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MINI-REVIEW

Unicellular cyanobacteria with a new mode of life: the lack of photosynthetic oxygen evolution allows nitrogen fixation to proceed

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Abstract Some unicellular N_2-fixing cyanobacteria have recently been found to lack a functional photosystem II of photosynthesis. Such organisms, provisionally termed UCYN-A, of the oceanic picoplanktion are major contributors to the global marine N-input by N_2-fixation. Since their photosystem II is inactive, they can perform N_2-fixation during the day. UCYN-A organisms cannot be cultivated as yet. Their genomic analysis indicates that they lack genes coding for enzymes of the Calvin cycle, the tricarboxylic acid cycle and for the biosynthesis of several amino acids. The carbon source in the ocean that allows them to thrive in such high abundance has not been identified. Their genomic analysis implies that they metabolize organic carbon by a new mode of life. These unicellular N_2-fixing cyanobacteria of the oceanic picoplankton are evolutionarily related to spheroid bodies present in diatoms of the family Epithemiaceae, such as *Rhopalodia gibba*. More recently, spheroid bodies were ultimately proven to be related to cyanobacteria and to express nitrogenase. They have been reported to be completely inactive in all photosynthetic reactions despite the presence of thylakoids. Sequence data show that *R. gibba* and its spheroid bodies are an evolutionarily young symbiosis that might serve as a model system to unravel early events in the evolution of chloroplasts. The cell metabolism of UCYN-A and the spheroid bodies may be related to that of the acetate photoassimilating green alga *Chlamydobotrys*.

Keywords Unicellular cyanobacteria without photosystem II · Spheroid bodies of diatoms · Symbiotic nitrogen fixation · Marine nitrogen-fixing cyanobacteria · *Rhopalodia gibba* · *Chlamydobotrys* · UCYN-A cyanobacteria

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Introduction

Most filamentous cyanobacteria that perform N_2-fixation during the day develop specialized cells, called heterocysts, that do not evolve O_2 photosynthetically. N_2-fixation also occurs in unicellular cyanobacteria, and these have to reconcile the incompatible reactions of N_2-fixation catalyzed by the O_2-sensitive nitrogenase and photosynthetic O_2-evolution. Some of the unicellular cyanobacteria separate the two processes by performing N_2-fixation in darkness and photosynthetic O_2-evolution and CO_2-fixation during the day. However, few other unicellular cyanobacteria can fix both CO_2 and N_2 in light. These cells must be able to protect their nitrogenase from damage by O_2, but the mechanisms for this are largely unknown. The subject on unicellular N_2-fixing cyanobacteria has extensively been reviewed (Fay 1992; Turner et al. 2001; Berman-Frank et al. 2003; Stal and Zehr 2008) and shall not be repeated here. As discussed in the following, some N_2-fixing unicellular cyanobacteria have recently been discovered to have an inactive photosynthetic reaction II, the O_2-evolving part of the photosynthetic apparatus in cyanobacteria, eukaryotic algae, and plants. Thus, the nitrogenase of these unicellular cyanobacteria is not exposed to the O_2 generated photosynthetically. Such organisms offer

fascinating perspectives both in cell metabolism, ecology, evolution, and application and will, therefore, be discussed in the present minireview.

A group of unicellular N_2-fixing cyanobacteria of the oceanic picoplankton reveals a new mode of life

Approximately 50% of the global biological N_2-fixation proceeds in the oceans of the world (Galloway et al. 2004; Stal 2009). The free-living filamentous *Trichodesmium* and the heterocystous *Richelia intracellularis* within the diatoms such as *Rhizoselenia* were thought, until recently, to be major players for this marine N-input (Zehr et al. 2008). *Trichodesmium* and *Richelia* prefer warm, tropical and subtropical areas, whereas in the temperate regions, e.g. in the Baltic Sea, genera such as *Nodularia* and *Aphanizomenon* seasonally form the major N_2-fixing blooms (Diez et al. 2008).

Additional contributors in tropical and subtropical regions are unicellular cyanobacteria with a diameter between 2 and 8 μm, such as *Cyanothece* of more coastal and benthic areas and *Crocosphaera watsonii* (belonging to the unicellular N_2-fixing cyanobacterial group B) in the open oceans (Zehr et al. 2001; Mazard et al. 2004). Cells with a diameter <1 μm, thus belonging to the picoplankton, are even more important N_2-fixing species (unicellular cyanobacteria group A = UCYN-A) in these areas (Falcon et al. 2004; Langlois et al. 2005; Goebel et al. 2008; Church et al. 2009; DeLong 2010). These cyanobacteria were not previously detected, since they are smaller than the related *Cyanothece* and *Crocosphaera* and lack phycoerythrin (Goebel et al. 2008). These UCYN-A organisms were discovered by amplification of the nitrogenase gene (*nifH*) and its transcripts from oceanic water samples (Zehr et al. 1998, 2001) and also are characterized by their 16S rRNA gene sequences (Zehr et al. 2008). They are found to be small dim cells by flow cytometry coupled with the quantitative polymerase chain reaction (Goebel et al. 2008). It has previously been suggested from *nifH* sequences that UCYN-A cells are most closely related to those from the marine unicellular cyanobacterium *Cyanothece* sp. strain ATCC 51142 and from the spheroid bodies of *R. gibba* (Zehr et al. 1998). However, the phylogenetic tree both of 16S rRNA (Fig. 1a) and the *nifH* (Fig. 1b) gene indicates that these organisms, though being in the same group, cluster on somewhat different lineages in the phylogram. Despite being identified as unicellular N_2-fixing cyanobacteria, the UCYN-A have yet to be cultivated. Using molecular techniques (the quantitative polymerase chain reaction), they have been found to be widely distributed in tropical and subtropical waters and appear to inhabit cooler waters than *Trichodesmium* or *Crocosphaera* (Langlois et al. 2005; Church et al. 2008; Moisander et al. 2010).

