

Carotenoid composition in the cyanobacterium *Phormidium laminosum*

Effect of nitrogen starvation

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When pigments of the non- N_2 -fixing cyanobacterium *Phormidium laminosum* were carefully extracted and analyzed in a completely O_2 -free atmosphere, by either high performance liquid chromatography (HPLC) or thin layer chromatography (TLC), the presence of only two carotenoids (namely, β -carotene and nostoxanthin) was detected. However, exposure of pigments to an air atmosphere during their manipulation led to the rapid appearance in the organic extracts of at least three additional carotenoids (identified as caloxanthin, zeaxanthin and β -cryptoxanthin). This fact could explain the presence in cyanobacteria of such hydroxylated derivatives of β -carotene widely reported in the literature. Nitrogen starvation also resulted in an important decrease on the relative β -carotene/nostoxanthin content of cells, suggesting that this nutritional condition affects thylakoid membranes more drastically than cytoplasmic membranes.

β -Carotene; Nostoxanthin; HPLC; Nitrogen-starvation; *Phormidium laminosum*

1. INTRODUCTION

Cyanobacteria (blue-green algae) represent an important group of prokaryotes capable of carrying out, like higher plants and green algae, oxygenic photosynthesis. The typical visible coloration of these organisms is due to the presence of chlorophyll (only chlorophyll *a* is present), phycobiliproteins and carotenoids. Chlorophyll and carotenoids form complexes with specific proteins which are embedded in the thylakoid membranes constituting the photosynthetic apparatus where carotenoids play important roles as light-harvesting pigments and protecting chlorophyll against oxidation. Carotenoids also form part of the cyanobacterial cytoplasmic membranes [1,2]. Hladík et al. [3] suggested that carotenoids can mediate a part of the interactions which stabilize the structure of pigment/protein complexes.

Nitrogen starvation results in a rapid degradation of the photosynthetic apparatus, although the size and shape of cells remain unchanged in most cases. Thus, after a 24-h starvation period *Anacystis nidulans* cells showed an almost complete loss of thylakoid structure

but still possessed intact cytoplasmic membranes [4]. During nitrogen-starvation all pigments (mainly phycobiliproteins and to a minor degree chlorophyll) are catabolized originating the rapid and progressive bleaching of cultures, classically known as nitrogen chlorosis. With respect to the changes of carotenoid content, the number of studies is scanty and the results are contradictory.

The main carotenoids present in cyanobacteria are β -carotene, nostoxanthin, caloxanthin, echinenone, myxoxanthophyll and zeaxanthin [5,6]. In nitrate-grown cells of *A. nidulans* the major carotenoids present are β -carotene (52%) and zeaxanthin (38%) [4], although the presence of very small amounts of caloxanthin, nostoxanthin and cryptoxanthin were also reported [2,4]. Moreover, Omata and Murata [2] found that the purified cytoplasmic membranes of this cyanobacterium contained zeaxanthin (60%), caloxanthin (25%) and nostoxanthin (11%), but only 3% of the β -carotene and trace amounts of cryptoxanthin. In contrast, the thylakoidal membranes contained β -carotene (53%), zeaxanthin (24%) and caloxanthin (15%), whereas the relative content of nostoxanthin (6%) and cryptoxanthin (2%) was very low. β -Carotene is the dominant carotenoid present in all fractions of thylakoid membranes from *Plectonema boryanum* [3] where myxoxanthin, cryptoxanthin and zeaxanthin are also present at lower levels, but nostoxanthin and caloxanthin are absent.

High performance liquid chromatography (HPLC),

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Abbreviations: HPLC, high performance liquid chromatography; TLC, thin layer chromatography

because of its reproducibility and quick operation, constitutes a very powerful tool in pigment analysis in general, and in carotenoid studies in particular. Most carotenoids are extremely unstable when illuminated in an air atmosphere and after extraction can be rapidly oxidised, producing a number of oxidised artifacts [7] which may be absent in cells.

This paper shows that in the thermophilic, non- N_2 -fixing cyanobacterium *Phormidium laminosum* only two carotenoid pigments are present in vivo, and give evidence that the reported presence of several carotenoids in a number of cyanobacteria could arise from artifacts produced during the extraction or chromatographic separation of pigments in an aerobic atmosphere.

2. MATERIALS AND METHODS

Silica gel (Aluchrom) plates, HPLC grade acetonitrile, ethyl acetate and methanol, and analytical grade organic solvents were from Scharlau (Barcelona, Spain). Glass fibre (GF/C) filters were from Whatman Biochemicals (Maidstone, UK). Other reagents were obtained from Merck (Darmstadt, Germany).

