

# The central body of the cyanelles of *Cyanophora paradoxa*: a eukaryotic carboxysome?<sup>1</sup>

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**Abstract:** The cyanelles of the glaucocystophyte *Cyanophora paradoxa* combine two prokaryotic features not found in other phototrophic eukaryotes: a peptidoglycan wall and a putative carboxysome. Both of them would be indispensable when an inorganic carbon concentrating mechanism involving high accumulation of bicarbonate in the cyanelle stroma is assumed. Two approaches were used. (i) An expressed sequence tag library was generated allowing access to interesting genes and microarray technology. Hybridization of the microarrays to RNA from cells grown at high and low CO<sub>2</sub> yielded 97 genes that were upregulated under CO<sub>2</sub> stress whereas 87 genes were found to be downregulated. (ii) Cyanelle central bodies were isolated and protein components other than Rubisco were investigated by mass spectrometry. So far, mass spectrometric analysis of putative carboxysomal proteins yielded only sequences with no match in the databases. Rubisco activase could be shown via in vitro import and Western blotting to be copackaged with Rubisco in isolated purified central bodies. While our data support the presence of an inorganic carbon concentrating mechanism in cyanelles, they do not allow us to distinguish the microcompartment as a carboxysome or pyrenoid.

**Key words:** *Cyanophora paradoxa*, cyanelles, carboxysome, Rubisco activase, carbon-concentrating mechanism, microarrays.

**Résumé :** Les cyanelles de la glaucocystophyte, *Cyanophora paradoxa*, combinent deux caractéristiques procaryotiques qu'on ne retrouve pas chez d'autres eucaryotes phototrophes; une paroi de peptidoglucans et un présumé carboxysome. Ces deux caractéristiques seraient indispensables, si on assume qu'un mécanisme de concentration du CO<sub>2</sub> est impliqué dans l'accumulation du bicarbonate dans le stroma des cyanelles. Les auteurs ont utilisé deux approches. (i) Ils ont généré une librairie des séquences génomiques exprimées qui permet d'avoir accès à des gènes intéressants et à la technologie des puces à ADN. L'hybridation de puces à ADN avec l'ARN de cellules cultivées en présence de faibles ou de fortes teneurs en CO<sub>2</sub>, montre que 97 gènes ont été activés, alors que 87 ont été réprimés. (ii) Ils ont isolé les corps centraux des cyanelles et ils ont examiné les constituants protéiques, autres que la Rubisco, par spectroscopie de masse. Jusqu'ici, les analyses par spectroscopie de masse des protéines des carboxysomes ne conduit qu'à des séquences sans correspondance dans les banques de données. On a pu montrer que l'activase de la Rubisco, via importations in vitro et les transferts Western, est attachée à la Rubisco, dans des corps centraux isolés et purifiés. Bien que les données supportent la présence d'un mécanisme de concentration du CO<sub>2</sub> dans les cyanelles, elles ne permettent pas de distinguer le microcompartment comme un carboxysome ou comme un pyrénioïde.

**Mots clés :** *Cyanophora paradoxa*, cyanelles, carboxysome, activase de la Rubisco, mécanisme de concentration du CO<sub>2</sub>, puces à ADN.

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## Introduction

*Cyanophora paradoxa* is the most investigated member of the glaucocystophytes, an evolutionarily interesting algal phylum whose plastids (cyanelles) are surrounded by a peptidoglycan layer, a clear indication of their descent from

endosymbiotic cyanobacteria (Löffelhardt and Bohnert 2001). As is the case for many aquatic photosynthetic microorganisms, *C. paradoxa* is expected to possess an inorganic carbon concentrating mechanism (CCM) on the following grounds: preliminary mass spectrometric measurements of carbon isotope discrimination indicate the presence of a

