Symbiosis (2012) 57:51–55
DOI 10.1007/s13199-012-0174-2

Ultrastructural and molecular characterization of cyanobacterial symbionts in *Dictyocoryne profunda* (polycystine radiolaria)

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Received: 10 April 2012 / Accepted: 4 June 2012 / Published online: 16 June 2012

Abstract Cyanobacterial symbionts were detected in the extracytoplasm of the polycystine radiolarian *Dictyocoryne profunda* Ehrenberg. The bacterial symbionts were observed as numerous spherical bodies ~0.5–1.0 μm in diameter under transmission electron microscopy. They were present in a very restricted location close to the periphery of the host radiolarian shell, adjacent to the central capsular wall. Several cells of them may have been in the process of cell division or just divided. The symbionts had thylakoid-like structures, which ran around the cell periphery in two or three concentric layers. Based on the small subunit ribosomal DNA (16S rDNA) phylogenetic analyses, the intracellular symbiotic bacteria grouped with cyanobacteria belonging to the genus *Synechococcus*. Three sequences, one from each of three specimens of *D. profunda*, collected in March/October 2009 and March 2010 from the East China Sea, were the same and branched within *Synechococcus* clade II, that is characterized by strains with low amounts of phycourobilin (PUB).

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Keywords Cyanobacteria · *Dictyocoryne profunda* · Radiolaria · Symbiont · *Synechococcus*.

1 Introduction

Radiolarians are amoeboid, planktonic marine protists, commonly found from arctic to tropical waters and throughout the water column from the surface to the greatest depths (Casey 1971). Radiolarians are important components of epipelagic communities in oligotrophic oceans because they prey upon algae and other planktonic organisms and they contribute significantly to local primary production because of their algal symbionts.

Dinoflagellates, prasinophytes, and haptophytes have all been identified as symbionts of radiolarians (e.g. Anderson 1983; Gast and Caron 1996). Bacterial symbionts are also present in natural populations of some radiolarian species (Anderson 1983; Anderson and Matsuoka 1992; Carpenter and Foster 2002; Foster et al. 2006a,b). Although a wide range of algae has been reported as symbionts of radiolarians since the end of the 19th century (e.g. Brandt 1881), the bacterial symbionts of radiolarians are still poorly known.

The cyanobacterial symbionts of radiolarians were previously identified as bacteria or brown microalgae (e.g. Anderson and Matsuoka 1992). Recently, the bacterial symbionts of the radiolarian *Dictyocoryne* sp. belonged to the cyanobacterial genus *Prochlorococcus,* based on the 16S ribosomal RNA gene sequences (Foster et al. 2006a). Moreover, the ultrastructural features of cyanobacterial symbionts of *Dictyocoryne truncatum* (Ehrenberg), including the cell diameter (0.5–0.8 μm), the typical peripheral thylakoids, and other features were reminiscent of *Prochlorococcus* sp. (Foster et al. 2006b).

Here we report some newly discovered intracellular bacteria which are found in the extracytoplasm of the

solitary polycystine radiolarian *Dictyocoryne profunda* Ehrenberg, based on 16S rDNA phylogenetic analyses and fine structural characteristics.

2 Materials and method

2.1 Sample collection

Cells of the polycystine radiolarian *Dictyocoryne profunda* Ehrenberg (Fig. 1) were collected from the East China Sea at Site 990528 (26°37′N, 127°47′E), located approximately 5 km northwest of Okinawa Island, Japan, in March/October 2009 and March 2010 using a plankton net (60 cm diameter opening with 37 μm mesh). The net was towed obliquely just under the surface for about 5 min. The collected samples were stored in plastic bottles containing seawater from the field, maintained at about 25 °C. The bottles were taken immediately to the laboratory at the Tropical Biosphere Research Center, University of the Ryukyus. Radiolarian species were identified and isolated from other plankton. They were then transferred to six-well culturing dishes containing seawater filtered by a 0.2 mm filter (Millipore Corporation, Billerica, MA, USA), which were stored until ultrastructural studies and 16S rDNA region amplifications were done. Each radiolarian specimen used for analysis was photographed.

