PHOTOSYNTHETICA 38 (2): 233-241, 2000

Morphological and physiological differences in *Synechococcus elongatus* during continuous cultivation at high iron, low iron, and iron deficient medium

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Abstract

Thermophilic unicellular cyanobacterium *Synechococcus elongatus* Näg. var. *thermalis* Geitl. strain Kovrov 1972/8 was cultivated in continuous flow reactor to simulate conditions occurring in nature in regions with low iron concentration. Two degrees of iron deprivation were established: (*a*) low iron (LI) conditions (9.0 μM Fe) when cells still maintained maximal growth rate but already exhibited changes in photosynthetic apparatus, and (*b*) iron deficient (ID) conditions (0.9 μM Fe) when cell growth rate decreased and extensive morphological and functional changes were observed. A decrease in the cellular content of phycobilin antenna was observed in both ID and LI cells and an increase of carotenoid concentration only in the ID culture. Morphologically, ID cells showed a decrease in the amount of phycobilins and in the number of thylakoid membranes. This suggests that *S. elongatus* responds to decrease in iron availability by substitution of the phycobilisomes by antennae containing chlorophyll (Chl) and carotenoids. Photochemical activity of photosystem (PS) 2, determined as F_v/F_m ratio was similar in high iron (HI) and LI cultures and approximately five times lower in ID culture. On the other hand, the activity of the whole electron transport chain showed the opposite tendency: the relative rates of the CO_2-dependent oxygen evolution in HI : LI : ID cultures were approximately 1 : 2 : 4. Thus in nutrient stress the photosynthetic apparatus preserved its activity despite the decrease in the amount of both Chl-binding complexes and thylakoid membranes.

Introduction

Although Fe is the fourth most abundant element in Earth's crust, its biological availability is critically dependent on its redox state. At physiological pH levels in aqueous, oxic ecosystems iron forms poorly soluble ferric hydroxide in particulate and colloidal form (Wetzel 1975). Cyanobacteria, in particular those of genus *Synechococcus*, are able to adapt to conditions of substantially decreased iron concentration (Hutber *et al.* 1977, Sandmann 1985; see Boyer *et al.* 1987). This flexibility allows them to prosper in a number of biotopes, such as high nutrient-low Chl (HNLC) regions in the oceans (Greene *et al.* 1992, Behrenfeld and Kolber 1999), as well as in freshwater biotopes (Gunnars and Blomqvist 1997).

In freshwater environments, ferric compounds are slightly more soluble than in seawater. But the iron concentration may be limited by chemical and physico-chemical processes, which accompany reactions of iron with phosphorus and sulphide and absorption of phosphate to colloidal ferric oxohydroxide (Gunnars and

Received 21 February 2000, *accepted* 23 June 2000.
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Abbreviations: OD_{750} – optical density, the apparent absorbance including the loss of scattered irradiation at λ 750 nm, which is used as a characteristic of algal (cyanobacterial) particle (cell) concentration; Chl *a* - chlorophyll *a*; CP34 – PS2 chlorophyll binding protein, *isiA* gene product; F_0, F_v, F_m - fluorescence parameters; HI - high iron culture/medium; HPLC - high performance liquid chromatography; ID - iron deficient culture/medium; LI - iron limited culture/medium; POE - photosynthetic oxygen evolution; PS - photosystem; SDS - sodium dodecylsulphate.
Acknowledgements: Authors are grateful to Drs. Ivan Šetlík, Eva Šetlíková, Josef Komenda, and Ondřej Prášil for their advices, tricks, and tips. Authors would also like to thank Dr. Josef Sulek for EM pictures. This work was supported by the grant from the Ministry of Education, Youth, and Physical Training of the Czech Republic (Grant no. VS96085).

Blomqvist 1997). In natural environment iron is present in more than one order lower concentrations than in laboratory growth media.

The molecular basis of low-iron protection in cyanobacteria is complex. An easily understandable mechanism is based on replacement of iron-containing proteins, such as ferredoxin, by alternate flavodoxin (Straus 1994). Often discussed is also the low iron-induced production of a Chl-binding protein CP34 (Straus 1994), product of the *isi* gene. This protein has been reported as Chl storage protein (Burnap *et al.* 1993) or an excitation energy quencher (Lovčinský *et al.* 1999). There may appear one consistently observed change, induced by iron stress, the increase of the emission maximum at 686 nm in the low-temperature fluores-cence emission. Recently, other alterations of cell metabolism induced by iron stress have been also reported, such as production of iron-deficiency-induced protein A (IdiA) with a calculated molecular mass of 35 kD (Michel *et al.* 1998). The effect of iron stress was recently also reported in *Synechococcus* 7942 (Park 1999).