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CCM (D. Sültemeyer, personal communication), and the central body of cyanelles constitutes a microcompartment that in all known cases is associated with a functional CCM (Badger et al. 1998). Increase of the CO<sub>2</sub> level in CCM microcompartments harboring Rubisco increases the efficiency of photosynthetic carbon fixation. Operation of the CCM is triggered by growth at ambient (0.0376%, Keeling and Whorf 2004) CO<sub>2</sub> concentrations with concomitant induction of CO<sub>2</sub> and bicarbonate transporters and components of the microcompartments (Kaplan and Reinhold 1999). These electron-dense structures are carboxysomes in prokaryotes and pyrenoids in eukaryotic algae. We favour the hypothesis that the cyanelles of *C. paradoxa* (belonging to the first phototrophic eukaryotes) retained yet another prokaryotic feature in addition to the peptidoglycan wall: the unique case of a eukaryotic carboxysome as exemplified by the conspicuous Rubisco-containing (Mangeney and Gibbs 1987) central body. An interesting hypothesis (Raven 2003) recently linked these two prominent characteristics of cyanelles together: the carboxysomal type of CCM involves accumulation of HCO<sub>3</sub><sup>-</sup> in the cyanelles to such an extent that the cytosolic osmolarity of the fresh water alga would not be able to counterbalance it. The stabilizing peptidoglycan layer prevents the cyanelles from bursting. Consequently, in the course of plastid evolution, both of these features, the peptidoglycan layer and carboxysomes, were abandoned together. However, an alternative hypothesis suggests that carboxysomes in cyanobacteria and pyrenoids in algae originated independently, together with their respective CCMs, about 400 million years ago, in response to a reduction in CO<sub>2</sub> levels and an increase in oxygen levels in the atmosphere (Badger and Price 2003). Thus, the possibility still exists that we are dealing with a pyrenoid in the cyanelles of *C. paradoxa*. In *C. paradoxa*, the central body, in general, has rounded boundaries without a recognizable shell, possibly because of its intimate association with cyanelle DNA and thylakoid membranes (Löffelhardt and Bohnert 2001). However, in some cases a polyhedral appearance can be noted. Moreover, in the cyanelles of the glaucocystophytes *Gloeochaete wittrockiana* and *Cyanoptylche gloeocystis* large polyhedral central bodies are confined by distinct shells. In this paper, we report the results of microarray-based gene expression analyses in response to low CO<sub>2</sub> levels and of attempts to characterize the cyanelle microcompartment.

## Materials and methods

### Algal cultures

*Cyanophora paradoxa* strain 1555 (Breiteneder et al. 1988) was grown as previously described (Steiner et al. 2000).

### cDNA libraries

*Cyanophora paradoxa* strain 1555 was grown under 6% CO<sub>2</sub> to the exponential growth phase and harvested at timepoint 0 h (for the high-CO<sub>2</sub> library) while parallel cultures were shifted to ambient (= low) CO<sub>2</sub> at 0 h and individually harvested after 2, 12, and 24 h (equal amounts of polyA<sup>+</sup>-RNA were pooled for the low-CO<sub>2</sub> library). cDNA libraries were constructed from 3 µg polyA<sup>+</sup>-RNA (cDNA

Synthesis Kit, Stratagene, La Jolla, California), whereby fragments larger than 500 bp were selected by cDNA size fractionation columns (Invitrogen, Carlsbad, California) and ligated into pBluescript II SK (+) (Stratagene) for directional cloning.

### DNA sequencing

Plasmids were isolated from bacterial pellets using the QIAprep 96 Turbo Miniprep Kit (Qiagen Inc, Valencia, California) and Qiagen 9600 BioRobots. Sequencing of 4992 clones was performed from the 5'-end using M13 reverse or SK primers and ABI Prism BigDye<sup>TM</sup> Terminators version 3.0/3.1 (Perkin-Elmer, Wellesley, Massachusetts). Unincorporated dye terminators and primers were removed by ethanol precipitation, and sequencing reaction products were analyzed on the ABI 3700 capillary array automated DNA sequencing system (Applied Biosystems Group, Foster City, California).