2.2 Ultrastructural observation

For ultrastructural studies, single cell of *D. profunda,* which was collected in March 2009 at the site, was embedded in 1.5 % low-temperature-gelling agarose (Merck, Darmstadt, Germany) made up with seawater. A piece of the agarose gel with an embedded *D. profunda* cell was initially fixed in 2.0 % glutaraldehyde made up with 0.1 M sodium cacodylate buffer (pH 7.0) with 0.1 M sucrose. The piece of agarose gel was then rinsed three times in 0.1 M sodium cacodylate buffer (pH 7.0) with 0.1 M sucrose before postfixation in 1.0 % OsO_4 at room temperature for 2 h. After dehydration through an ethanol series (30, 50, 80, 90, and 100 %), the specimen was embedded in Spurr's resin (Agar Scientific, Essex, UK) and sectioned by diamond knife. The sections were picked up on Formvar-coated grids, and then double stained with uranyl acetate and lead citrate (Reynolds 1963). These sections were examined with a JEM-100CX II transmission electron microscope (JEOL, Tokyo, Japan). Micrographs were taken by a film camera.

2.3 16S rDNA extraction and amplification

Single cells of *D. profunda* were rinsed twice in filtered seawater, and the ectocytoplasm containing cyanobacterial symbionts was physically separated from the central capsule by a sterilized razor blade. The ectocytoplasm was rinsed twice in distilled water, and then it was used as a template for the amplification of 16S rRNA coding regions. Polymerase chain reaction (PCR) was accomplished using a eubacterial-specific forward primer 8 F (Edwards et al. 1989): 5′- AGAGTTTGATCCTGGCTCAG-3′ and a reverse primer

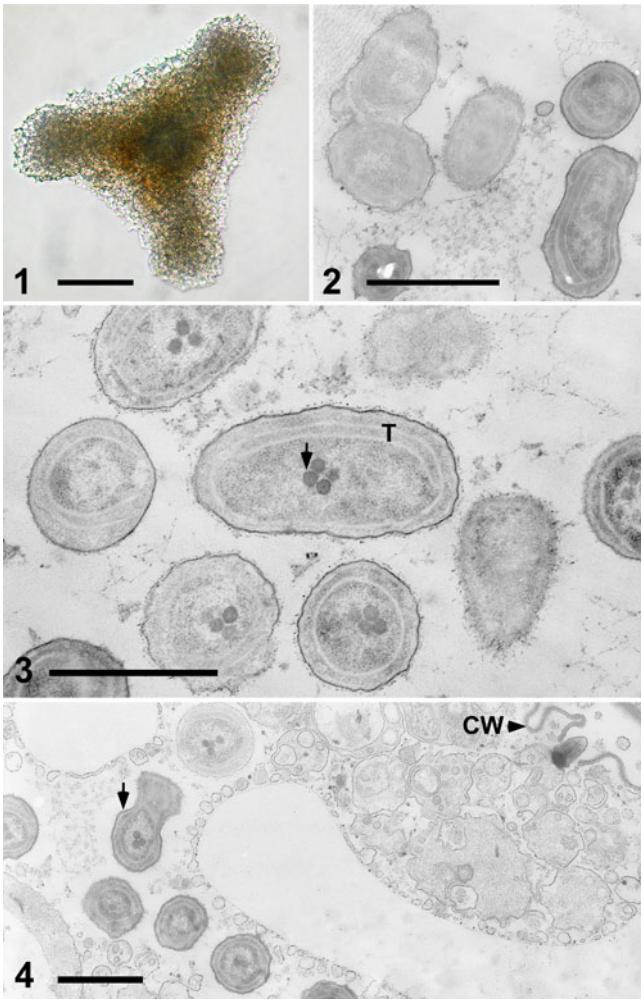

Fig. 1 Light micrographs of *Dictyocoryne profunda* Ehrenberg, collected at Site 990528 (26°37′N, 127°47′E), East China Sea. Scale bar =50 μm. **Fig. 2** Transmission electron micrographs of cyanobacterial symbionts in *Dictyocoryne profunda*. The symbionts have spherical, slightly elongated or constricted outlines. Several cells may have been in the process of cell division or just divided. Scale bar= 1.0 μm. **Fig. 3** Transmission electron micrographs of cyanobacterial symbionts in *Dictyocoryne profunda*. The symbionts have thylakoid-like structures (T), which run around the cell periphery ranging in two or three concentric layers. Carboxysomes are often present at the center of the cells (arrow). Scale bar =1.0 μm. **Fig. 4** Transmission electron micrographs of cyanobacterial symbionts in *Dictyocoryne profunda*. The symbionts are observed as numerous spherical bodies ~0.5–1.0 μm in diameter (arrow). They are found in a very restricted location close to the periphery of the host radiolarian shell adjacent to the central capsular wall (CW). Scale bar =1.0 μm

