Cytomorphology of six halotolerant coccoid cyanobacteria using DAPI fluorescent and transmission electron microscopy, compared with molecular data

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Abstract: Cytomorphological characters of six strains of unicellular cyanophytes, all belonging to "*Euhalothece/ Halothece*" group in the sense of Garcia–Pichel et al. (1998), but of different hypersaline origin, have been studied using DAPI fluorescent staining and light and transmission electron microscopy. They can be divided into two clades, which are well separated according to phenotypic taxonomy (morphology, cytology and ultrastructure). The first group (four strains) could be included in the subcluster "*Euhalothece"* (on the generic level, but not yet validly described). Broad oval cells of ca 4–6 μm breadth with indistinct chromatoplasm, net like nucleoids and parallel lengthwise–arranged thylakoids often in fascicles are characteristic for this group. The second subcluster should be classified into another genus because of oblong or rod–shaped cells of smaller size (cca 2–4 μm wide), peripheral "chromatoplasm" (position of mostly lengthwise parietal thylakoids) and more or less compact nucleoid. These strains belong into the vicinity of the genus *Cyanobium*.

Key words: Cyanobacteria, *Cyanobium*, *Cyanothece*, DAPI staining, *Halothece*, *Euhalothece*, nucleoid, taxonomy, TEM

Introduction

Contemporary cyanobacterial taxonomy often follows combination of two methodological approaches. The "classical" method, based on visible characters (morphological, cytological, physiological and ultrastructural) together with ecology should be combined with increasingly employed modern molecular methods, e.g. DNA base composition (Herdman et al. 1979), DNA/DNA hybridisation (Stam & Stulp 1988), sequence–specific deoxyribonucleases (Waard & Duyvesteyn 1980), nucleotide sequence of the 16S rRNA (Wilmotte et al. 1992) and amplified rDNA restriction analysis (Garcia–Pichel et al. 1998). However, most studies utilize only one approach for two reasons. First, many researchers lack certain phylogenetically important strains. Second, information relating to original or natural materials is often difficult or impossible to obtain. Further, molecular data are not always in agreement with classical botanical characterization and the synthesis of such results is difficult in some cases.

Garcia–Pichel and colleagues studied 13 strains of extremely halotolerant coccoid cyanobacteria on the basis of 16S rRNA gene sequences (Garcia–Pichel et al. 1998). They separated the *"Halothece"* subcluster (represented by only one strain originating from Mexico), which was later validated as a genus by Margheri et al. (2008), due to a complex evaluation of genetics, morphology, ecophysiology and ultrastructure. The second subcluster "*Euhalothece"* contains 12 strains, which were all more than 95% similar in 16S rRNA gene sequence one to another, but they were not yet validly described on the generic level. Margheri et al. (1999) assessed genetic diversity of a set of novel 12 strains (using amplified 16S rDNA restriction analysis), and after comparison with the dendrogram depicted by Garcia–Pichel et al. (1998) they concluded that these strains belong also to *Euhalothece* subcluster (cf. Fig. 13).

The aim of our study is to provide phenotypic characterisation and diversity of some strains studied by Margheri et al. (1999) and compare them with the genetic diversity represented by 16S

rRNA gene sequence analysis. We conclude that results obtained by both approaches are congruent (the botanical approach is in agreement with the genetic one).

Materials and methods

Organisms and culture conditions. The strains CE 9, TP 5, 16Som 2, CA 3, VI 22, and PE 14 were obtained from Dr. M.C. Margheri and their origin is listed elsewhere (MARGHERI et al. 1999). All of the strains originated from hypersaline environments. The cultures were grown in test tubes (10 ml) in liquid medium (commercial seawater salts – 33.33 g.l^{-1}, $K_2HPO_4.3H_20$ – 0.04 g.l^{-1}, $NaHCO_3$ – 0.1 g.l^{-1}, citric acid – 0.003g.l^{-1}, ferric ammonium citrate – 0.003 g.l^{-1}, EDTA sodium salt – 0.0005 g.l^{-1}, trace elements of BG–11 medium – 0.5 ml per litre). Cultures were slightly aerated, temperature maintained at 30°C and continuous white light adjusted to about 50 μmol m^2.s^{-1}.