In this work we describe changes in the morphology of *S. elongatus* grown in continuous culture that models the natural conditions where (*a*) the decrease of iron concentration in medium induces spectral and morphological changes but does not limit growth (LI), and (*b*) iron concentration limits the rate and the extent of cell division (ID). In comparison to the cells grown in laboratory medium, LI cells show differences in the absorption spectra and exhibit less ordered thylakoid membranes. In the ID culture chains of multiple, incompletely divided cells were observed, major changes in thylakoid organisation occurred, and an increase in the amount of carotenoids increased.

Materials and methods

Growth of the cells: Thermophilic cyanobacterium *Synechococcus elongatus* Näg. var. *thermalis* Geitl. strain Kovrov 1972/8, from the collection of Biophysical Laboratory, Academy of Sciences, Krasnojarsk, Russia, was grown at 56 °C in the nutrient medium A (Kratz and Myers 1955) modified by addition of 10.0 mM $NaHCO_3$. The medium and all stock solutions were prepared from de-ionised water (*Millipore*). Cultures were grown in glass flat-cuvette bioreactors with 1000 cm^3 working volume. Bioreactors were irradiated by 50 W halogen light bulbs (*Osram*, Chemnitz, Germany) from one side (irradiance of 560 $\mu mol\ m^{-2}\ s^{-1}$) and aerated by a mixture of air with 2 % ($cm^3\ m^{-3}$) of CO_2. At the beginning of the cultivation, the cells were grown in a batch culture with high iron concentration (200.0 μM). The continual cultivation started at the end of the lag phase. When the culture has stabilised, cells were harvested, washed with medium without addition of iron sodium chelate, and diluted into three growth media: high-iron (HI) culture, starting optical density (OD_{750}) = 0.66, iron concentration 200.0 μM, dilution rate 0.045 h^{-1}. LI culture: starting OD_{750} = 0.75, iron concentration 9.0 μM, dilution rate 0.042 h^{-1}. ID culture: starting OD_{750} = 0.75, iron concentration 0.9 μM, dilution rate 0.047 h^{-1}. Continual cultivation started in all cultures (HI, LI, ID) simultaneously. The culture in the standard (HI) medium served as a control. All cultures were monitored by measurements of absorbance and dry mass. Samples from all cultures in the bioreactors were collected once a day in order to evaluate changes in cell morphology and photosynthetic performance.

OD_{750} measurements: Cell suspension was diluted five times with distilled water and absorbance was measured at 750 nm by *Spekol 11* (*Carl Zeiss*, Jena, Germany) spectrophotometer.

Dry mass: This parameter was chosen instead of cell counting due to the difficulties caused by the cell aggregation. 10 cm^3 of suspension was centrifuged at 2 200×*g* for 20 min at 4 °C, dried at 105 °C for 24 h, and weighted. The control experiment with 36 h of drying was performed. Dry mass was constant after 24 h.

Absorption spectra of samples diluted 5 times (with medium) were recorded on *Shimadzu UV-3000* (Japan) spectrophotometer with medium as a reference in the wavelength range from 350 to 800 nm.

Photosynthetic activity was measured as oxygen production of whole electron transport chain. Oxygen chamber (Bartoš *et al.* 1975) with the volume of 5 cm^3 was kept at 30 °C. Clark-type oxygen electrode (*YSI/5331* Oxygen Probe, Yellow Springs, USA, polarization 0.7 V) was connected to *Oxy-recorder* (*Photon System Instruments*, Brno, Czech Republic). Current from the electrode was recorded by Oxywin software (*Photon System Instruments*, Brno, Czech Republic). Electrode was calibrated to steady state of oxygen in water at 30 °C (230 $mol\ m^{-3}$), zero level was estimated by bubbling water with gas nitrogen (99.9 %). Suspensions of HI, LI, and ID cells were diluted to OD_{750} = 0.13 with appropriate medium. After 3 min of dark adaptation, the actinic saturating radiation was switched on (580 $\mu mol\ m^{-2}\ s^{-1}$) for 250 s.

