3*R*,2′*S*)-myxol 2′-α-L-fucoside (Fig. 3). The IUPAC-IUBMB semi-systematic name is (3*R*,2′*S*)-2′-(α-L-fucopyranosyloxy)-3′,4′-didehydro-1′,2′-dihydro-*β*,*ψ*-carotene-3,1′-diol.

Composition of Pigments

The composition of the pigments in the cells cultured under light for 2 weeks was 50% *β*-carotene (mol% of total carotenoids), <1% *β*-cryptoxanthin, 2% zeaxanthin, 5% caloxanthin, 11% nostoxanthin, 20% echinenone, 2% canthaxanthin, 4% myxol 2′-fucoside, and 5% 2-hydroxymyxol 2′-fucoside. The molar ratio of total carotenoids to Chl *a* was 0.44, which was comparable to those of some *Anabaena* and *Nostoc* species [25]. These values varied slightly as a function of the batch and the culture conditions.

Colonies of *N. commune* collected from a natural field (Kanazawa, Japan) contained the same pigments, but the composition was different from that of *N. commune* NIES-24: 18% *β*-carotene, 1% zeaxanthin, 3% caloxanthin, 4% nostoxanthin, 43% echinenone, 12% canthaxanthin, 9% myxol 2′-fucoside, and 11% 2-hydroxymyxol 2′-fucoside. The molar ratio of total carotenoids to Chl *a* was 0.53. The growth conditions in the natural field might be very different from the laboratory conditions.

Table 1 ^{1}H-NMR data of nostoxanthin, myxol 2′-fucoside, and 2-hydroxymyxol 2′-fucoside in $CDCl_3$

Protons[a]	Nostoxanthin (2)[b]	Myxol 2′-fucoside (3)	2-Hydroxymyxol 2′-fucoside (1)
3-Hydroxy-β-end group			
H_3-16,17		1.077 s	
H_3-18		1.739 s	
H_3-19,20		1.974 s	
H-2ax		1.48 dd (11, 11)	
H-2eq		1.77 d (12)	
H-3ax		4.01 m	
H-4ax		2.05 dd (15, 8)	
H-4eq		2.39 dd (15, 6)	
H-7		6.10 d (16)	
H-8		6.14 d (15)	
H-10		6.16 d (11)	
H-11		6.64 m	
H-12		6.37 d (16)	
H-14		6.26 d (10)	
H-15		6.64 m	
2,3-Dihydroxy-β-end group			
H_3-16	1.005 s		1.005 s
H_3-17	1.130 s		1.130 s
H_3-18	1.718 s		1.717 s
H_3-19,20	1.972, 1.975 s		1.973 s
H-2ax	3.33 dd (9, 3)		3.33 d (9)
H-3ax	3.84 dd (15, 8)		3.84
H-4ax	2.14 dd (16, 9)		2.14 dd (16, 11)
H-4eq	2.48 dd (16, 6)		2.48 dd (16, 6)
H-7	6.06 d (15)		6.06 d (15)
H-8	6.13 d (14)		6.12 d (14)
H-10	6.17 d (10)		6.17 d (11)
H-11	6.64 m		6.64 m
H-12	6.37 d (14)		6.37 d (14)
H-14	6.26 d (9)		6.26 d (11)
H-15	6.64 m		6.64 m
1,2-Dihydroxy-3,4-didehydro-1,2-dihydro-ψ-end group			
H_3-16		1.189 s	1.187 s
H_3-17		1.199 s	1.197 s
H_3-18		1.923 s	1.920 s
H_3-19,20		1.974 s	1.973 s
H-2		3.89 d (7)	3.89 d (7)
H-3		5.63 dd (14, 7)	5.63 dd (14, 7)
H-4		6.40 d (14)	6.40 d (14)
H-6		6.20 d (10)	6.20 d (10)
H-7		6.59 dd (14, 10)	6.59 dd (14, 10)
H-8		6.40 d (14)	6.40 d (14)
H-10		6.20 d (10)	6.20 d (10)
H-11		6.64 m	6.64 m
H-12		6.37 d (16)	6.37 d (14)
H-14		6.29 d (10)	6.29 d (10)
H-15		6.64 m	6.64 m
Fucoside moiety			
H-1″		5.06 d (3)	5.06 d (3)
H-2″		3.87	3.87
H-3″		3.85	3.84
H-4″		3.80	3.80
H-5″		4.07 dd (12, 6)	4.07 dd (12, 6)
H_3-6″		1.188 d (6.5)	1.186 d (6.5)