DAPI staining and fluorescence microscopy. Month–old cells were centrifuged, the resulting pellet resuspended in Tris buffer (pH=7.2), and cells fixed in 1% glutaraldehyde at 20°C for 30 min. DNA was stained with DAPI (1 μg ml^{-1} in Tris buffer) as described earlier (CEPÁK 1996a). The preparations were examined with an epifluorescence microscope (OLYMPUS BX 60, Japan) connected to a COHU High Performance LLD.

Transmission electron microscopy. Samples preserved with 2% of formaldehyde were washed, postfixed with glutaraldehyde in cacodylate buffer and stained with OsO_4. After dehydration in an acetone series the samples were embedded in Spur resin, cut, and then investigated in a transmission electron microscope (Jeol JEM 1010, Japan).

Results and discussion

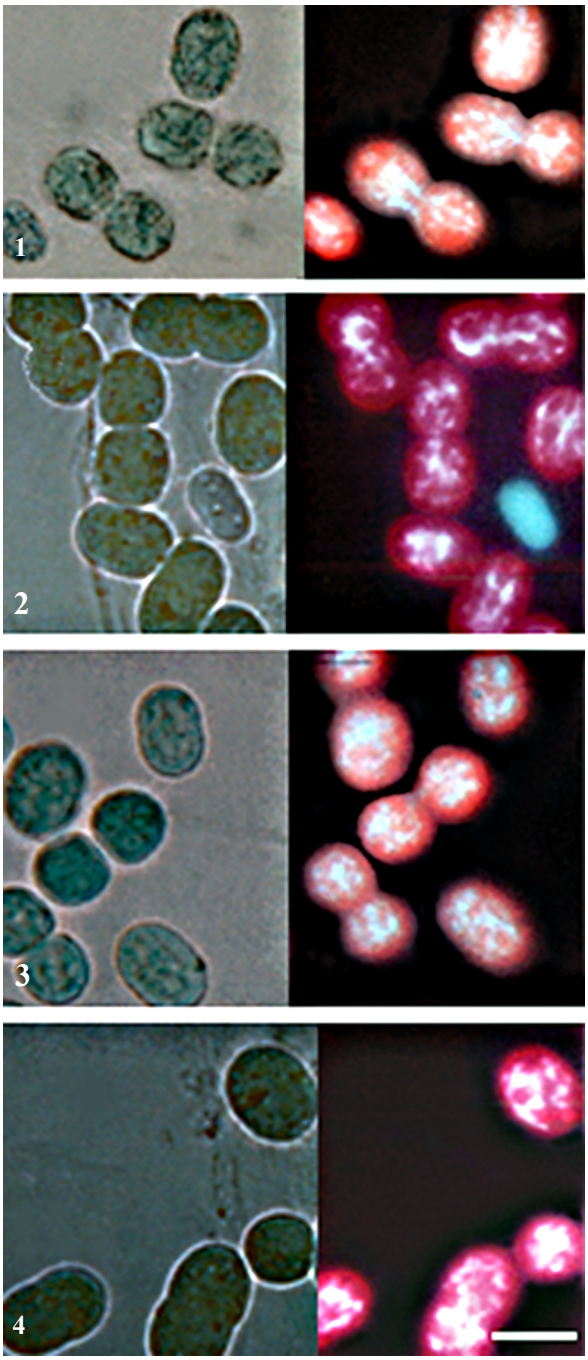
We examined six strains of extremely halotolerant coccoid cyanobacteria isolated from hypersaline environments (MARGHERI et al. 1999). Using traditional taxonomic approaches, we report in more detail the cytomorphology of nucleoids using DAPI fluorescence staining and thylakoid patterns employing transmission electron microscopy. Our results were compared with the most closely related genera *Cyanobium, Cyanobacterium, Halothece* and *Cyanothece* (Table 1). We conclude that the strains examined can be divided into two groups, which are well separated with their cytomorphological and ultrastructural characters (I and II in Table 1).