Fluorescence measurements: The minimum (F_0) and maximum (F_m) components of Chl *a* fluorescence were

measured by pulse modulated fluorometer (*PAM101*) designed by Schreiber (1986) and produced by *Walz* (Effeltrich, Germany), and connected to *Oxy-recorder* (*Photon System Instruments*, Brno, Czech Republic). After 3 min of dark adaptation, 8 cm^3 of cell suspension (OD_{750} = 0.13) was injected into a temperature-controlled cuvette with a 2 mm thickness of the suspension layer (Komenda *et al.* 1992), and measured. Values were recorded by Oxywin software (*Photon System Instruments*, Brno, Czech Republic). For the measurement of F_m, the suspension of the cells was irradiated with 1-s saturating flash from halogen illuminator of 5 000 to 75 000 μmol m^{-2} s^{-1} of photosynthetic active radiation. The fluorometer was used with a standard combination of filters designed by the supplier. F_v/F_m values were calculated according to formulae given in Krause and Weis (1991).

Non-denaturing gel electrophoresis: For analysis of pigment-proteins, cells were ultrasonicated 5 times for 30 s at 10 kHz, centrifuged for 3 min at 240 000×*g* and the supernatant was solubilised in dodecylmaltoside (detergent : Chl *a* = 10 : 1) for 20 min. Extracts were analysed by non-denaturing electrophoresis in 5 to 10 % gradient acrylamide gel according to the method of Laemmli (1970), except that SDS was replaced by 0.2 % *Deriphat 160* in upper electrophoretic buffer, the gel contained no detergent. Samples were loaded on the gel in two concentrations: high (14 μg Chl *a*) and low (7 μg Chl *a*), respectively.

Pigment extraction and analysis by high performance liquid chromatography (HPLC)

Sample preparation: 5 cm^3 of cell suspension was centrifuged at 42 000×*g* for 5 min at 4 °C. The pellet was re-suspended on *Vortex* in 5 cm^3 of 100 % (cm^3 m^{-3}) methanol, pigments were extracted for 5 min, and then the mixture was centrifuged at 4 200×*g* for 10 min at 4 °C.

Chl concentration: Absorbance of supernatant was measured on *Shimadzu UV-3000* at 665.2 nm (A_{750} = 0). The extinction coefficient $\varepsilon_{665.2}$ = 79.95 l g^{-1} cm^{-1} (Porra *et al.* 1989) was used to measure the total concentration c_{Chla}.

HPLC setup: Pigments were separated on reversed-phase column (*Separon SGX C 18*; particle size 10 μm; 150×3 mm; *Tessek*, Praha, Czech Republic). Mobile phase was pumped by *LCP 3001* pump (*Ecom*, Praha, Czech Republic) at flow rate 33 mm^3 s^{-1}. Column was equilibrated by 90 % (cm^3 m^{-3}) methanol in water for 20 min. Pigments were eluted by linear gradient (*GP3* gradient maker; *Ecom*, Praha, Czech Republic): 0 to 9 min 90 to 100% methanol, and then isocratically with 100 % methanol until β-carotene was eluted from the column. Pigments were detected by UV-VIS diode array detector (model *996*, *Waters*, Milford, USA). Data were collected and processed by *Millennium 32* software (*Waters*, Milford, USA).

Electron microscopy and membrane counting: Samples of cells were fixed in a mixture of 2.5 % glutaraldehyde and 2 % OsO_4 for 3 h, dehydrated through ascending concentrations of acetone, and embedded in Spurr (*Polysciences*, USA). Ultrathin sections were cut on ultramicrotome (*Leica ultracut UCT, Leica*, Wien, Austria), stained with 2 % uranyl acetate and lead citrate, and examined by a *JEM 1010* transmission electron microscope (*JEOL*, Tokyo, Japan) using 80 kV. Thylakoid membranes in 100 cells of each culture (HI, LI, ID) were counted at 150×150 nm squares. Statistical analysis was performed by one-way parametric ANOVA test and by Tukey HSD honest test.

Results

S. elongatus was cultivated in continuous flow reactor at high iron (HI), low iron (LI) and iron deficient (ID) conditions. The cultivation regimes were as follows:

(*1*) In HI culture (Fe concentration 200.0 μM) cells were grown in the medium optimised for batch cultivation (Kratz and Myers 1955). In this medium, cells were able to multiply without any observable physiological changes and thus there was no nutrient limitation in this continuous culture. The culture reached a stable density, optimal for given irradiance and the flow rate of the medium within two days. At given irradiance (560 μmol m^{-2} s^{-1}) the growth of the culture was limited by radiant energy. In the experiments with higher irradiances, cells showed increasing tendency to aggregate and the culture was not homogenous (values not shown).