Examples of their occurrence in high abundances are cooler, nutrient-poorer areas, e.g. between 14°N und 29°N in a region between 152°W and 170°W of the Pacific Ocean (Church et al. 2008) and along a transect westwards and in parallel to Japan (Kitajima et al. 2009). UCYN-A organisms have recently been reported in particularly high abundances at substantially higher latitudes including in deeper (up to 100 m depth) subsurface waters in the South Pacific Ocean (Moisander et al. 2010).

UCYN-A cyanobacteria express the *nifH* gene with maximal transcript abundance during daytime (Zehr et al. 2007). They apparently can fix N_2 in the light which is unlike several unicellular N_2-fixing cyanobacteria that separate photosynthetic O_2-evolution and N_2-fixation by performing the former process during the day and the latter in darkness (Gallon 2001; Trepel et al. 2009). UCYN-A cells have nitrogenase gene arrangement and composition similar to those of *Cyanothece* sp. ATCC 51142 and of the spheroid bodies of *Rhopalodia gibba* (Zehr et al. 2008). A metagenomic analysis of cells concentrated by flow cytometry did not reveal any genes coding for phycocyanin, phycoerythrin or associated linkers which explains that they have not been detected earlier in the oceans by phycoerythrin fluorescence microscopy or flow cytometry. In addition, genes coding for the Calvin-Benson cycle and for photosystem II were not detected on the genomic contig, and target genes of these two photosynthetic part reactions (Rubisco and *psb*A, respectively) could not be amplified by PCR (Zehr et al. 2008). Thus, UCYN-A cyanobacteria cannot synthesize organic carbon by photosynthesis but are strictly dependent on extracellular sources. Due to the absence of photosystem II, their nitrogenase is not exposed to O_2 generated photosythetically. The genomic contig revealed photosystem I genes. UCYN-A cells apparently meet their energy demand by cyclic photophosphorylation. It has not been determined yet which type of thylakoids they possess.

The recently obtained metagenomic and metatranscriptomic data sets showed that the genome of UCYN-A is highly conserved (>97% sequence identity) for samples taken from Pacific, Indian and Atlantic Oceans which contrasts strikingly with other marine bacteria such as *Pelagibacter* or the photosynthetic *Prochlorococcus* that show a high sequence diversity (Tripp et al. 2010). The assembly of the complete genome (Tripp et al. 2010) indicated that UCYN-A contains complete metabolic pathways such as glycolysis (from glucose-6-P to pyruvate), pentosephosphate pathway, fatty acid biosynthesis, pigment biosynthesis and assimilatory sulfate reduction besides N_2-fixation. In contrast, no Calvin cycle or any other of the five known CO_2-fixation pathways (Thauer 2007) are present, and they lack the genes coding for enzymes of the tricarboxylic cycle, the biosynthesis of several amino acids such as