The first group contains a uniform type of cells which are broadly oval–shaped and 4–6 μm in size (Figs 1–4). The size and outer shape of nucleoids coincide well with the dimensions and shape of the cells. DNA is concentrated into small skeins of various shape–oval, elongated or globular, which are connected by thin filaments of DNA. In the fluorescent microscope indistinct or narrow peripheral layer was observed. Nucleoids similar in appearance were observed previously in *Cyanothece aeruginosa* (CEPÁK 1993, 1996a) and *Cyanothece halobia* (CEPÁK 1996a). Contrary to the strains above, which contain parallel and lengthwise–arranged thylakoids (indistinct fascicles) spread throughout the whole cell (Figs 7–10), the type species *Cyanothece aeruginosa* (KOMÁREK 1976, KOMÁREK et al. 2004) is distinguished by radially–arranged fascicles of thylakoids, small intrathylakoidal spaces, and distinctly larger dimensions. In addition, they differ in ecology, as *C. aeruginosa* was isolated from acidic marshes. For this reason the strains studied were compared with the halophilic strain CH1, designated as *C. halobia* (MARGHERI et al. 1999), which should be separated also from the genus *Cyanothece* and shifted into another genus. *Cyanothece halobia* (strain CH1) was studied previously by ROUSSOMOUSTAKAKI & ANAGNOSTIDIS (1991), CEPÁK (1996a) and KOMÁREK et al. (2004). According to GARCIA–PICHEL et al. (1998) and MARGHERI et al. (1999), the cluster of "*Euhalothece"*, to which belong also the studied strain (Table 1, group 1) seems to be suitable genus different from *Cyanothece* in the original sense for such cyanobacteria, but has not yet been validly described and published. "*Euhalothece*" and "*Cyanothece* sensu Rippka" contain more then 30 strains deposited in various collections and need further studies. The validation of these genera under the Botanical Code, containing these strains (it concerns also the related strain PCC8305 of "*Dactylococcopsis salina*") is expected, but needs further data and therefore will be solved in the next study.

The second group of strains posesses smaller–sized cells, which are oblong or rod–shaped and about 2–3(4) μm long. In contrast to the first group, we have observed more compact nucleoids with big skeins of DNA without visible DNA threads (Figs 5,6). A distinct layer of "chromatoplasm" was visible and parietal thylakoids concentrated along the cell walls are typical for these strains (Figs 11,12). Both strains fit the description of the *Cyanobium* type according to cell size and shape and nucleoid morphology

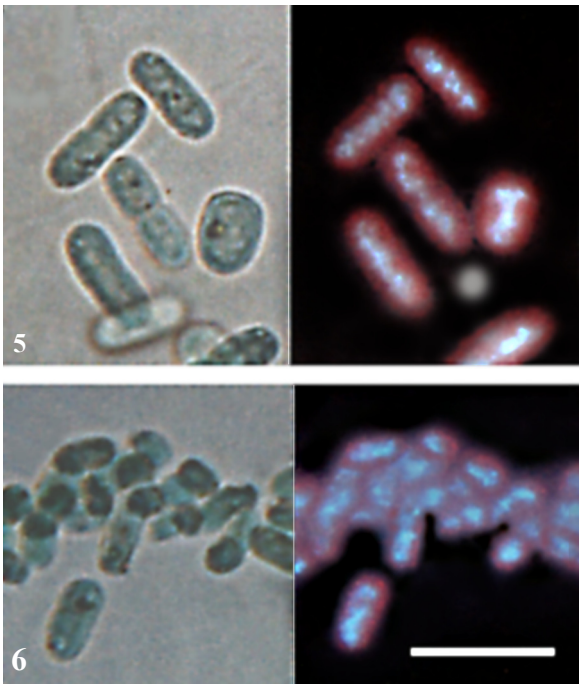
Table 1. Cytomorphological characters of strains studied (I and II) compared with the most related generic units (III and VII)