[a] δ (ppm), multiplicity (d: doublet, m: multiplet, s: singlet) and coupling constants (Hz)

[b] Numbers in parentheses indicate the peak numbers in Fig. 1

Discussion

In this study, we identified the molecular structures, including the stereochemistry, of all the carotenoids in *N. commune* NIES-24 (IAM M-13). The major carotenoid was β-carotene. Its hydroxyl derivatives were (3*R*)-β-cryptoxanthin, (3*R*,3′*R*)-zeaxanthin, (2*R*,3*R*,3′*R*)-caloxanthin, and (2*R*,3*R*,2′*R*,3′*R*)-nostoxanthin. The unique keto derivatives were echinenone and canthaxanthin; the unique myxol glycosides were (3*R*,2′*S*)-myxol 2′-fucoside and (2*R*,3*R*,2′*S*)-2-hydroxymyxol 2′-fucoside. This is only the second species found to contain 2-hydroxymyxol [1, 8]. Although these carotenoids, including their stereochemistry, have been found in other cyanobacteria, only the species of the study contained nine kinds of carotenoid or had such a variety of carotenoids compared with other cyanobacteria [22, 23]. 4-Ketomyxol 2′-methylpentoside was, however, absent.

The carotenoids found in *Nostoc* were only reported in a few species with insufficient identification [5, 7]. *Nostoc commune* strain B 1453-5 was reported to contain β-carotene, zeaxanthin, caloxanthin, nostoxanthin, echinenone, canthaxanthin, 4-hydroxy-4′-keto-β-carotene, and myxoxanthophyll [20]. Since 4-hydroxy-4′-keto-β-carotene was only found in animals [1], this structure might be disputable. The structure of myxoxanthophyll (myxol glycoside) was unknown, and 2-hydroxymyxol 2′-methylpentoside was absent. Further, a *Nostoc* sp. was reported to contain β-carotene, zeaxanthin, echinenone, and myxoxanthophyll [4]. We have previously found that *N. punctiforme* PCC 73102 and *Anabaena* sp. PCC 7120 (also known as *Nostoc* sp. PCC 7120) contain β-carotene, echinenone, canthaxanthin, myxol 2′-fucoside, and 4-ketomyxol 2′-fucoside [25].

The presence of glycosides (mostly methylpentoside) of myxol and its derivatives is limited to cyanobacteria, with no reports of their presence in any other bacteria or

eukaryotic algae [1, 5, 22, 23]. Further free myxol has been found in only two marine bacteria, strain P99-3 (MBIC 03313; previously *Flavobacterium* sp.) [28] and strain YM6-073 (MBIC 06409, *Flavobacteriaceae*) [17].