(*2*) LI culture (Fe concentration 9.0 μM). The iron concentration was set to the lower limit of interval in which cells were capable to maintain the same cell density and dry mass as in standard medium, *i.e.*, cell growth was not limited by nutrients at given irradiance. However, under these conditions significant structural changes of photosynthetic apparatus accompanying iron deficiency have already occurred.

(*3*) ID culture (iron concentration 0.9 μM), where the dilution rate of the medium had to be decreased in order to maintain stable OD and dry mass. Dry mass in the culture was lower than that in HI or LI cultures. In this culture we examined changes occurring at concentrations of iron limiting cell growth.

Cells in LI and ID cultures doubled at almost the same

rate as HI cells (*ca.* 15 h). Comparisons of dry mass, Chl *a* concentration, and oxygen evolution are summarised in Table 1. The numbers of cells are not given since these were obscured by high tendency of cells to aggregate in HI and also by high number of undivided cells in LI and ID (Fig. 1).

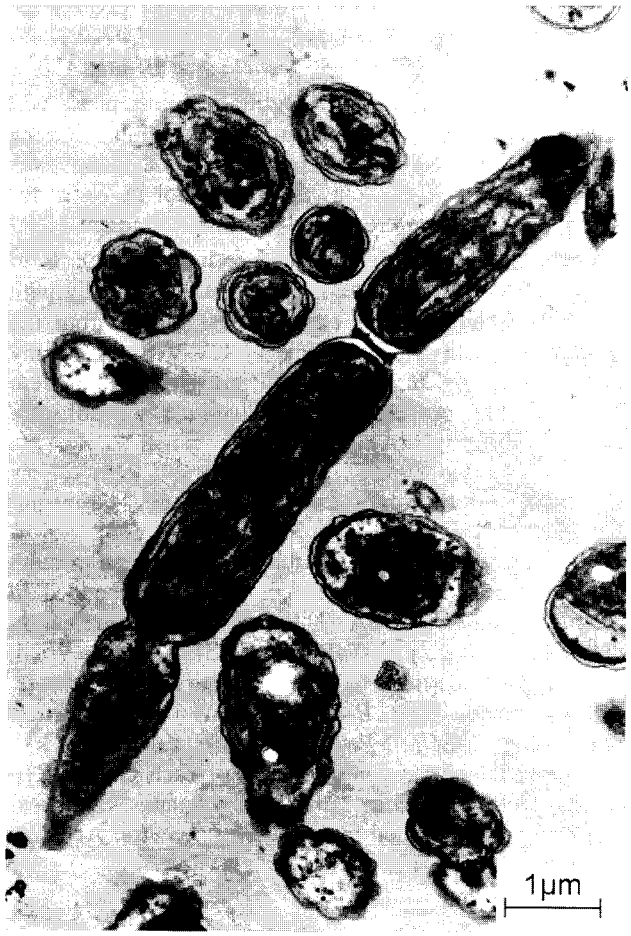

Fig. 1. Electron microscopic picture of thin layer sections of undivided cells in ID culture. Bar represents 1 μm.

The Chl concentration (Table 1) represents the amount of pigments in the lipid bilayer of thylakoid membrane which are bound to photosynthetic complexes. The rate of oxygen evolution per cell (cultures of the same OD_{750}) decreased in the order of HI>LI>ID. However, when the values were normalised to Chl concentration, the values showed the opposite tendency. Yet, when the O_2 evolution was normalised to the dry mass of the culture, no simple tendency was found. The relative rate of oxygen evolution of the ID culture was approximately 5-fold lower than that of the HI culture and 9-fold lower than that of LI culture.

The activity of PS2 measured as the F_v/F_m ratio (Table 2) decreased in the order HI>LI>ID. Comparing to LI and HI cells, the ID cells had also much higher relative concentrations of all carotenoids (Fig. 2).