Phormidium laminosum (strain OH-1-pCl₂) cells were grown autotrophically at 45°C in medium D [8] supplemented with 0.5 g·l⁻¹ of NaHCO₃. Cultures were continuously stirred with an air stream (about 1 liter of air·min⁻¹·l⁻¹ of culture) or mechanically (100 cycles·min⁻¹) in an orbital shaker (Gallenkamp) and continuously illuminated by white fluorescent lamps at a light intensity on the surface vessels of about 100 µmol photons·m⁻²·s⁻¹. Cell growth was estimated as the DNA (measured as deoxyribose) content of cultures. Deoxyribose was evaluated after extraction for 15 min at 70°C with 5% (v/v) perchloric acid [9]. Cells growing exponentially were collected at 4°C by centrifugation (10 min at 15000×g) or by filtration on glass fibre filters under reduced pressure. Cells were washed with distilled water and then resuspended in fresh medium D containing either nitrate or no nitrogen source (starvation medium).

The water-soluble phycobiliproteins were extracted and assayed as reported by Bennett and Bogorad [10]. Organic solvent-soluble pigments were extracted from cells sonicated with 8 vols of acetone in an ice-bath. Chlorophyll and carotenoids were estimated in the acetone extracts as described by Allen and Smith [11]. For further chromatographic analysis, extracts were transferred to 3 vols of *n*-hexane, washed with distilled water and, finally, completely dehydrated over anhydrous Na₂SO₄ and evaporated to dryness under an N₂ stream. Samples were stored at -20°C in sealed vials under an N₂ atmosphere until use. Then samples were dissolved in ethyl acetate and filtered through 0.45 µm pore nylon membranes (SS-Disk Nylon-66, Scharlau) before analysis.

For HPLC analysis samples were filtered through silica-cart C-18 RPS filters (Scharlau) and 20 µl aliquots injected into an HPLC system composed by a high pressure pump (LKB 2150), a gradient control valve (LKB 2040/203), an injection valve (Rheodyne 7125), a low pressure mixing chamber (LKB-Ultrograd 11300) and a photodiode array detector (Waters 990). A reversed-phase (Ultrapac LKB Lichrosorb C18, 5 µm particle size) octadecylsilane column (250×4 mm) was used as the stationary phase. The mobile phase consisted of a linear (0% to 100% ethyl acetate in acetonitrile/methanol 9:1, v/v) gradient performed with a programmable solvent delivery controller (LKB 2125) and degassed with a stream of He. Chromatographies were carried out for 30 min at a flow-rate of 1 ml·min⁻¹ and a pressure of 35–48 bar. Pigments in the effluent were continuously monitored at wavelengths from 300 to 600 nm with the photodiode array detector connected to a NEC APC III computer for storing and processing data of chromatograms and spectra. The peaks

Table I

Pigment composition of *P. laminosum* cells at different physiological status

Pigment (mg/mg deoxyribose)	With nitrogen supply at growth phase ^a		After 3 days of nitrogen starvation
	Exponential	Stationary	
Chlorophyll	10.9 ± 0.9	9.05 ± 1.1	4.6
Phycocyanin	69.0 ± 3.0	77.3 ± 2.5	2.4
Allophycocyanin	12.5 ± 2.5	22.3 ± 2.5	1.2
Carotenoids	3.95 ± 0.55	5.93 ± 0.35	1.9

^a Figures represent mean values estimated at the beginning and at the end of each growth phase.

were identified according to their retention times and by their visible spectra obtained with the photodiode-array detector.

Carotenoids were also separated by TLC on silica gel plates as described by Murata et al. [12] using petroleum ether/2-propanol (20:1, v/v) as the developing solvent. All the procedures were carried out in the dark or in dim light and under a N₂ atmosphere to avoid pigment damages. Carotenoids were identified by their mobilities (*R_f*) and visible spectra of samples scraped off from thin-layer plates and extracted in *n*-hexane (β -carotene and β -cryptoxanthin) or acetone (other carotenoids).

3. RESULTS AND DISCUSSION

3.1. Pigment composition of cells

Cultures of *P. laminosum*, growing in media containing suitable nitrogen sources, show a blue-green coloration which is typical of cyanobacteria. The major pigments responsible for this coloration are phycobiliproteins, chlorophyll and carotenoids. Although these pigments are present in all the growth phases, their relative cellular content among them varies. Table I summarizes the pigment content of cells growing in a nitrate-containing medium during the exponential and stationary phases, and after 3 days in a nitrogen-free medium. The chlorophyll content of cells during the exponential phase of growth is slightly higher than in older cultures, whereas the other pigments reach their highest cellular levels during the stationary phase. The content of carotenoids (as mg pigment per litre of culture) continuously increased for more than 400 h of culture (i.e. during the exponential and stationary phases of growth).