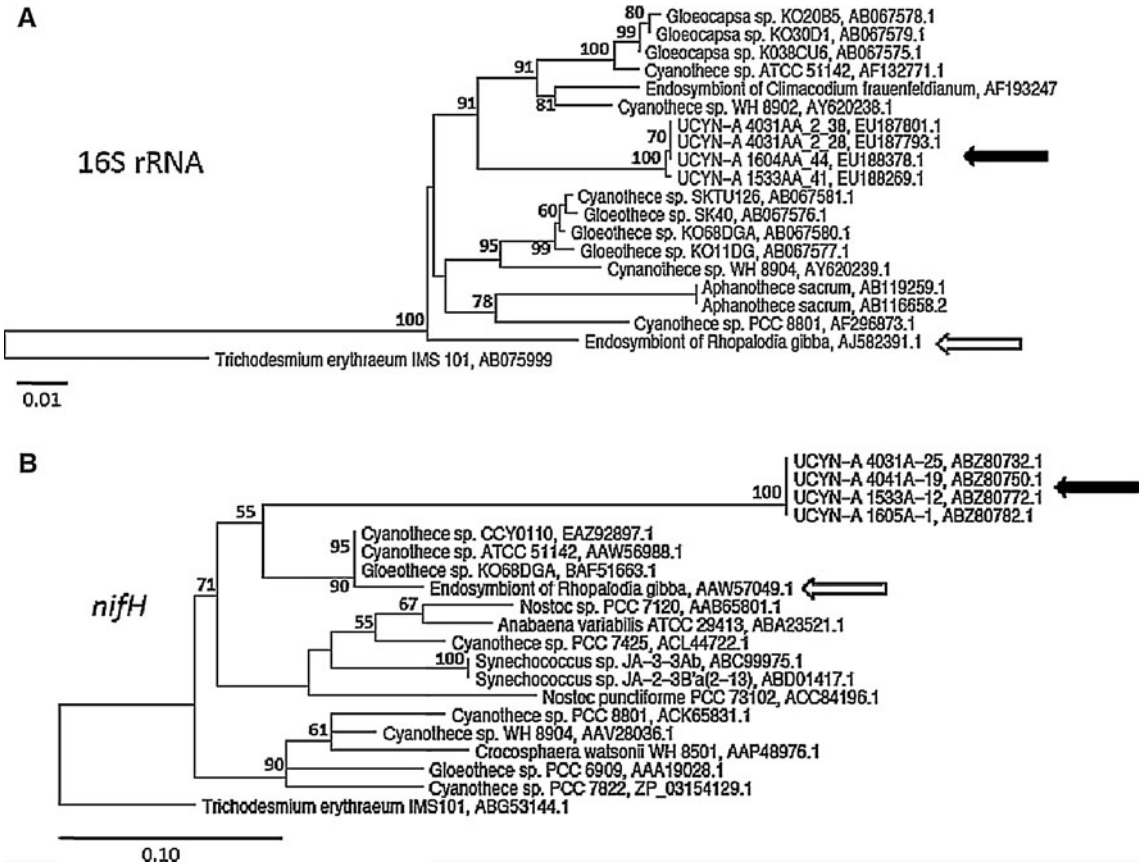

Fig. 1 **a** *Phylogenetic tree* of unicellular cyanobacterial 16S rRNA nucleotide gene sequences. The *arrow* indicates representative UCYN-A sequences. Sequences were aligned using the SILVA aligner (http://www.arb-silva.de/aligner/) (Pruesse et al. 2007). The *tree* was constructed in MEGA4 (Tamura et al. 2007) using a Neighbor-Joining method and evolutionary distances were computed using a Jukes-Cantor correction. Bootstrap values were inferred from 1,000 replicates. Due to the short length of EU187793 and the complete deletion of positions containing gaps or missing data, there were 614 positions in the final data set. **b** *Phylogenetic tree* of *nifH* protein sequences from unicellular cyanobacteria. The *arrow* indicates representative UCYN-A sequences. Protein sequences were aligned using a hidden Markov model from the Pfam database (http://pfam.sanger.ac.uk/) (Finn et al. 2010). The *tree* was constructed using a Neighbor-Joining method, a Poisson correction was used to compute the evolutionary distances, and a bootstrap test was conducted with hahah 1,000 replicates. There were 108 positions in the final data set

valine, leucine or isoleucine and of purine (Tripp et al. 2010). They possess transporters for sugars and dicarboxylic acids. It is therefore tempting to assume that they assimilate dicarboxylic acids, unidentified sugars and/or amino acids from ocean waters despite its low nutrient content. Sugars would then be degraded via glycolysis to pyruvate. Also other non-photosynthetic bacteria of the oceans, e. g. the uncultured SAR11 group, apparently utilize monosaccharides by modifications of the glycolytic pathway (Schwalbach et al. 2010). In UCYN-A, the two pyruvate:ferredoxin oxidoreductases described for cyanobacteria (Schmitz et al. 2001) are absent, but the genes coding for all three enzymes of the pyruvate:dehydrogenase complex (pyruvate dehydrogenase; lipoamide acetyltransferase, lipoamide dehydrogenase) have been detected on the genome (unpublished data). Thus, the UCYN-A cells might be able to cleave pyruvate in the presence of coenzyme A to acetyl coenzyme A and CO_2, but the fate of the remaining two electrons (of NADH) remains uncertain. They have no bidirectional hydrogenase to dispose of the reducing equivalents (but uptake hydrogenase is present). To get rid of reductant, they must produce a fermentative endproduct such as malate (malate dehydrogenase is present), lactate, formate or any other compound formed in NADH oxidation. Excreted energy-rich compound (malate, lactate) would likely be rapidly consumed by other microorganisms, which could symbiotically supply the missing amino acids to UCYNA. Despite attempts to observe symbiotic partners (Tripp et al. 2010), no organism accompanying UCYN-A has been detected as yet. In addition, genes coding for lactate dehydrogenase or formate deydrogenase are not apparent in the genome. A transfer of the reducing equivalents from NAD(P)H to an enzyme and then directly to O_2 is unlikely.

The pennate diatom *Rhopalodia gibba* and its endosymbiotic spheroid bodies

The UCYN-A cyanobacteria of the picoplankton bear resemblances to previously reported endosymbiotic microorganisms. Diatoms of the family Epithemiceae contain oblong inclusions, the so-called spheroid bodies (Pfitzer 1869, as quoted by Klebahn 1896). There are 1–4 (maximally 16) per cell within the genera *Rhopalodia* and *Epithemia* and with *Denticula vanheurcki*, however not with *D. tenuis* (Geitler 1977). The latter author also reported that the fission of the spheroid bodies is largely independent of the multiplication of the host and that at least one spheroid body is transferred to the next generation in each case. The spheroid bodies are not pyrenoids, as assumed originally (Klebahn 1896; Fritsch 1945), since they are outside of the chloroplast of the Epithemiaceae. A relationship to unicellular cyanobacteria was inferred from electron microscopic investigations (Drum and Pankratz 1965). Thus, the spheroid bodies were suspected to be peculiar organelles or intracellular organisms. However, any evidence for a relatedness to unicellular cyanobacteria was questioned by Geitler (1977).