Group	Strain	Cell breadth (μm)	Cell shape	Thylakoid pattern	Nucleoid morphology	Ecology
I. *"Euhalothece"*	CE 9 CA 3 VI 22 16Som 2	4.5 – 6.5	broad oval	parallel lengthwise arranged thylakoids through the whole cell volume (fascicles distinct or indistinct)	Net–like many fine skeins of DNA connected by thin filaments of DNA	halophilic or halotolerant
II. *Cyanobium*–like strains	TP 5 PE 14	2.0 – 3.5	oblong or rod–shaped	thylakoids concentric, parietal	Compact–granular several indistinct big skeins of DNA	halophilic strains (the genus contains both, freshwater and halotolerant types)
III. *Cyanothece aeruginosa* (type species)	NC–258 (reference strain) SAG 87.79	7.0 – 36.0	oval	thylakoids radially arranged, irregular fascicles	Net–like several large skeins of DNA connected by filaments of DNA	acidic (peaty) waters
IV. *Cyanobium gracile* (type species)	PCC6307 (type strain)	0.2 – 0.4	oblong or rod–shaped	thylakoids arranged concentrical, parietal	Compact rod–like skeins of DNA	probably freshwater (described from culture)
V. *Halothece californica* (type species)	MPI 96P605 (type strain)	2.6 – 7.8	oval to cylindrical	irregularly lengthwise in cells	Net–like agglomerated skeins of DNA connected by filaments	halophilic
VI. *Cyanobacterium stanieri* (type species)	PCC 7202 (type strain)	(1.0)2.0–4.5(7.0)	cylindrical or slightly oval	parallely, densely along the whole cell volume (not fasciculated)	Granular to net–like irregular in centres of cells	freshwater (described from culture)
VII. freshwater "*Cyanothece*" sensu Rippka	PCC 8303 PCC 8995 PCC 6910 PCC 8801	1.8–4	widely oval, rarely to almost ± spherical	thylakoids ± concentrated parietally with irregularities	Net–like	freshwater, paddy fields



Figs 1–4. Cells under the light microscope (left row) and the same DAPI fluorescent micrographs of "*Euhalothece"* strains (right row). Cells are large broad oval with net–like nucleoids: (1) CE 9; (2) VI 22; (3) 16Som 2; (4) CA 3. Scale bar 5 μm.