### Microarray construction

ESTs were batch-blasted against the National Center for Biotechnology Information (NCBI) nonredundant protein database using blastx (NCBI) for preliminary annotation. Using the online resource PipeOnline (Ayoubi et al. 2002), ESTs were assembled into 2322 contigs that aided the generation of a list of clones for microarray printing. A total of 2378 cDNA inserts were PCR amplified by colony PCR with M13 forward and reverse universal primers. PCR products were printed in triplicate onto micro slide glasses of the type SuperAmine Substrates (ArrayIt<sup>TM</sup>, Sunnyvale, California) using the microarray-stamping machine OmniGrid 100 (Gene Machines, Athens, Georgia). High-throughput sequencing and microarray printing were performed at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois, Urbana-Champaign, Illinois.

### Target preparation

Exponential cultures (6% CO<sub>2</sub>) were harvested at timepoint 0 h or shifted to ambient CO<sub>2</sub> at 0 h and harvested after 0.5, 1, 3, 6, and 24 h. Total RNA was extracted twice for each timepoint from different cultures. The direct labeling protocol from the Microarray Centre at the Ontario Cancer Institute, University Health Network, Toronto, Ontario, using 25 µg total RNA was performed as described with subsequent isopropanol precipitation for probe cleanup.

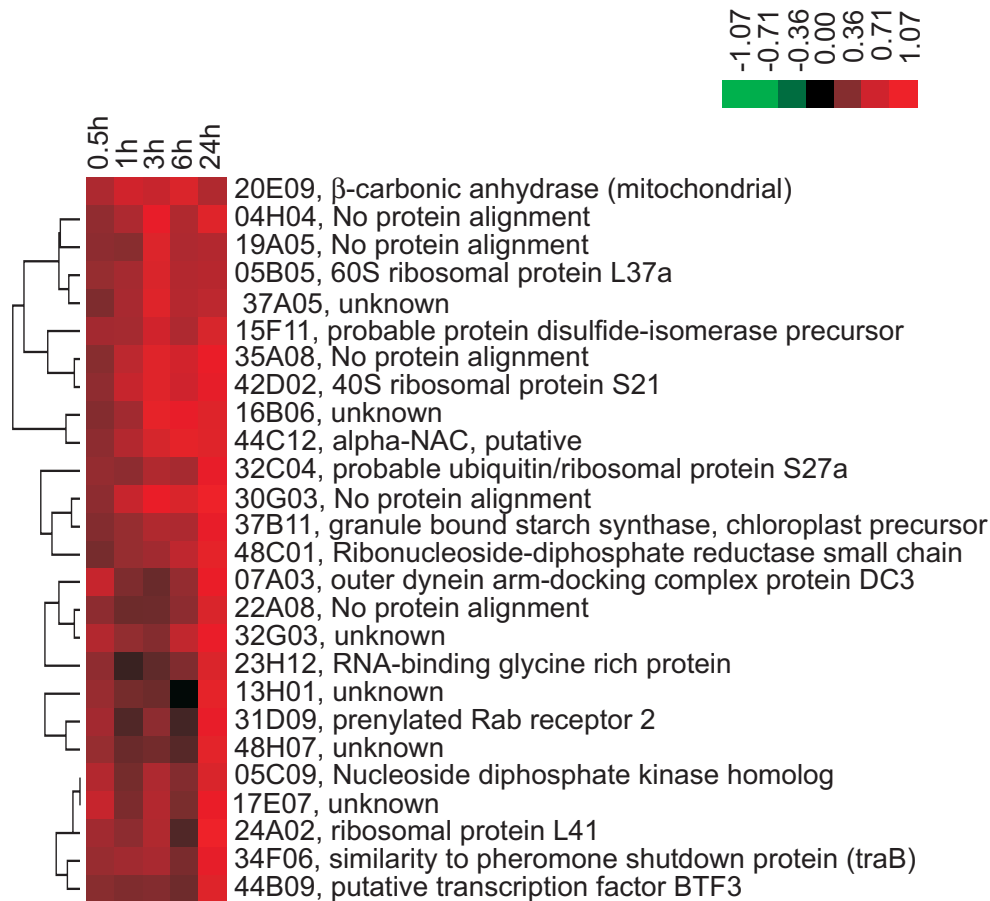
### Microarray hybridization

Hybridization was performed at 37 °C for 18 h with DIG Easy Hyb solution (Roche, Basel, Switzerland) and hering sperm DNA (10 mg·mL<sup>-1</sup>, 7.5 µL added to 100 µL Hyb solution). After removal of the cover slip in 1× SSC (0.15 mol·L<sup>-1</sup> NaCl, 0.15 mol·L<sup>-1</sup> sodium citrate), slides were washed at 50 °C in prewarmed 1× SSC, 0.1% SDS with gentle agitation for 20 min, and spun dry for 5 min at 500 r·min<sup>-1</sup>. For each timepoint, a dye swap and a control hybridization with the second isolated total RNA was performed.