The electron microscopic image (Fig. 2a) shows two spheroid bodies in *Rhopalodia gibba* that resemble coccoid cyanobacteria (Floener and Bothe 1980). The spheroid bodies are enclosed by a thick cell wall and are surrounded by a membrane of the host. As with the *Rhizobium*–legume and mycorrhiza–plant symbioses, host and spheroid bodies are strictly separated, and often an optically empty space is seen in between them, as already noted by Drum and Pankratz (1965). Thylakoids are discernible and extend from the inner layer (probably the cytoplasmic membrane) radially to the center of the spheroid bodies (Fig. 2b). Almost all cyanobacteria and also the cyanelles of glaucophyta (Löffelhardt et al. 1997; Reyes-Prieto and Bhattacharya 2007) possess concentric thylakoids. However, a radial thylakoid arrangement was described for the cyanobacteria *Phormidium retzi* and *Oscillatoria limosa* (Golecki and Drews 1982). Since their cultures grew independently of the addition of combined nitrogen, Drum and Pankratz (1965) speculated that *Rhopalodia gibba* may meet its N-demands by N_2-fixation.

To our knowledge, members of the Epithemiaceae are not available in culture collections currently. These organisms are apparently not very rare in freshwaters. *Rhopalodia gibba* has been described for the Große Plöner See in Northern Germany (Geitler 1977), and this species and members of the genus *Epithemia* can readily be isolated from ponds of the botanical gardens of the Universities of Vienna, Austria (Geitler 1977) and Marburg, Germany (H. A. von Stosch, K. Wenderoth, personal communication). In 1978, the doctoral student L. Floener of Cologne isolated *R. gibba* in Marburg with the help of the late H. A. von Stosch. This diatom can be grown in liquid media on all conventional N-sources (NO_3^-, NH_4^+ or N_2 as the only N-source) with essentially the same generation time of 2–3 days (Floener 1982). However, they were strictly epiphytic, meaning that they could be grown only on the surface of 2% agar in liquid culture medium. After 4–6 weeks of growth, they were scratched off with a bent glass rod for physiologic experiments. Although it was impossible to get enough cells for a detailed biochemical analysis, the material was sufficient for performing N_2-fixation (C_2H_2-reduction) experiments (Fig. 3).

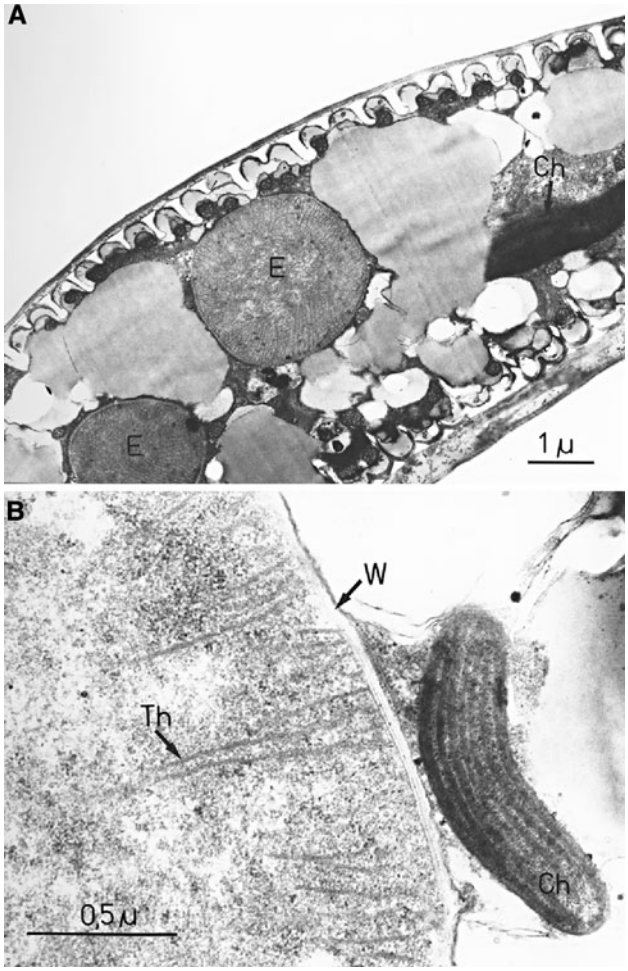

Fig. 2 *Longitudinal sections* through *Rhopalodia gibba*. **a**. The electron microscopic image reveals two spheroid bodies (*marked with E*) which show a thick cell wall and thylakoids. A sausage-like chloroplast (Ch) is also present. **b**. Section of a spheroid body showing the radially orientated thylakoids. Part of the neighboring chloroplasts with its thylakoids is seen. *W* thick wall of the spheroid body. The *Rhapolodia* cells were fixed with glutaraldehyde—OsO_4 and prepared as described by Schnepf and Deichgräber (1978). For further details, see Floener (1982) from where the originals of the photos were taken. The *bar* represents µm

The *Rhopalodia* cultures reduced C_2H_2 immediately, without any lag phase, continuously and strictly light