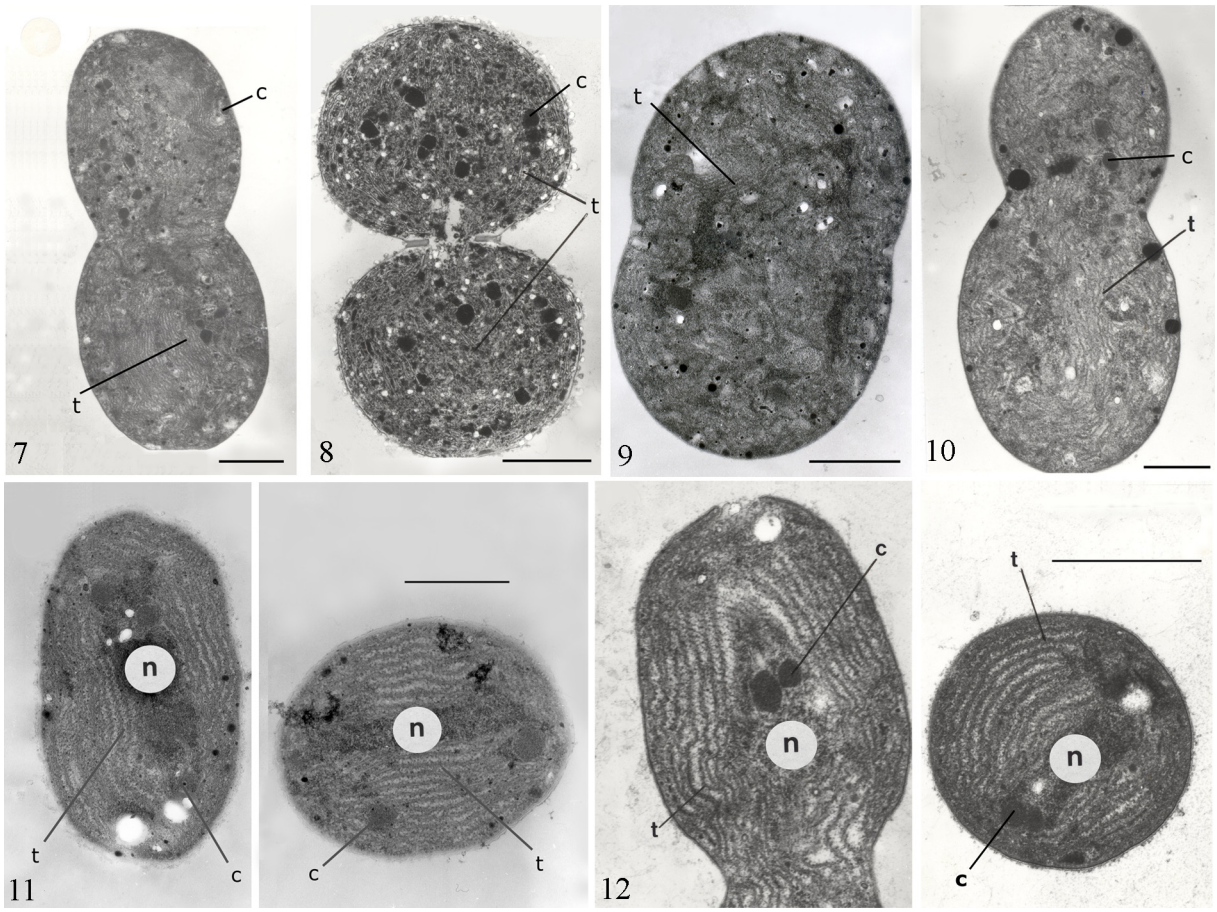
(KOMÁREK et al. 1999). Thylakoids are arranged lengthwise and mostly concentrically (parietal arrangement).



Figs 5, 6. Cells under the light microscope (left row) and the same DAPI fluorescent micrographs of *Cyanobium*–like strains (right row). Strains are smaller with oblong or rod–shaped cells and granular, more or less compact nucleoids: (5) TP5; (6) PE 14. Scale bar 10 μm.

Summary: *"Halothece/Euhalothece"* cluster *sensu lato* was defined by GARCIA–PICHEL et al. (1998) on the basis of 16S rRNA gene sequence analysis as a group of extremely halotolerant cyanobacteria (sustained growth in the range 6–16% salinity). However this monophyletic cluster includes strains belonging to different genera according to the traditional botanical system as well as molecular evaluation (deviations in the phylogenetic tree). The designation of "*Halothece*" as a separate cluster was restricted to one strain MPI 96P605 (CASTENHOLZ 1969, MARGHERI et al. 1999, TURNER et al. 2001, KOMÁREK et al. 2004); its generic status and relations to "*Euhalothece*" were confirmed by MARGHERI et al. (2008).

Other "*Euhalothece*" strains and mostly halophilic "*Cyanothece*" strains sensu RIPPKA & COHEN–BAZIRE (1983) evidently also belong into few separated clusters. They are similar particularly with respect to main cell morphology (solitary oval cells) and nucleoid division. The cells divide only by binary fission (pinching), they do not form any chains (in contradiction to *Halothece* under special conditions in cultures) and newly formed daughter cells are alike. Nucleoid division is obviously passive because it occurred simultaneously with cell fission. This cluster is evidently different from typical *Cyanothece*, which is based on the species *C. aeruginosa.* According to our results, the generic status of *"Euhalothece"*, to which our strains CE9, CA3, VI 22 and 16Som 2 belong, is



Figs 7–10. Longitudinal section of cells of "*Euhalothece*"–like strains: (7) CE 9; (8) VI 22; (9) 16Som 2; (10) CA 3. Thylakoids are mostly longitudinally and irregularly spread throughout the whole cell volume, often in fascicles. Scale bar 1 μm.
Figs 11, 12. Lengthwise– (a) and cross–sections (b) of cyanobacterial cells of *Cyanobium*–like strains: (11) PE 14; (12) TP5. Thylakoids (t) are lengthwise oriented, parietally and concentric; (n) „nucleoplasm", (c) carboxysomes. Scale bar 1 μm.