When cells growing exponentially with a nitrogen supply were transferred to a medium lacking the nitrogen source, cells rapidly changed to a green-yellowish coloration (nitrogen chlorosis) which is characteristic of the nitrogen-deficiency. These nitrogen-starved cells underwent an important decrease in the cellular content of all pigments, which is more drastic in the case of phycobiliproteins. The carotenoids content of the nitrogen-starved culture (as mg pigment per litre) remained more or less constant for at least 150 h. After 3 days of nitrogen starvation the content (as mg

Table II
Carotenoids present in *P. laminosum* cells analyzed by TLC and HPLC

Carotenoid	TLC		HPLC			
	Air	N ₂	R _f	Air (after hydrolysis)	N ₂	t _{min}
β -Carotene	+	+	0.96	+	+	21.6
Unidentified I	±	-	0.84	-	-	-
β -Cryptoxanthin	+	-	0.73	+	-	15.5
Zeaxanthin	+	-	0.48	+	-	12.1
Caloxanthin	-	-	-	+	-	11.1
Nostoxanthin	+	+	0.26	+	+	10.1
Unidentified II	±	-	0.14	-	-	-

Carotenoids were extracted and analyzed by TLC and HPLC as described in Materials and Methods in an air or an N₂ atmosphere. Only carotenoids analyzed by HPLC whose relative proportion (figures in parentheses) is higher than 0.8% are included. (+), present; (-), not detected; (±), traces.

pigment per mg deoxyribose) of chlorophyll and carotenoids was, respectively, about 46% and 56% of the initial contents at the beginning of the starvation treatment whereas those of phycocyanin and allophycocyanin were about 3.6% and 11%, respectively.

3.2. Carotenoid composition

When pigment extracts of the whole cells of *P. laminosum*, grown either in a nitrate-containing or a nitrogen-lacking medium, were analyzed by HPLC or TLC, the presence of only two carotenoids (namely β -

carotene and nostoxanthin) was observed (Table II). These pigments were identified according to their retention times and by their visible spectra when analyzed by HPLC (Fig. 1A). Alternatively, carotenoids were identified by their mobilities and visible spectra when analyzed by TLC in dim light and under a strict O₂-free atmosphere.

The presence in *P. laminosum* of only two carotenoids strongly contrasts with the data in the literature where, besides β -carotene and nostoxanthin, a number of oxidized derivatives of β -carotene (namely, calo-

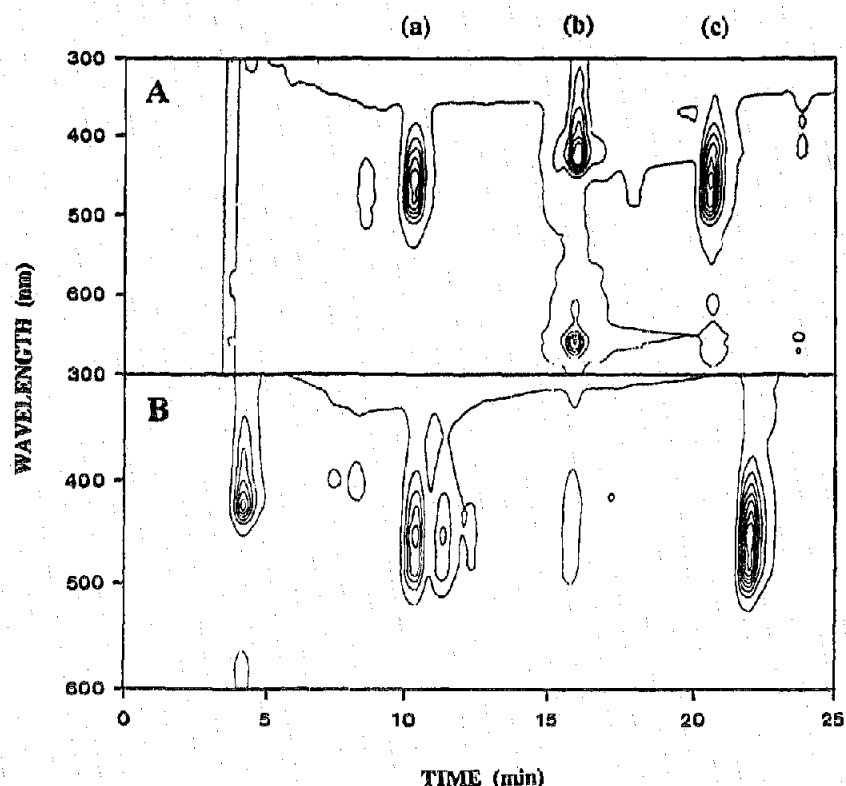


Fig. 1. Chromatographic analysis of *P. laminosum* pigments obtained by HPLC. (A) Pigments were extracted and chromatographed in an O₂-free atmosphere and dim light. (B) Pigments were chromatographed after hydrolysis with KOH in the dark. (a) Nostoxanthin; (b) chlorophyll and derivatives; and (c) β -carotene.