The electron microscopy pictures of the thylakoid membranes (Fig. 3) show that both LI (Fig. 3*B*) and ID

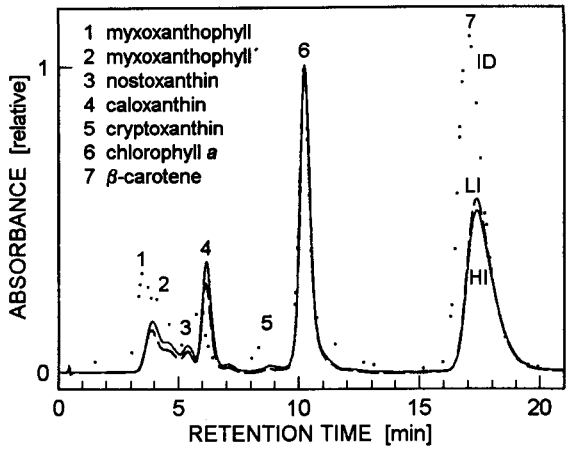

Fig. 2. HPLC chromatogram of pigment extracts from HI culture (*solid line*), LI culture (*dashed line*), and ID culture (*dotted line*). Curves were normalised on Chl *a* concentration. Significant increase of myxoxanthophylls (*1, 2*), cryptoxanthin (*5*), and β-carotene (*7*) was detectable in the ID culture.

(Fig. 3*C*) cells were less ordered and visually thinner than those of HI (Fig. 3*A*). The careful examination of multiple pictures revealed that the ID cells had significantly less ($p<<0.01$) thylakoid membranes than the HI and LI cells, respectively (Table 3). The ID cells (Fig. 3*C*) also differed from LI cells (Fig. 3*B*) by clear disconnection of the cell wall from the rest of the cell.

The absorption spectra (Fig. 4) show a decrease in the signal of phycobilins (maximum at 634 nm) in both ID and LI cells and a change in the Soret band, mainly visible as the increase at the 485 nm shoulder. This can be related to the increase of the carotenoid content of cells.

The non-denaturing gel electrophoresis pattern (Fig. 5) was in HI culture identical to those found generally in cultures from batch cultivations (Komenda and Masojídek 1995). Three main differences were found in ID culture: (*1*) a proportion of phycobilins was not disaggregated by the detergent and was present as a high molecular mass complex on the gel, (*2*) a new Chl-binding protein of molecular mass lower than that of phycobiliproteins was observed, and (*3*) a very low molecular mass carotenoid-containing band appeared on the gel. In the same position, a protein is detectable by Coomassie blue staining (not shown). The identity of this band as a carotenoid-binding protein has to be subject of further experiments. In the LI culture, the carotenoid band is a major one. The second major difference between the non-denaturing gel of ID and of the other cultures is the absence of distinct bands in the region of PS1 and PS2. On the other hand, in LI culture there was higher concentration of PS1 than in the cells from the HI culture.