### Image processing and data analysis

Scanning was performed on a GenePix 4000B scanner

**Fig. 1.** A subcluster of *Cyanophora paradoxa* genes upregulated by low CO<sub>2</sub>. RNA from five timepoints from 0.5 to 24 h after the shift were used for hybridizations.



**Table 1.** Examples for genes upregulated upon shift to low CO<sub>2</sub> (97 genes).

Functional category	Array ID	Gene product
Photosynthesis (CCM)	37F12	Ribulosebiphosphate carboxylase–oxygenase activase
	20E09	Beta carbonic anhydrase (mitochondrial)
ROS-inactivating enzymes	28B08	Thioredoxin peroxidase (antioxidant enzyme)
	27E09	Catalase isozyme 1
Protein degradation	19B05	Ubiquitin conjugating enzyme UBC1
Chaperones	15F11	Protein disulfide isomerase-like protein
	44C12	Alpha NAC-like protein, nascent polypeptide associated complex alpha chain
Starch synthesis	37B11	Granule bound starch synthase, chloroplast precursor
Nitrite transport	31G04	Nitrite transporter NAR1

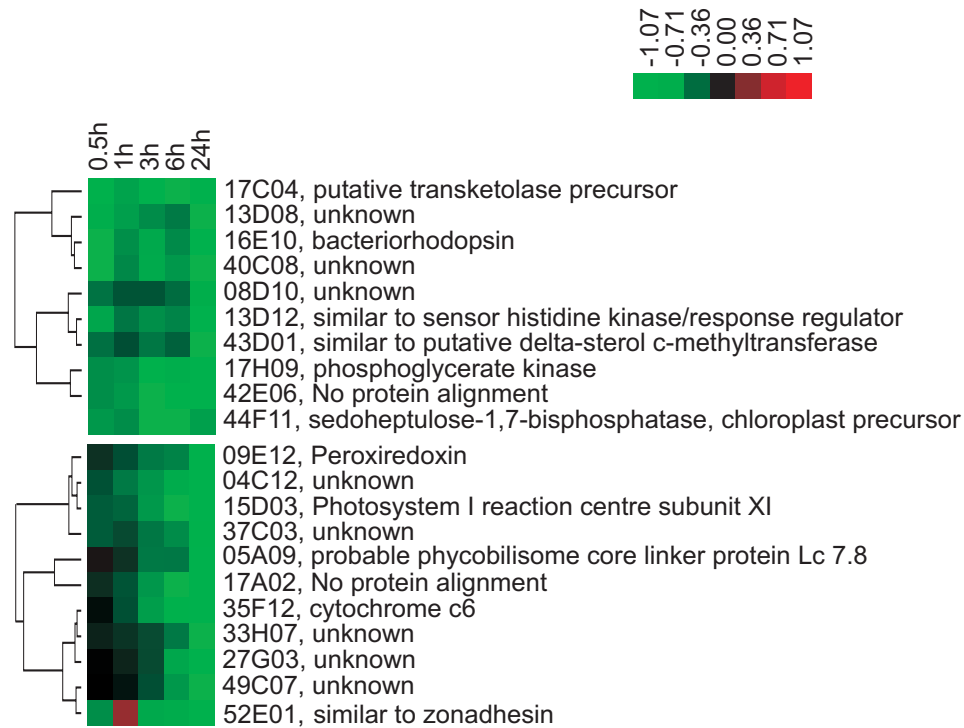
with GenePix Pro 4.1 software (Axon, California). The photomultiplier tube gain was adjusted to avoid saturation of spots. The local background signal was subtracted automatically from the hybridization signal of each spot for both wavelengths. Poor-quality spots, either flagged manually or by the software, as well as spots where background signals were higher than hybridization signals, were excluded from normalization and data analysis. Global normalization was performed using the corresponding GenePix default normalization factor. For individual transcripts to be considered differentially regulated, the mean of the median of ratios of all included spots needed to be  $\geq 1.66$  or  $\leq 0.6$ , and the mean of the median of ratios of the included spots of at least one of