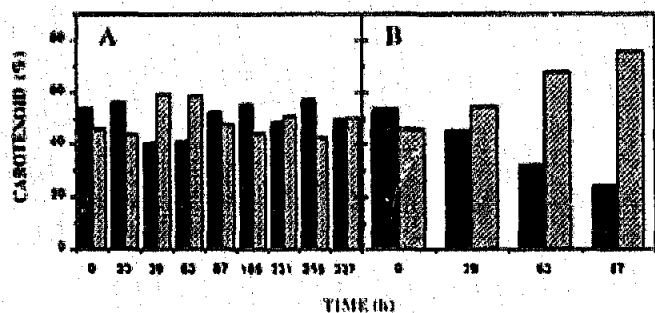


Fig. 2. Changes in the relative proportion between carotenoids present in *P. laminosum*. Cells were grown in a nitrate-containing (A) or in a nitrate-free (B) medium. Carotenoids were evaluated from chromatograms recorded at 430 nm. Nostoxanthin (dashed bars), β -carotene (solid bars).

xanthin, echinenone, myxoxanthin, zeaxanthin, cryptoxanthin, among others) were reported in cyanobacteria [2-4,6]. Because of the well-known instability of some carotenoids in the light and under an aerobic atmosphere [7], and because only TLC techniques were used to analyze the carotenoid composition in all the previous studies [2-4], we analyzed the *P. laminosum* pigments by TLC after their extraction and chromatography in an air atmosphere and maintaining the rest of the analytical conditions unchanged. As shown in Table II, the exposure (1 h) to air of pigment extracts was sufficient to visualize on the TLC plates the yellow spots corresponding to zeaxanthin, cryptoxanthin and traces of other unidentified carotenoids. Similar results were obtained when an aliquot was hydrolyzed with KOH in an air atmosphere and analyzed by HPLC (Fig. 1B). In this case, the β -carotene content of the sample decreased slightly whereas the nostoxanthin content decreased more than 50%, producing appreciable amounts of caloxanthin, zeaxanthin and β -cryptoxanthin (Table II). In all cases, the loss of nostoxanthin corresponded to the sum of the carotenoids produced by the exposure to air of pigment extracts, suggesting that these three carotenoids probably originate from nostoxanthin.

The evolution of carotenoid percentage compositions in *P. laminosum* cells during the time of culture in a nitrate-containing or a nitrogen-lacking medium is shown in Fig. 2. In nitrate-grown cells the relative content of β -carotene and nostoxanthin showed unspecific variations along the culture time (Fig. 2A), the ratio β -carotene/nostoxanthin being near unity. However, significant changes in the latter ratio were observed in the pigment composition of nitrogen-starved cells (Fig. 2B), which varied from about 1.1 (at zero time) to about 0.3 (after 87 h of starvation). The calculated (about 4-fold) decrease in the β -carotene/nostoxanthin ratio was mainly due to a β -carotene decrease rather than to a nostoxanthin increase. Since the total content of carotenoids remains nearly constant and the content

of deoxyribose increased, the nostoxanthin content of nitrogen-starved cells diminished slightly but the decrease in the β -carotene content represented about 70% of the total decrease of carotenoids.

Similar results of the effect of nitrogen starvation on the content of β -carotene and xanthophylls were reported in *A. nidulans*, in which β -carotene gradually decreased whereas the levels of xanthophylls (mainly zeaxanthin and in minor extent caloxanthin and nostoxanthin) were greatly enhanced [4]. It is also remarkable that in *A. nidulans* nostoxanthin only represents (at maximum) 6% of the total carotenoids, this percentage only being observed after 72 h of nitrogen starvation [4].

In cyanobacteria the major carotenoid component in the thylakoid membranes is β -carotene, whereas xanthophylls are associated with cytoplasmic membranes [1,2,4,13]. The presence in cells of *P. laminosum* of only one xanthophyll (nostoxanthin) implies that there is only one such species in the cytoplasmic membrane of this cyanobacterium. Moreover, nitrogen starvation results in important ultrastructural changes of the intracytoplasmic membranes accompanied by thylakoid swelling and vesiculation [14,15] and culminates in the disappearance of the thylakoid membrane system [4]. The fact that nitrogen-starvation affected mainly the levels of β -carotene and that this carotenoid seemed specifically associated to the thylakoid membranes, together with the fact that nitrogen-starvation resulted mainly in the disappearance of the photosynthetic membranes, supports the idea that carotenoids can stabilize the structure of the protein/pigment complexes playing a decisive structural role, as suggested by Hladik et al. [3].

This hypothesis could also be valid for *P. laminosum*, whose nitrogen-starved cells showed similar size and shape to nitrate-grown cells when observed under the scanning electron microscope (data not shown), although an exocellular layer of mucilage appeared and rapidly increased with the starvation time.

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