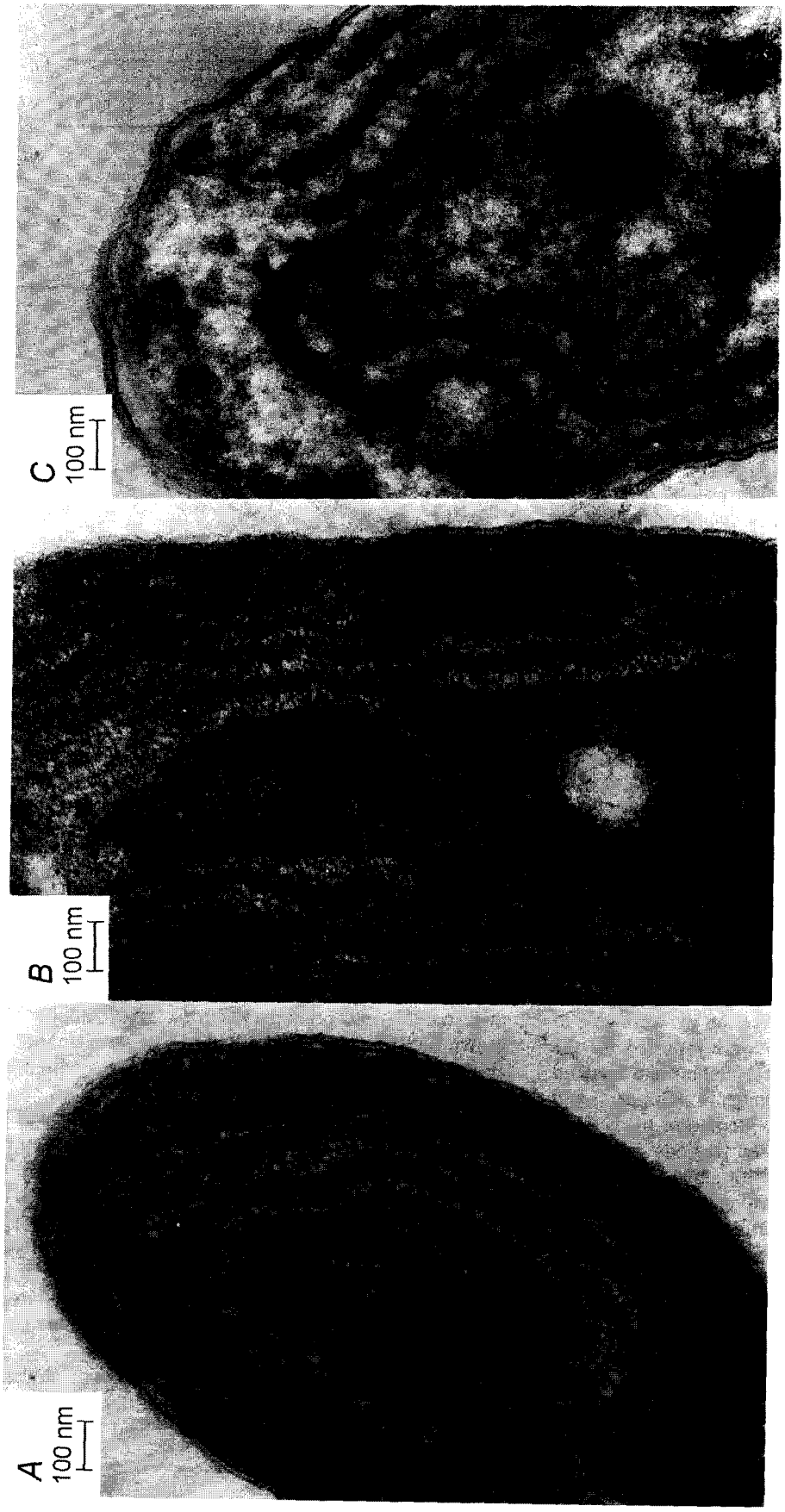

Fig. 3. Electron microscopic picture of thin layer sections of cells of HI (*A*), LI (*B*), and ID (*C*). The number of thylakoid membrane lamellae per square unit of cell interior was identical in HI and LI cultures. In the ID culture the number of membranes was 1.5 times lower. Bar represents 100 nm.

Table 1. Characteristics of high iron (HI), low iron (LI), and iron deficient (ID) cultures of thermophilic cyanobacterium *Synechococcus elongatus*. All values were obtained as described in the Material and methods section.

	Dry mass [kg m^{-3}]	Chl *a* [g m^{-3}]	Photosynthetic oxygen evolution (POE) [μmol(O_2) s^{-1}]	POE per dry mass [mmol(O_2) kg^{-1} s^{-1}]	POE per Chl *a* [mmol(O_2) kg^{-1}(Chl) s^{-1}]
HI	1.595 ± 0.02	25.450 ± 0.4	2.323 ± 0.03	1.457 ± 0.02	91.306 ± 2.2
LI	1.320 ± 0.03	18.207 ± 0.4	3.120 ± 0.03	2.364 ± 0.02	171.389 ± 1.4
ID	0.645 ± 0.01	0.504 ± 0.03	0.178 ± 0.01	0.276 ± 0.01	352.577 ± 15.4

Table 2. Chl *a* fluorescence parameters.

	F_0	F_m	F_v/F_m
HI	0.2993 ± 0.02	0.4967 ± 0.07	0.3900 ± 0.05
LI	0.3133 ± 0.01	0.4467 ± 0.02	0.2983 ± 0.01
ID	0.2350 ± 0.03	0.2565 ± 0.02	0.0751 ± 0.01

Table 3. Thylakoid membrane number comparison after the statistical evaluation by one-way parametric ANOVA test and Tukey HSD honest test (F values are given).