three hybridizations needed to  $\geq 2.0$  or  $\leq 0.5$ . Hierarchical cluster analysis of differentially regulated genes was performed using CLUSTER and visualized with TREEVIEW (Eisen et al. 1998).

#### Isolation of import-competent cyanelles

*Cyanophora* cells suspended in 25 mmol·L<sup>-1</sup> HEPES buffer, pH 7.6, 0.35 mol·L<sup>-1</sup> sucrose, 2 mmol·L<sup>-1</sup> EDTA were broken in a Waring Blender: five times for 1 min at full speed with 1 min of cooling in ice water in between blendings (Steiner et al. 2003). Inspection under the light microscope revealed more than 90% broken cells. Cyanelles were pelleted at 1500g (2 min in a Sorvall centrifuge, GSA

**Fig. 2.** Subclusters of *Cyanophora paradoxa* genes downregulated by low CO<sub>2</sub>. RNA from five timepoints from 0.5 to 24 h after the shift were used for hybridizations.



**Table 2.** Examples for genes downregulated upon shift to low CO<sub>2</sub> (87 genes).

Functional category	Array ID	Gene product
Phycobilisome antenna	39H12	Phycobilisome core linker protein Lc 7.8
Calvin cycle enzymes	44F11	Sedoheptulose-1,7-bisphosphatase chloroplast precursor
	17H09	Phosphoglycerate kinase (chloroplast) precursor
	17C04	Putative transketolase precursor
Photosynthetic electron transport	26B08	Photosystem I reaction centre subunit II precursor
	15D03	Photosystem I reaction centre subunit XI
	31G11	Photosystem I reaction centre subunit X (psaK)
	35F12	Cytochrome c6
Protein synthesis	24B02	Translation elongation factor 1 beta 2
Cytoskeletal components	01B09	Tubulin beta-1 chain
	08A08	Alpha-1 tubulin

rotor, with the brake off). The pellet was carefully suspended in 8 mL of SRM buffer (50 mmol·L<sup>-1</sup> HEPES, 0.33 mol·L<sup>-1</sup> sorbitol, pH 8.0) using a fine brush. Cyanelle suspension (4 mL) was layered on top of a cushion of 40% Percoll (in SRM buffer, 4 mL). After centrifugation for 7.5 min in a Sorvall SS34 rotor at 2000g, class II cyanelles (i.e., those not competent for protein import) and residual mitochondria remained on top of the cushion whereas intact cyanelles were pelleted. The blue-green pellet was gently dissolved in 1 mL of SRM buffer, diluted to 40 mL with SRM buffer, and pelleted at 1000g for 2 min. This washing procedure was repeated twice.

#### Import assay

The radiolabeled precursor of Rubisco activase was synthesized by in vitro transcription and translation of the respective cDNA cloned into the pBAT vector as described

previously (Steiner et al. 2000). A cyanelle suspension in SRM buffer, equivalent to 40 µg of chlorophyll, was incubated with the translation mixture for 7–25 min at 25 °C in a total volume of 150 µL. Cyanelles were then isolated again by centrifugation for 2 min at 800g and eventually treated with thermolysin to remove precursor adjacent to the envelope.