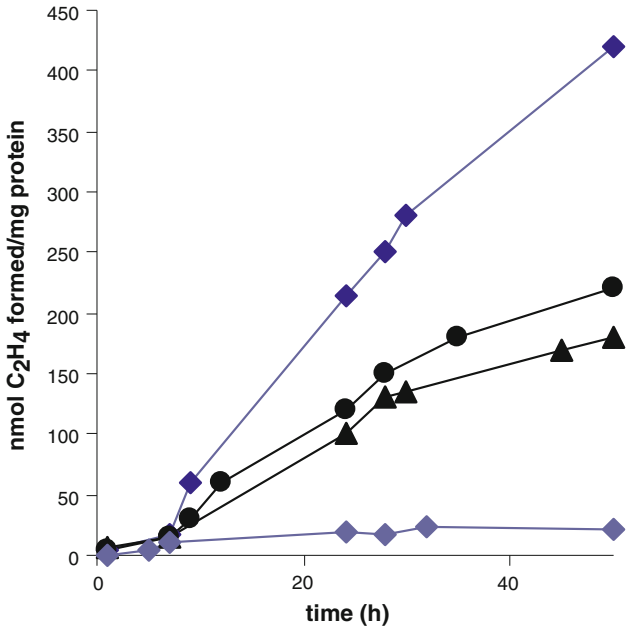

Fig. 3 C_2H_2–reduction (N_2-fixation) by *Rhopalodia gibba* The experiment was performed in seven 2-ml Fernbach flasks containing intact cells of *R. gibba* with 0.3 mg protein in 3 ml nutrient solution, 4% O_2 in the gas phase of the vessels and low light intensities at their surface (~3,500 lux). Prior to the assay, the cells had been grown under N_2-fixing conditions, scratched off from the agar surface of the culture vessels, centrifuged and taken up in the assay medium. The amount of C_2H_4 formed was determined by gas chromatography. The original data are from Floener (1982). *Lines between two filled squares* denotes under N_2-fixing conditions (without combined nitrogen in the medium). *Lines between two filled circles* denotes with nitrate in the medium. *Lines between two filled triangles* denotes with ammonium ions in the medium. *Lines between two filled diamonds* denotes in darkness, under N_2-fixing conditions

dependent with a rate of approximately 10 nmol C_2H_4 formed/h x mg total diatom protein. The activity was distinctly higher when the cells were grown on N_2 and not NO_3^- or NH_4^+ as N-source (Fig. 3). Generally, the addition of combined nitrogen to cultures completely blocks nitrogenase synthesis, but here this N-source possibly did not reach the spheroid bodies quantitatively. Maximal C_2H_2-reduction activity required 2–4% O_2 in the gas space of the vessels (Floener and Bothe 1980). Thus, as with several unicellular cyanobacteria (Fay 1992), N_2-fixation by *R. gibba* required reduced O_2-tensions (microaerobic conditions). The C_2H_2-reduction rate was maximal at the low light intensity of 5,000 lux (approximately) at the vessels, but higher light intensities were not inhibitory (Floener 1982). The *R. gibba* culture was not free from contaminant bacteria. An enrichment culture of the contaminant bacteria did not fix N_2 (Floener 1982). The spheroid bodies are permanently incorporated into the cells of the Epithemiaceae, and are not like the kleptoplastids where heterotrophic euglenids temporarily exploit cyanobacteria for performing photosynthesis prior to their digestion (Schnepf et al. 2002). The spheroid bodies also do not resemble any of the other known endosymbioses within protists (Nowack and Melkonian 2010). Besides being cyanobacteria they are totally unrelated to the marine heterocystous cyanobacterium *Richelia intracellularis* that lives endosymbiotically in the diatoms of the genera *Rhizosolenia* or *Hemiaulus* (Zeev et al. 2008).

N_2-fixation is restricted to prokaryotes, and no chloroplast of eukaryotes has this capability. The relevance of the spheroid bodies is that they are N_2-fixing entities within diatoms, thus within eukaryotic cells. The occurrence of thylakoids indicates the relatedness of spheroid bodies to cyanobacteria. However, no typical cyanobacterial pigments such as phycobilins, echinenone or myxoxanthophyll could be extracted, but diatoxanthin and diadinoxanthin of the Bacillariophyceae (Floener 1982) were present. Spheroid bodies are definitively not related to cyanelles of glaucophyta that do not possess chloroplasts. In glaucophyta, only the cyanelles perform photosynthesis. Cyanelles possess concentrically arranged thylakoids and phycobilins. They cannot perform N_2-fixation (Floener et al. 1982).

A unicellular, N_2-fixing cyanobacterium tightly integrated into a eukaryote could serve as a fascinating model organism for future research and applications. In the past, there have been many attempts with limited success to artificially construct a stable symbiosis between a crop plant and a symbiotic N_2-fixing microorganism to make the plant independent of a supply with combined N (Tikhonovich and Provorov 2009). Mainly due to the lack to obtain sufficient amounts of cells, the research on *R. gibba* had to be abandoned then by the Cologne laboratory, but the results obtained so far were published in a proceedings volume (Floener and Bothe 1980). In addition, cells became smaller and smaller with the generations, since sexual states of *R. gibba* were not available. The definitive proof that spheroid bodies are cyanobacteria-related could not be obtained since molecular techniques were not available 30 years ago.