	HI	LI	ID
HI	x	0.993056	0.000022
LI	0.993056	x	0.000022
ID	0.000022	0.000022	x

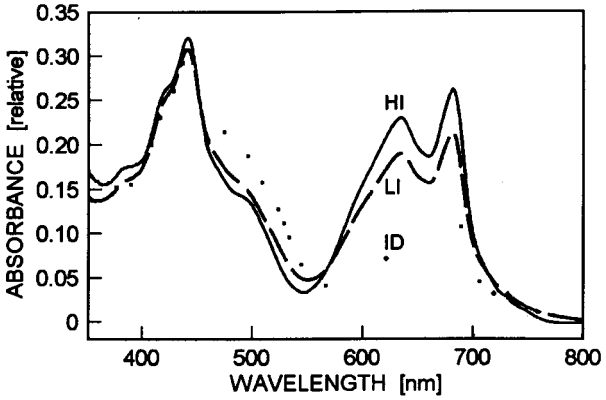

Fig. 4. Changes in absorption spectra accompanying the decrease of iron concentration in the medium. Spectra were recorded from HI culture (*solid line*), LI culture (*dashed line*), and ID culture (*dotted line*). Decrease at the maximum 634 nm was caused by the decrease of phycobilins, increase of the shoulder at 485 nm indicated the increase of carotenoids.

Discussion

Continuous cultivation was performed under conditions ensuring a steady, high supply of all nutrients except iron. This is in contrast to most previous studies (Vassiliev *et al.* 1995, Henley and Yin 1998) where cyanobacteria were cultivated in batch cultures under steady depletion of nutrients. In the continuous cultivation we were able to simulate natural conditions which were reported for the coastal zones of the Pacific Ocean (Hutchins and Bruland 1996) and in freshwater environments (Gunnars and Blomqvist 1997). In these regions depletion by N or P was not reported, although in the regions with less intensive mixing there might be areas of uneven distribution of iron. According to Gunnars and Blomqvist (1997) the removal of phosphorus after the positive redox-turnover seems to be controlled by the availability of precipitating iron in both freshwater and marine systems.

There are no data concerning nutrient concentration in the natural environment where the studied strain of *S. elongatus* was collected. However, it has been successfully cultivated in our laboratory for more than twenty years. Cyanobacteria growing in natural conditions use aggregation as one of the protective mechanisms against high irradiance (Pechar and Masojídek 1995). However, most cyanobacterial strains after several generations of laboratory cultivation lose their ability to aggregate. In this respect, the strain used in this study is exceptional (Koblížek *et al.* 2000). Cells grown in HI medium are still able to aggregate, but this ability is lost after their transfer into the medium with lowered iron concentration.

S. elongatus is capable to maintain the activity of PS2 in oxygen evolution per Chl unit in a wide range of conditions including those where the cell growth is limited by the concentration of the essential element, iron (as shown in this study). This is achieved by at least two adaptation strategies that are expressed in variations in PS2 efficiency relative to Chl and dry mass. Experiments with the aim to find the lowest concentration of iron at which stable cultivation conditions can occur are in progress.

Most of the carotenoids co-purify with thylakoid membranes (Komenda and Masojídek 1995) but carotenoid-binding proteins were detected also in plasma

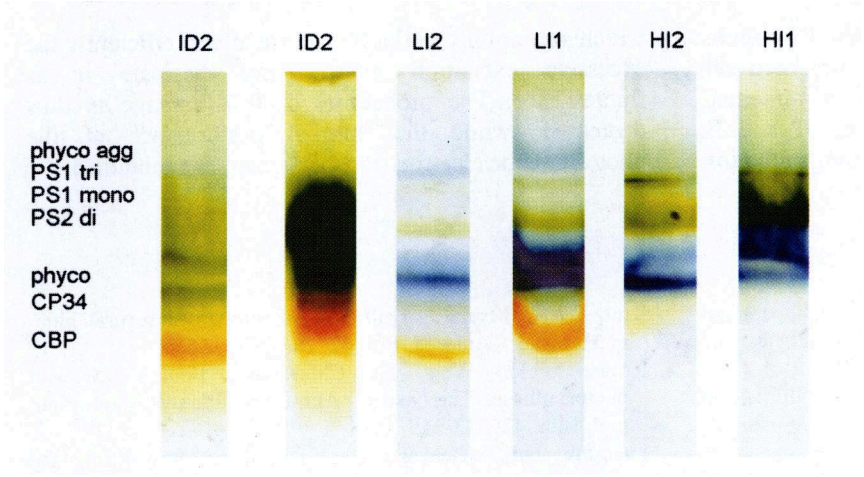

Fig. 5. Non-denaturing gel of detergent extracts of sonicated HI, LI, and ID cells. Lanes 1 and 2 of each sample represent high (14 μg Chl *a*) and low (7 μg Chl *a*) loading, respectively. Individual bands are denoted as follows phyco agg - nondesagreggated phycobilins; PS1 tri - photosystem 1 trimers; PS1 mono - photosystem 1 monomers; PS2 di - photosystem 2 dimers; phyco - phycobilins; CP34 – PS2 chlorophyll-binding protein, *isiA* gene product; CBP – carotenoid-binding protein and free pigments.