#### Isolation of central bodies

The import reaction was stopped by the addition of 1 mL ice-cold SRM buffer followed by centrifugation at 800g and 4 °C for 2 min. The cyanelle pellet (without thermolysin treatment) was washed in SRM, resuspended in 1 mL 2× SRM, and incubated for 25 min at room temperature with 30 µL of a 10 mg·mL<sup>-1</sup> lysozyme stock solution, which led to digestion of the peptidoglycan wall and cyanelle lysis. After DNase treatment, the lysate was layered on top of a step

**Table 3.** Radioactivity incorporated into various cyanelle subfractions after in vitro import of  $^{35}\text{S}$ -labeled pre-Rubisco activase.

Fraction	% incorporation into mature protein (precursor: 100)	
	7-min incubation	25-min incubation
Intact cyanelles	95	100
Stroma	55	40
Thylakoids + central bodies	40	60
Central bodies	10	20

gradient consisting of two Percoll layers (10% and 40%, respectively) and centrifuged. Thylakoid membranes with bound central bodies banded at the interface. The thylakoid layer was carefully removed, treated with SRM buffer containing 0.1% Triton X-100, and loaded on top of a second gradient of analogous composition. After centrifugation, a whitish pellet was obtained indicating that mild detergent treatment resulted in partial release of the central bodies from the thylakoid membranes.

### Gel electrophoresis

Proteins were separated on SDS-polyacrylamide gradient gels (10–18%) for better resolution in the low molecular mass range (Laemmli 1970). DNA fragments were separated on 0.8%–1.5% agarose gels in 50 mmol·L<sup>-1</sup> Tris, 20 mmol·L<sup>-1</sup> sodium acetate, 27 mmol·L<sup>-1</sup> acetic acid, 2 mmol·L<sup>-1</sup> Na<sub>2</sub>EDTA (Sambrook et al. 1989). Import data were analyzed using a PhosphoImager and the Molecular Dynamics IMAGE QUANT version 3.3 program, ensuring that all the signals remained in the linear detection range.

## Results

### Changes in gene expression depending on the CO<sub>2</sub> level

One cDNA library each was established from cells grown under ambient (low) or high (6%) CO<sub>2</sub> for sequencing and annotation of more than 5000 ESTs. Of these, 2300 unique ESTs were chosen for microarray printing. Hybridizations with RNA from cells grown in high and low CO<sub>2</sub> (harvested at various timepoints after downward shift) were performed. A total of 184 genes were found to be differentially expressed under high and low CO<sub>2</sub>. Upon shift to low CO<sub>2</sub>, 97 genes were upregulated (Fig. 1, Table 1) and 87 genes (Fig. 2, Table 2) were downregulated by a factor of 1.5–3.

The functions of 30% of these genes are not known. However, the extent of the effects was smaller than what has been observed for *Synechocystis* sp. strain PCC6803 under similar conditions (Wang et al. 2004). Several groups of genes were downregulated similarly in cyanelles and the cyanobacterium, for example genes for photosystems I and II and phycobilisome components, Calvin cycle enzymes, and proteins of the translation apparatus. Others (e.g., genes for a nitrite transporter subunit, starch synthase, mitochondrial carbonic anhydrase (CA)) followed an induction pattern as observed in *Chlamydomonas reinhardtii* (Miura et al. 2004) although with less pronounced increases.

### Characterization of the central body of cyanelles

An isolation procedure for central bodies via two successive Percoll step gradients was developed. The size of the