1520R (Edwards et al. 1989): 5′-AAGGAGGTGAT CCAGCCGCA-3′. PCR amplification was performed using a MiniCycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 1 min, at 52 °C for 1 min, and at 72 °C for 1 min. Each PCR product was purified by Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and then cloned in the pGEM-T Easy Vector System (Promega) using *E. coli* JM109 Competent Cells (Promega). Five clones for each amplified fragment from the inserts were sequenced using flanking vector primers. The partial sequences of the 16S rDNA were performed with the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit and analyzed with an ABI 3130 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.4 Phylogenetic analyses

We analyzed three 16S rDNA sequences of symbiotic bacteria obtained from each of three specimens of *Dictyocoryne profunda* collected in March/October 2009 (the *D. profunda* 1 cyanobiont and the *D. profunda* 2 cyanobiont, respectively) and March 2010 (the *D. profunda* 3 cyanobiont). The obtained sequences were aligned with 39 other sequences of cyanobacteria retrieved from GenBank using ClustalW ver. 1.81 (Thompson et al. 1994). Subsequently, the alignment was manually refined using the nucleotide sequence editor Se-Al ver. 1.0a1 (Rambaut 1996). Regions with gaps were excluded from our analyses, resulting in a total of 1332 nucleotide characters. The accession numbers of the 16S rDNA sequences used in this study are indicated in Fig. 5. Molecular phylogenetic relationships were inferred from the neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-likelihood (ML) (Felsenstein 1981) methods using PAUP* ver. 4.0b10 (Swofford 2002). A data set of 42 taxa (1332 bp) was used for the NJ and ML analyses. The best-fitting nucleotide substitution model was selected by using Modeltest ver. 3.06 (Posada and Crandall 1998) was used. The evolutionary distance decided from the GTR+G+I distance matrices models was used for NJ and ML analyses. The ML analysis was initially obtained from the transition/transversion (ts/tv) ratio of the GTR+G+I distance matrices model, which was estimated by maximizing the likelihood value for the NJ topology. The ML tree was then analyzed using a heuristic search method with a TBR branch-swapping option and random taxon addition. Relative levels of support for nodes of the NJ and ML trees were assessed by calculating full heuristic bootstrap proportion values (Felsenstein 1985) based on 1000 and 100 replicates in the NJ and ML analyses, respectively. Only the ML tree with bootstrap values for both NJ and ML is shown in Fig. 5.

3 Results and discussion

3.1 Transmission electron microscopy

Dictyocoryne profunda cells were characterized by the presence of numerous spherical bodies, ~0.5–1.0 μm in diameter. They were restricted to the periphery of the shell adjacent to the central capsular wall (Figs. 2, 3 and 4). They had spherical, slightly elongated or constricted outlines. Several cells may have been in the process of cell division or just divided (Fig. 2). Thylakoid-like structures were present which ran around the cell periphery in two or three concentric layers (Fig. 3). Carboxysomes were often present at the center of the cells (Fig. 3).

In the present study, TEM showed bacterial symbionts within the extracytoplasm of *Dictyocoryne profunda* that were similar to those from *D. truncatum* reported by Anderson and Matsuoka (1992) and Foster et al. (2006b). As was initially observed by Anderson and Matsuoka (1992) under TEM observation, *D. truncatum* also contains a number of "bacteroids," which are 0.2–0.5 μm and present throughout the intracapsular cytoplasm. On the basis of 16S rDNA sequence analyses and the ultrastructural observations, these bacterial symbionts in *Dictyocoryne* sp. and *D. truncatum*, which were found in the endoplasmic reticulum, have peripheral thylakoids and belong to the cyanobacteria genus *Prochlorococcus* (Foster et al. 2006a,b). We therefore analyzed the 16S ribosomal DNAs isolated from these bacterial symbionts of *D. profunda* in order to determine their identity.

3.2 16S rDNA-based phylogenetic analyses

The DDBJ/GenBank accession numbers for the 16S rDNA sequence data of these bacterial symbionts of *D. profunda* are AB693940, AB693941, and AB693942. BLAST sequence similarity searches (http://www.ncbi.nlm.nih.gov) confirmed that our sequences match those of the cyanobacteria *Synechococcus*. Phylogenetic analyses based on the 16S rDNA sequences of 39 cyanobacteria *Synechococcus* and their relatives (*Prochlorococcus*) placed our three sequences into the robust *Synechococcus* radiation (Fig. 5). Our sequences form a monophyly with the environmental clone sequences of *Synechococcus* strains WH 8002 and WH 8109, isolated from the tropical Sargasso Sea in the Atlantic Ocean; CC9605, isolated from the oligotrophic edge of the California Current; RS9902 and RS9904, isolated from the Gulf of Aquaba, Red Sea; and KORDI-12 and KORDI-20, isolated from the East Sea (Sea of Japan) (Fig. 5).