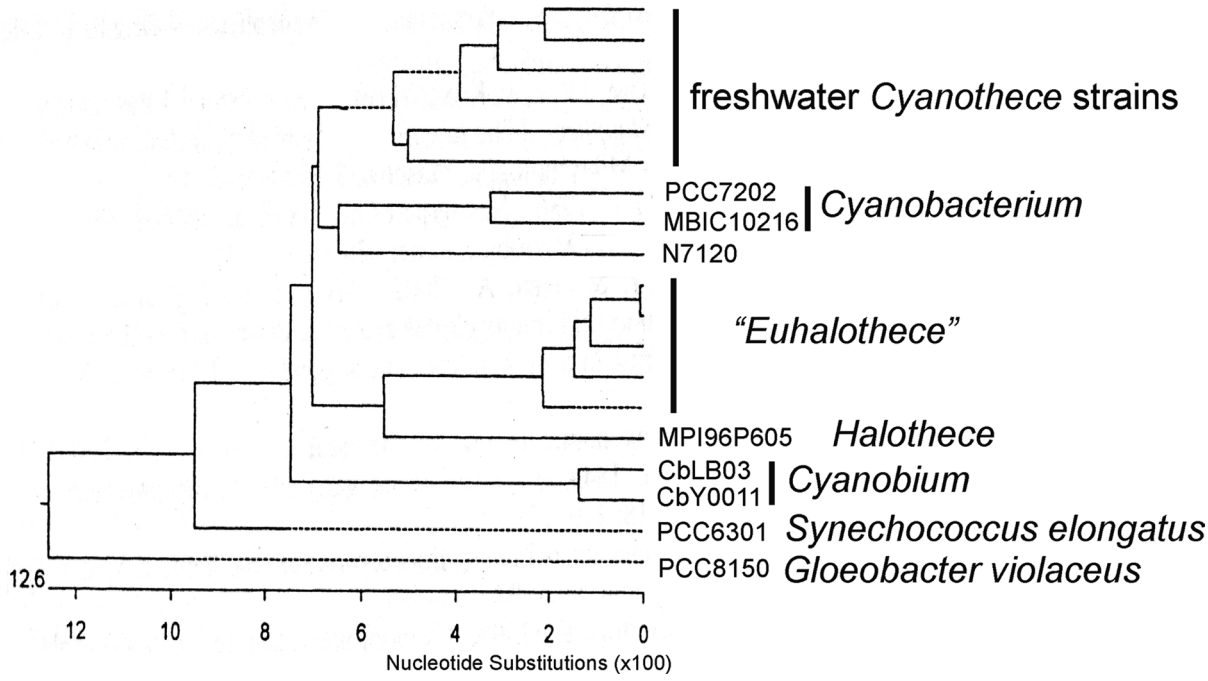


Fig 13. The phylogenetic tree derived from Megalign (DNA Star) from GenBank of NCBI, illustrating the clusters on the generic level ("*Euhalothece"*, *Halothece, Cyanobium* and *Cyanobacterium* strains). Partly derived from Margheri et al. (1999).

confirmed and should be classified as a special halophilic genus. Our studies also confirmed that the strain CH1 (*Cyanothece halobia*) belongs to this generic unit.

Two our other strains (TP5, PE14) belong in the genus *Cyanobium* Rippka et Cohen–Bazire 1983, and are mostly related to the halophilic species *Cyanobium bacillare*, which was, however, described only according the morphological characters (Komárek & Anagnostidis 1998). Our strains of "*Euhalothece*" and *Cyanobium* belong into the separate clusters, which are illustrated in Fig. 13 (cf. dendrogram in Margheri et al. 1999, Fig. 3). Little problematic is only the strain TP5. Their respective specific definition should be studied in future.

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