central body and its tight connection to cyanelle DNA and thylakoids necessitated a step involving mild detergent treatment of the thylakoid fraction to release part of the bound microcompartments. Isolated carboxysomes offered the potential for a proteomics approach and for the identification of carboxysome proteins other than Rubisco. After SDS-PAGE of the central body pellet, several bands ranging in size from 30 to 103 kDa were subjected to in-gel digestion. Peptide masses from the (major) 52- and 103-kDa bands and partial amino acid sequences generated via MS/MS of selected peptides corresponded to Rubisco and to the phycobilisome core-membrane anchor polypeptide ApcE (a contaminant), respectively. To date, no conclusive results have been obtained from database searches either with respect to peptide pattern or to partial sequences for the other bands investigated. We were more successful with Rubisco activase. The gene containing a typical cyanelle transit sequence was cloned and used to generate a labelled precursor that readily imported into isolated cyanelles and was shown to integrate into the putative carboxysomes in a time-dependent manner: after a 7-min incubation, 10% of the label was found in the central body pellet, which increased to approximately 20% after a 25-min incubation (Table 3). Heterologous antisera directed against Rubisco activase from spinach revealed a band of approximately 45 kDa upon Western blotting of SDS gels prepared from isolated central bodies (S. Fathi-Nejad, unpublished data).

## Discussion

We have no direct answer to the question: is there a carboxysomal CCM operating in the cyanelles of *C. paradoxa*? There are significant, though moderate, effects on gene expression upon shift to low CO<sub>2</sub>. *Cyanophora paradoxa* was grown under continuous light. Under these conditions, mitochondrial respiration plays a minor role only, explaining the much lower effect on expression of mitochondrial CA compared with that in *Chlamydomonas reinhardtii* where a factor of about 100 was reported (Miura et al. 2004).

We expect that a number of more highly regulated genes will also be found because the microarray platform used contained only approximately 20% of the total number of genes present in *C. paradoxa*. A future approach will be to further exploit the low CO<sub>2</sub> library by subtraction and to follow the expression pattern of interesting genes via RT-PCR. The bulk of Rubisco and activase seems to be concentrated in the central body, which should play a major role in the CCM.

Among cyanobacteria, Rubisco activase is found in filamentous, nitrogen-fixing species (the likely ancestors to chloroplasts) but not in unicellular ones, with the exception of *Gloeobacter violaceus* strain PCC7421 (CyanoBase, <http://www.kazusa.or.jp/cyano/cyano.html>). The catalytic mechanism might differ from that of the eukaryotic enzyme (Portis 2003). The *Cyanophora* enzyme resembles those from higher plants and green algae in possessing a N-terminal extension and missing the C-terminal extension found in cyanobacteria, whereas the central part is well conserved for all of the oxygenic phototrophs with Rubisco activase that have been examined. The localization of the cyanobacterial activase is uncertain, whereas immunoelectron microscopy showed an association of this enzyme with pyrenoids in algae (McKay et al. 1991). Here, we could unequivocally demonstrate a tight association of Rubisco activase with its substrate, Rubisco, in the cyanelle microcompartment. Rubisco large subunit is highly conserved, that is, the identity scores between the cyanelle protein and its cyanobacterial counterparts lie between 83% and 84%. Nevertheless, when a BLAST search of CyanoBase was done with cyanelle Rubisco large subunit, the top ranking cyanobacterial proteins were those from *Anabaena* strain 7120, *Nostoc punctiforme*, *Trichodesmium erythraeum*, all filamentous, nitrogen-fixing and activase-containing species (data not shown).

At present, the isolation of central bodies is a compromise with respect to intactness of these delicate microcompartments and their purity. The sensitivity of the mass spectrometric method used is sufficient to characterize even minor bands. The discrimination between bona fide components of the central body and contaminating proteins will remain difficult until a higher percentage of *C. paradoxa* genes is covered by ESTs.

One of our goals, the isolation of the gene(s) for cyanellar CA could not be achieved. A gene with bipartite presequence consisting of a transit sequence followed by a signal sequence would point towards a luminal cyanelle CA and thus a pyrenoid. A CA gene containing a transit sequence only would allow us to investigate the in vitro import of the precursor into cyanelles. In the case of incorporation of CA into the central body, the latter then could be considered a carboxysome. In summary, our data do not yet allow strict designation of the central bodies of *C. paradoxa* as either carboxysomes or pyrenoids. They might even represent an intermediate step in the evolution from a carboxysomal-type CCM towards a pyrenoidal-type CCM. The moderate effects on gene expression observed upon shift to low CO<sub>2</sub> and the eukaryotic-type Rubisco activase point in that direction.

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