In the past few years, U.G. Maier and coworkers at Marburg University, Germany, reisolated *R. gibba* and used the same culture conditions as Floener and Bothe (1980). They were then able to definitively prove by molecular methods that spheroid bodies possess nitrogenase and are related to free-living cyanobacteria (Prechtl et al. 2004; Kneip et al. 2007, 2008). These authors were able to isolate spheroid bodies and their DNA from *R. gibba*. Sequencing and the phylogenetic analysis of the 16S rDNA gene revealed a close relatedness of the spheroid bodies to the free-living N_2-fixing cyanobacterium *Cyanothece* sp, ATTC 51142 (Prechtl et al. 2004). Preparations of spheroid bodies were used to detect N_2-fixation by use of *nifD* gene, one of the two genes that code for the MoFe-protein (larger subunit) of nitrogenase. DNA hybridizations proved the presence of the gene in spheroid-DNA. Immunogold-labeling

experiments with nitrogenase antibodies showed that the enzyme was expressed in spheroid bodies. Phylogenetic analysis of the 16S rDNA- and *nifD* genes showed that the branches that separate free-living cyanobacteria and spheroid bodies are very short. Thus, the symbiosis of *R. gibba* with its endosymbiotic spheroid bodies has evolved rather recently (Kneip et al. 2007). Spheroid bodies possess all genes that are essential for N_2-fixation with some genes being more related to those in *Cyanothece* and others to those in *Gloeothece* (Kneip et al. 2008). The non-essential *fdx*N-gene has been changed to a pseudogene by insertion of stop codons. Inactivation by truncation occurs in *nifU*, another gene which is also not absolutely required for the expression of nitrogenase. A lot of genes coding for cell metabolism enzymes underwent deletion or inactivation by insertion of A and T nucleotides. Examples of this are genes coding for photosynthesis proteins such as *petJ*, (encoding cytochrome c_6), *petE* (for plastocyanin), *psb*C und *psb*D (both of photosystem II). The spheroid bodies apparently retained only the thylakoids, but the loss of photosynthetic pigmentation (Kneip et al. 2008) might indicate that they are unable to perform any part reaction of photosynthesis. This includes photosystem-I dependent cyclic photophosphorylation, since none of the genes of this pathway has been detected in the fosmid library. However, this will finally be proven when the complete sequenced genome will be available. For the generation of ATP and their own carbon skeleton, the spheroid bodies must be strictly dependent on a supply with organic carbon from the host.

The presumed sole task of the spheroid bodies is to supply the host with a product of N_2-fixation (NH_4^+?). The estimated genome size of the spheroid bodies is 2.6 Mb and is thus less than half than that of *Cyanothece* sp. CCY0110 (5.8 Mb). Due to the loss of quite a number of genes, the spheroid bodies are non-autonomous and cannot be grown independently of their diatom host. Remarkably, the two DNA-repair genes *rec*A and *rec*F are intact, in contrast to the situation with other tightly incorporated symbiotic systems (Kneip et al. 2008). *R. gibba* with its unicellular cyanobacteria-related spheroid bodies represents a new model system that might provide insights into early events in the evolution of chloroplasts (Kneip et al. 2008).

Is the cell metabolism of the unicellular UCYN-A cyanobacteria related to that of the acetate photoassimilating green alga *Chlamydobotrys*?

The cell metabolism of the UCYN-A cyanobacteria of the picoplankton, indeed, shows some analogous features to that of the acetate photoassimilating green alga *Chlamydobotrys* sp. of the Volvocales, an organism taxonomically very distant from cyanobacteria. A series of papers in the early 1960s established that *Chlamydobotrys* is capable of a particular form of photoheterotrophy that came to be known as photoassimilation of acetate (Pringsheim and Wiessner 1960, 1961; Wiessner and Gaffron 1964). *Chlamydobotrys* grows on acetate as the sole carbon source in the light but not in darkness. Under aerobic conditions, the rate of carbon assimilation is about 25% higher than under O_2-exclusion (Wiessner 1962). The carbon assimilated into the cell material mainly comes from the methyl group of acetate (Wiessner 1962). The rate of CO_2-fixation is at best very low and not sufficient to meet the organic C-demands of the cells (Wiessner and Gaffron 1964). Photosystem II seems to participate only in the regulation (redox poising) of the photosynthetic electron flow and does not serve as principal source of reductant in linear photosynthetic electron transport (Mende and Wiessner 1983). Energy (ATP) is generated mainly by cyclic photophosphorylation around photosystem I. In contrast to UCYN-A, *Chlamydobotrys* does not perform N_2-fixation.

The metabolic pathway leading from acetate to carbohydrates has never been entirely elucidated in *Chlamydobotrys*. Cyclic photophosphorylation seems to be necessary to activate acetate, perhaps to acetyl coenzyme A or acetyl phosphate, and then enzymes of the glyoxylate cycle are apparently involved in the incorporation of the methyl-group of acetate into the carbon of the cells (Wiessner 1962). From pyruvate on, gluconeogenesis might generate monosaccharides. Growth on acetate does not require the disposal of excess of reductant, and *Chlamydobotrys* is not dependent on the co-culture with another organism.

It remains to be shown whether acetate or any other chemically simple organic carbon source is sufficiently available in the oligotrophic regions of the open oceans to sufficiently allow growth of the N_2-fixing picoplankton. However, some abundant marine cyanobacteria such as *Prochlorococcus* release significant amounts of carboxylic acids into media (9 to 45 μg C L^{-1} in the form of acetic acid; Bertilsson and Jones 2003, 2005). The low population density (generally less than 1,000 cells ml^{-1}) and small size of UCYN-A (approximately 0.75 μm) imply a maximum carbon demand for biomass around 38 ng C L^{-1}, or three orders of magnitude less than that which could theoretically be provided by *Prochlorococcus.* This estimation is based on the assumption that the cell density of UCYN-A is 1.03 g cm^{-1}, its dry weight equals a third of wet weight, and C demand for biomass is half of dry weight. The division times of UCYN-A and *Prochlorococcus* are likely to be similar, although the growth rates of UCYN-A are not yet known. It is currently speculative to assume that UCYN-A utilizes acetate as *Chlamydobotrys* does and to what extent the pathways of acetate assimilation are similar in *Chlamydobotrys* and UCYN-A. This cyanobacterium

possesses an acetate kinase also and could use the ATP formed by cyclic photophosphorylation to activate acetate. Genes coding for enzymes of the glyoxylate cycle are, however, absent in UCYN-A. Thus, the photoassimilation pathway of acetate in UCYN-A, if occurring, must be other than in *Chlamydobotrys*.