membranes (Holt and Krogmann 1981). We have observed that significant amount of carotenoids transfers to the water phase upon sonication of the cells (data not shown).

Iron is also an important cofactor in Chl biosynthesis and inorganic nitrogen assimilation. Chlorosis is a universal indication of iron limitation (Öquist 1971). The response to lowered Fe concentration in the medium is also accompanied by a decrease in the phycobilin concentration. Phycocyanobilin and phycoerythrobilin are synthesised by the ferredoxin-dependent reduction of biliverdin, which is formed by the oxidative ring-opening activity of heme oxygenase on protoheme (Beale and Cornejo 1983). The resulting changes in colour are visible by naked eye and easily detectable by absorption spectroscopy (Fig. 4). Most probably, the decrease in the amount of phycobilins is responsible also for the less dense occupancy of cell interior by thylakoid membranes in ID cells and LI cells in comparison to HI cells (cf. Fig. 3*C*,*B* to 3*A*). On the negatively stained samples phycobilisomes cannot be seen, because their visuali-sation requires special staining (Bryant *et al.* 1979). The total number of membrane lamellae per square unit of the cell interior in ID cells decreased 2-fold in comparison to HI and LI cells. Sherman and Sherman (1983) reported 3- to 4-fold reduction in the number of thylakoid membranes seen in cross-sections of iron-deprived *A. nidulans*. However, the LI and ID cells were always shorter than HI cells, generally about two-thirds the length (for similar findings see Sherman and Sherman 1983). Therefore, although the absorbance was similar in all cultures, the number of cells in LI and ID cultures was probably substantially higher in comparison to HI cells.

The non-denaturing gel electrophoresis (Fig. 5) demonstrated that not only the amount of phycobilins, but also the composition and/or stability of the phycobilin complex was changed. It is manifested by the presence of high-molecular mass phycobilin complex in the gel of ID cells. In the same time, in ID cells a low-molecular mass Chl-binding protein was found, possibly the CP 34 protein (Michel and Pistorius 1992). The LI cells exhibited dramatical decrease in the concentration of phycobilins. Chl-binding proteins in the sample from the ID culture did not form distinct bands in the area between expected mobility of PS1 trimers, PS1 monomers, and PS2 dimers. This may be attributed either to gradual dissociation of PS1 trimers during the electrophoresis or to the existence of multiple complexes of photosystems with other membrane proteins, *i.e.*, the recently found small Cab-like unusual antenna proteins (Funk and Vermaas 1999). The most probable explanation is the continuous dissociation of PS1 trimers during the electrophoresis run. The low-molecular mass Chl-binding protein was still present as well as in ID cells, however, the dominant pigment became β-carotene, which is possibly bound to a protein. The increased amount of β-carotene and other carotenoids was nocfirmed by HPLC analysis (Fig. 2). Pigment proteins with individual complexes were identified earlier (Komenda and Masojídek 1995) by re-electrophoresis of bands excised from non-denaturing gel on denaturing electrophoresis. The lowest molecular mass band (CBP) could be formed of free pigments, although the staining by Coomassie blue indicated presence of some proteins in the same position (not shown).

The activity of photosynthetic apparatus measured by photosynthetic oxygen evolution normalised to Chl *a* concentration and by variable fluorescence exhibited the opposite tendencies. While the F_v/F_m was maximal in the HI culture, the relative oxygen evolution rate was maximal in ID culture. The decrease in F_v/F_m may be

also attributed to high relative F_0 level in the ID culture. This is, besides the changes observed in the green gel, another indication of reconstruction of Chl antennae apparatus in the ID culture compared to HI and IL cultures. We propose that the Chl-binding proteins, which appear only in the ID culture, divert efficiently the excessive excitation energy and dissipate it as fluorescence. The probability of PS2 damage is thus decreased, while the optimal performance of the photosynthetic electron transport chain is maintained.

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