The biosynthesis of carotenoids in some cyanobacteria has been previously reported [22, 23]. In the cyanobacterium *N. commune* NIES-24, the following pathway is proposed from its carotenoid composition (Fig. 4). Geranylgeranyl diphosphate synthase (CrtE) and phytoene synthase (CrtB) produce phytoene, and then phytoene desaturase (CrtP), ζ-carotene desaturase (CrtQ), and *cis*-carotene isomerase (CrtH) produce lycopene as in other cyanobacteria. Lycopene cyclase(s) produces γ-carotene and/or β-carotene from lycopene, but the enzyme(s) is unknown, except for *Synechococcus* sp. PCC 7002 (CruA and CruP) [11], *Synechococcus elongatus* PCC 7942 and *Prochlorococcus marinus* MED4 (CrtL) [3, 19]. β-Carotene is changed to zeaxanthin via β-cryptoxanthin by β-carotene hydroxylase (CrtR), and then changed to nostoxanthin (Fig. 3) via caloxanthin by 2,2′-β-hydroxylase (CrtG) in the hydroxyl pathway. CrtG has been recently functionally confirmed in *Brevundimonas* sp. SD212 (MBIC 03018) and in *T. elongatus* strain BP-1 [8, 14, 27]. β-Carotene is also changed to canthaxanthin (Fig. 3) via echinenone by β-carotene ketolase (CrtO or CrtW) in the keto pathway [12]. Although the synthesis of myxol 2′-fucoside (the myxol pathway) has yet to be elucidated [23], at least CrtR for myxol, which is the same enzyme described above, and GDP-fucose synthase (WcaG) for myxol 2′-fucoside are known to play a role [13]. Recently, the functions of 1′,2′-hydratase (CruF) and GDP-fucosyl transferase (CruG) have been confirmed in *Synechococcus* sp. PCC 7002 [6]. Finally, myxol 2′-fucoside is changed to 2-hydroxymyxol 2′-fucoside (Fig. 3) by the same CrtG described above, which has been functionally confirmed in *T. elongatus* strain BP-1 [8]. The functions of two CrtWs for canthaxanthin and 4-ketomyxol from *N. punctiforme* PCC 73102 [18]; those of CrtQ, CrtR for only myxol, CrtO for canthaxanthin, CrtW for 4-ketomyxol, and WcaG from *Anabaena* sp. PCC 7120 [9, 10, 12, 13]; and that of CrtR for myxol from *A. variabilis* ATCC 29413 [10] have been confirmed. Thus, this cyanobacterium contains three carotenogenesis pathways (Fig. 4). Further studies are needed for identification of these enzymes and genes.

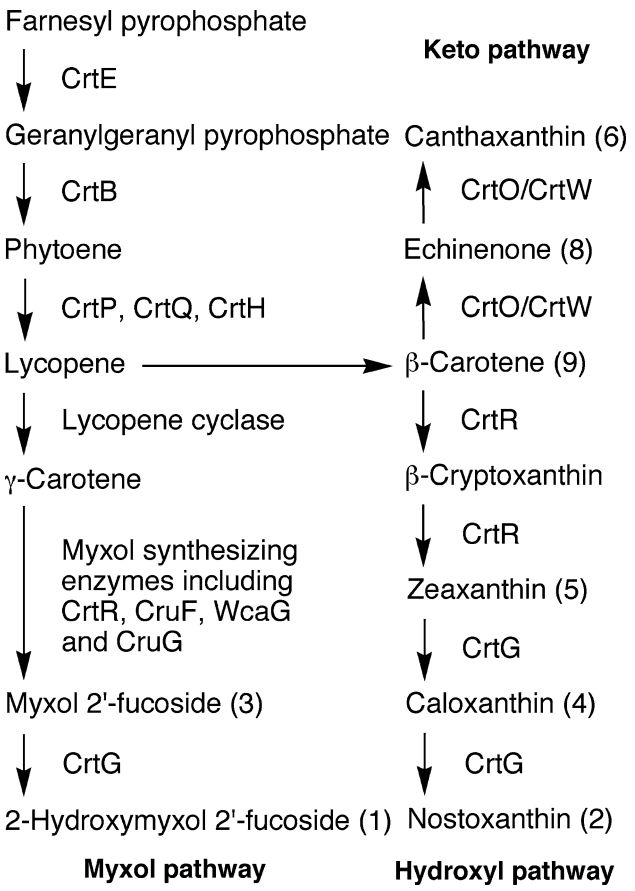

Fig. 4 Proposed biosynthetic pathways of carotenoids and their enzymes in *N. commune* NIES-24. Numbers in parentheses indicate the peak numbers in Fig. 1. See the text for further details

Variation in carotenoid compositions have been attributed to be due to the presence or absence of specific carotenogenesis genes and pathways, as well as to the characteristics of specific enzyme(s) [22, 23]. *Nostoc commune* NIES-24 contains many functional carotenogenesis enzymes and pathways, while other cyanobacteria may lack some enzymes. This species could be standard species for carotenoids of *Nostoc* and related genera, and may be useful for studying the characteristics of carotenoids and carotenogenesis enzymes, and regulation of carotenogenesis in each pathway. Further studies of carotenoids in some *Nostoc* species are in progress.

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