Concluding remarks

As heterocysts of filamentous cyanobacteria, UCYN-A organisms of the marine picoplankton and spheroid bodies of *R. gibba* lost photosystem II activity. The inability to evolve O_2 photosynthetically allows them to perform N_2-fixation. As with heterocysts, the catabolism of organic carbon is not fully understood in UCYN-A and spheroid bodies. Photoassimilation of acetate, as in the alga *Chlamydobotrys*, is only one of the possibilities that may happen in their carbon metabolism. Nature may hide further microorganisms with entirely new modes of life.

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References

Berman-Frank I, Lundgren P, Falkowski P (2003) Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Res Microbiol 154:157–164

Bertilsson S, Jones JBJ (2003) Supply of organic matter to aquatic ecosystems. In: Findlay SEG, Sinsabaugh RL (eds) Aquatic ecosystems: interactivity of dissolved organic matter. Academic Press, New York, pp 2–24

Bertilsson S, Berglund O, Pullin MJ, Chisholm SW (2005) Release of dissolved organic matter by *Prochlorococcus*. Vie et Millieu 55:225–231

Church MJ, Björkman KM, Karl DM, Saito MA, Zehr JP (2008) Regional distributions of nitrogen-fixing bacteria in the Pacific Ocean. Limn Oceanogr 53:63–77

Church MJ, Mahaffey C, Letelier RM et al (2009) Physical forcing of nitrogen fixation and diazotroph community structure in the North Pacific subtropical gyre. Global Biogeochem Cycles 23:GB2020. doi:2010.1029/2008GB003418.002009

DeLong EF (2010) Interesting things come up in small packages. Genome Biol 11:R118

Diez B, Bergman B, El-Shehawy R (2008) Marine diazotrophic cyanobacteria: Out of the blue. Plant Biotechnol 25:221–225

Drum RW, Pankratz S (1965) Fine structure of an unusual cytoplasmic inclusion in the diatom genus *Rhopalodia*. Protoplasma 60:141–149

Falcon LI, Carpenter EF, Cyprian OF, Bergman B, Capone DG (2004) N_2 fixation by unicellular bacterioplankton from the Atlantic and Pacific Oceans: phylogeny and in situ rates. Appl Environ Microbiol 70:765–770

Fay P (1992) Oxygen relations of nitrogen fixation in cyanobacteria. Microbiol Rev 56:340–373

Finn RD, Mistry J, Tate J et al (2010) The Pfam protein families database. Nucleic Acids Res Database Issue 38:D211–D222

Floener L (1982) Physiologische und biochemische Untersuchungen an dem Cyanellen enthaltenden Flagellat *Cyanophora paradoxa* und an *Rhopalodia gibba*, einer Diatomee mit blau-grünen Einschlüssen. Thesis, The University of Cologne, Germany

Floener L, Bothe H (1980) Nitrogen fixation in *Rhopalodia gibba*; a diatom containing blue-greenish inclusions symbiotically. In: Schwemmler W, Schenk HEA (eds) Endocytobiology, endosymbiosis and cell biology. Walter de Gruyter & Co, Berlin, pp 541–552

Floener L, Danneberg G, Bothe H (1982) Metabolic activities in *Cyanophora paradoxa* and its cyanelles, I. The enzymes of assimilatory nitrate reduction. Planta 156:70–77

Fritsch FE (1945) The structure and reproduction of algae II. Macmillan Co., New York

Gallon JR (2001) N_2 fixation in phototrophs: adaptation to a specialized way of life. Plant Soil 230:39–48

Galloway JN, Dentener FJ, Capone DG et al (2004) Nitrogen cycles: past, present and future. Biogeochem 70:153–226

Geitler L (1977) Zur Entwicklungsgeschichte der Epithemiaceen *Epithemia*, *Rhopalod*ia und *Denticula* (Diatomophyceae) und ihre vermutlich symbiotischen Sphäroidkörper. Plant Syst Evol 128:259–275

Goebel NL, Edwards CA, Carter BJ, Achilles KM, Zehr JP (2008) Growth and carbon content of three different sized diazotrophic cyanobacteria in the subtropical North Pacific. J Phycol 44:1212–1229

Golecki JR, Drews G (1982) Supramolecular organization and composition of membranes. In: Carr NG, Whitton BA (eds) The biology of cyanobacteria, Botanical monograph vol. 19. Blackwell, Oxford, pp 125–141

Kitajima S, Furuya KFH, Takeda S, Kanda J (2009) Latidudinal distribution of diazotrophs and their nitrogen fixation in the tropical and subtropical western North Pacific. Linm Oceanogr 54:537–547

Klebahn H (1896) Beiträge zur Kenntnis der Auxosporenbildung. I. Rhopalodia gibba. Pringsh Jahrb Wiss Bot 29:595–654

Kneip C, Lockhart PJ, Voß C, Maier UG (2007) Nitrogen fixation in eukaryotes- new models for symbiosis. BMC Evol Biol 7(55)