Based on 16S rDNA analyses, ten main clusters (clades I to X) have been recognized within the *Synechococcus* clade

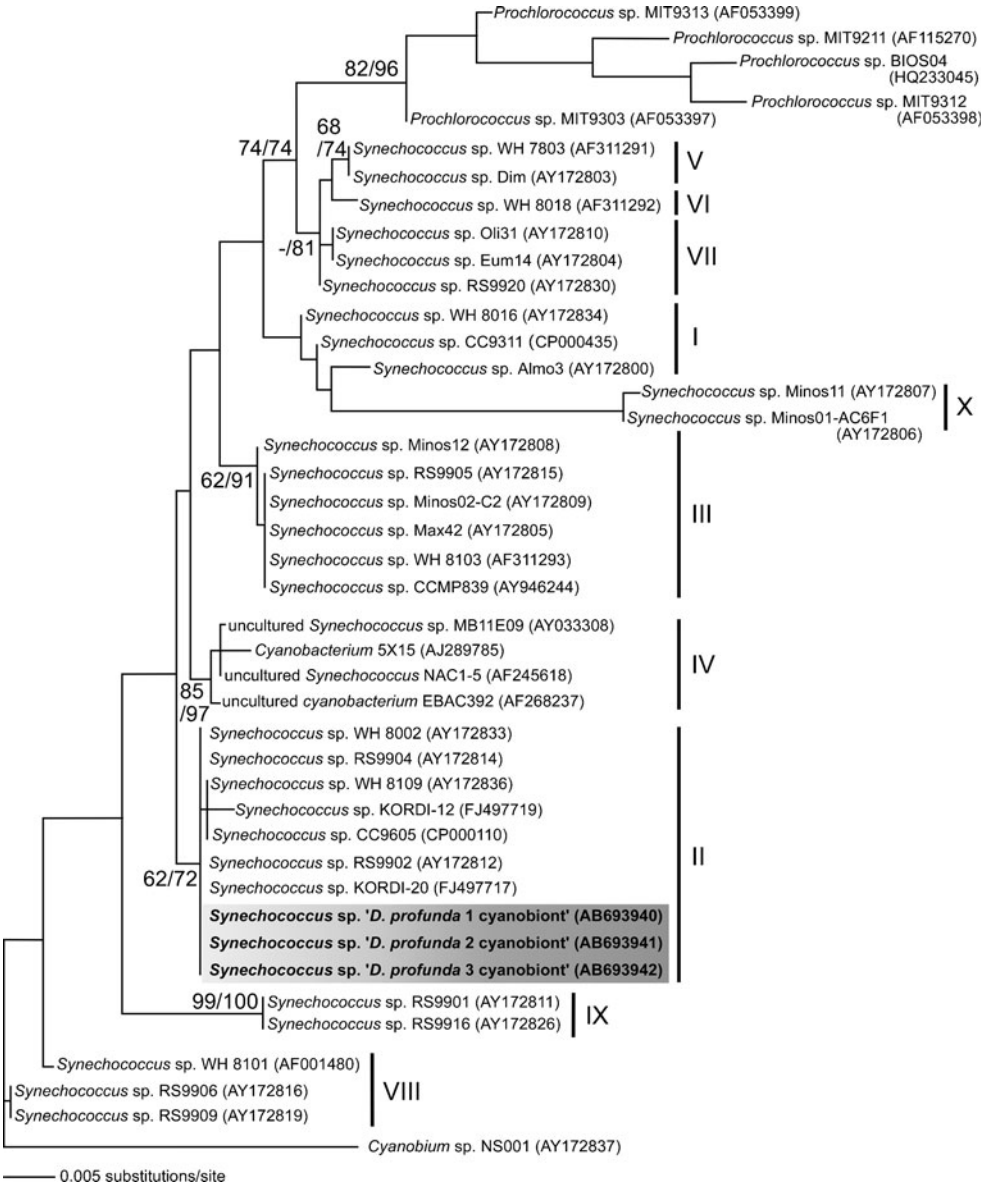

Fig. 5 16S rDNA phylogenetic tree based on the maximum-likelihood method (42 taxa, 1332 nucleotide sites) for our obtained sequences and other cyanobacterial strains already in the database. Bootstrap values exceeding 50 % are given at the respective nodes obtained from both maximum-likelihood and neighbor-joining methods (ML/NJ %). The *Synechococcus* strains fall into ten distinct clades

from cultures isolated from environmental samples (Rocap et al. 2002; Fuller et al. 2003). Our phylogenetic tree was largely congruent with other trees of *Synechococcus* lineage (Rocap et al. 2002; Fuller et al. 2003), and the cluster, including the three sequences we obtained, was composed of the members of the *Synechococcus* clade II (Rocap et al. 2002; Fuller et al. 2003; Tai and Palenik 2009). The *Synechococcus* clade II consists of marine phytoplankton abundant in the oligotrophic water column (e.g. Ferris and Palenik 1998), characterized by strains containing low levels of phycourobilin (PUB) (Rocap et al. 2002).