Kneip C, Voß C, Lockhart PJ, Maier UG (2008) The cyanobacterial endosymbiont of the unicellular algae Rhopalodia gibba shows reductive genome evolution. BMC Evol Biol 8:1–8

Langlois RJ, Laroche J, Raab PA (2005) Diazotrophic diversity and distribution in the tropical and subtropical Atlantic ocean. Appl Environ Microbiol 71:7910–7919

Löffelhardt W, Bohnert HJ, Bryant DA (1997) The cyanelles of *Cyanophora paradoxa*. Crit Rev Plant Sciences 16:393–413

Mazard SL, Fuller NJ, Orcutt KM et al (2004) PCR analysis of the distribution of unicellular cyanobacterial diazotrophs in the Arabian Sea. Appl Environ Microbiol 70:7355–7364

Mende D, Wiessner W (1983) Participation of photosystem II in regulation of photosynthetic electron transport in the green alga *Chlamydobotrys stellata*. Photobiochem Photobiophys 6:1–7

Moisander PH, Beinert RA, Hewson I et al (2010) Unicellular cyanobacterial distributions broadens the oceanic N_2 fixation domain. Science 327:1512–1514

Nowack ECM, Melkonian M (2010) Endosymbiotic associations within protists. Phil Trans Roy Soc B 365:699–712

Prechtl J, Kneip C, Voss C, Maier UG (2004) Intracellular spheroid bodies of *Rhopalodia gibba* have nitrogen fixing apparatus of cyanobacterial origin. Mol Biol Evol 21:1477–1481

Pringsheim EG, Wiessner W (1960) Photo-assimilation of acetate by green organisms. Nature 188:919–921

Pringsheim EG, Wiessner W (1961) Ernährung und Stoffwechsel von Chlamydobotrys (Volvocales). Arch Mikrobiol 40:231–246

Pruesse E, Quast C, Knittel K et al (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA

sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Reyes-Prieto A, Bhattacharya D (2007) Phylogeny of nuclear-encoded plastid-targeted proteins supports an early divergence of glaucophytes within plantae. Mol Biol Evol 24:2358–2361

Schmitz O, Gurke J, Bothe H (2001) Molecular evidence for the aerobic expression of nifJ, encoding pyruvate:ferredoxin oxidoreductase in cyanobacteria. FEMS Microbiol Lett 195:97–102

Schnepf E, Deichgräber G (1978) Development and ultrastructure of the marine, parasitic oomycete, *Lagenisma coscinodisci* Drebes (Lagenidiales). Arch Mikrobiol 166:133–139

Schnepf E, Schlegel I, Hepperle D (2002) *Petalomonas sphagnophila* (Euglenophyta) and its endocytobiotic cyanobacteria: a unique form of symbioses. Phycologia 41:153–157

Schwalbach MS, Tripp HJ, Steindler L, Smith DP, Giovannoni SJ (2010) The presence of the glycolysis operon in SAR11 genomes is positively correlated with ocean productivity. Environ Microbiol 12:490–500

Stal LJ (2009) Is the distribution of nitrogen-fixing cyanobacteria in the oceans related to temperature? Environ Microbiol 11:1632–1645

Stal LJ, Zehr JP (2008) Cyanobacterial nitrogen fixation in the ocean: diversity, regulation and ecology. In: Herrero A, Flores E (eds) The cyanobacteria, molecular ecology, genomics, evolution). Caister Academic Press, Norfolk, U.K, pp 423–446

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thauer RK (2007) A fifth pathway of C-fixation. Science 318:1732–1733

Tikhonovich IA, Provorov NA (2009) From plant-microbe interactions to symbiogenetics: a universal paradigm for the interspecies genetic integration. Annals Appl Biol 154:341–350

Trepel J, McDermott JE, Summerfield TC, Sherman LA (2009) Transcriptional analysis of the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 grown under short day/night cycles. J Phycol 45:610–620

Tripp HJ, Bench SR, Turk KA et al (2010) Metabolic streamlining in an open-ocean nitrogen-fixing cyanobacterium. Nature 464:90–94

Turner S, Huang T-C, Chaw S-M (2001) Molecular phylogeny of nitrogen-fixing unicellular cyanobacteria. Bot Bull Acad Sin 42:181–186

Wiessner W (1962) Kohlenstoffassimilation von *Chlamydobotrys* (Volvocales). Arch Mikrobiol 43:402–411

Wiessner W, Gaffron H (1964) Role of photosynthesis in the light-induced assimilation of acetate by *Chlamydobotrys*. Nature 201:725–726

Zeev EB, Yogev T, Man-Ahaoronovich D, Kress N, Herut B, Béjà O, Berman-Frank I (2008) Seasonal dynamics of the endosymbiotic, nitrogen-fixing cyanobacterium *Richelia intracellularis* in the eastern Mediterranean Sea. ISME J 2:911–923

Zehr JP, Mellon MT, Zani S (1998) New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (nifH) genes. Appl Environ Microbiol 64:3444–3450

Zehr JP, Waterbury JB, Turner PJ et al (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature 412:635–638

Zehr JP, Montoya JP, Jenkins BD et al (2007) Experiments linking nitrogenase gene expression to nitrogen fixation in the North Pacific subtropical gyre. Limn Oceanograph 52:169–183

Zehr JP, Bench SR, Carter BJ et al (2008) Globally distributed uncultured oceanic N_2-fixing cyanobacteria lack oxygenic photosystem II. Science 322:1110–1112