Molecular genetic studies have provided tools for the identification and classification of cyanobacteria in marine environments, making it possible to study the genetic diversity of natural populations (e.g. Rocap et al. 2002; Fuller et al. 2003; Choi and Noh 2009; Tai and Palenik 2009) and they were found even within the radiolarians. Radiolarians in the same genus (*D. truncatum* and *D. profunda*) harbored different types of cyanobacterial symbionts, whereas we found that *D. profunda* collected from the East China Sea in March/October 2009 and March 2010 harbored *Synechococcus* species that were indistinguishable from each other based on our 16S rDNA analyses. To resolve the specificity and diversity of the host-symbiont relationship in radiolarians, further information is required; particularly studies of the different host-cyanobacterial symbiont associations of radiolarians.

Acknowledgments We would like to acknowledge A. Takemura, Y. Nakano, and S. Nakamura of the University of the Ryukyus for their support and helpful discussions. We also wish to thank D. Honda of the Konan University for his valuable advice and suggestions. This study was financially supported by the grant-in-aid from JSPS Grants 19540491 and 23540545 to O. Takahashi.

References

Anderson OR (1983) Radiolaria. Springer–Verlag, New York

Anderson OR, Matsuoka A (1992) Endocytoplasmic microalgae and bacteroids within the central capsule of the radiolarian *Dictyocoryne truncatum*. Symbiosis 12:237–247

Brandt K (1881) Ueber das Zusammenleben von Thieren und Algen. Verh Physiol Ges Berlin 1881(1882):22–26

Carpenter EJ, Foster RA (2002) Marine cyanobacterial symbioses. In: Rai AN, Bergman B, Rasmussen U (eds) Cyanobacteria in symbiosis. Kluwer Academic Pub, Netherlands, pp 11–17

Casey RE (1971) Distribution of polycystine Radiolaria in the oceans in relation to physical and chemical conditions. In: Funnell BM, Riedel WR (eds) The Micropalaeontology of oceans. Cambridge University Press, Cambridge, pp 151–159

Choi DH, Noh JH (2009) Phylogenetic diversity of *Synechococcus* strains isolated from the East China Sea and the East Sea. FEMS Microbiol Ecol 69:439–448

Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17:368–376

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Ferris MJ, Palenik B (1998) Niche adaptation in ocean cyanobacteria. Nature 396:226–228

Foster RA, Collier JL, Carpenter EJ (2006a) Reverse transcription PCR amplification of cyanobacterial symbiont 16S rRNA sequences from single non-photosynthetic eukaryotic marine planktonic host cells. J Phycol 42:243–250

Foster RA, Carpenter EJ, Bergman B (2006b) Unicellular cyanobionts in open ocean dinoflagellates, radiolarians, and tintinnids: ultrastructural characterization and immuno-localization of phycoerythrin and nitrogenase. J Phycol 42:453–463

Fuller NJ, Marie D, Partensky F, Vaulot D, Post AF, Scanlan DJ (2003) Clade-specific 16S ribosomal DNA oligonucleotides reveal the predominance of a single marine *Synechococcus* clade throughout a stratified water column in the Red Sea. Appl Environ Microbiol 69:2430–2443

Gast RJ, Caron DA (1996) Molecular phylogeny of symbiotic dinoflagellates from planktonic foraminifera and radiolaria. Mol Biol Evol 13:1192–1197

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818

Rambaut A (1996) Se-Al. Sequence Alignment Editor, version1.0a1. Distributed by the author, Department of Zoology, University of Oxford

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212

Rocap G, Distel DL, Waterbury JB, Chisholm SW (2002) Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. Appl Environ Microbiol 68:1180–1191

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Swofford DL (2002) Phylogenetic Analysis Using Parsimony (PAUP*), ver 4.0b10. Sinauer Associates, Sunderland

Tai V, Palenik B (2009) Temporal variation of *Synechococcus* clades at a costal Pacific Ocean monitoring site. ISME J 3:903–915

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680