Marine Biology (2005) 148: 35–41
DOI 10.1007/s00227-005-0064-8

RESEARCH ARTICLE

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Transmission, plasticity and the molecular identification of cyanobacterial symbionts in the Red Sea sponge *Diacarnus erythraenus*

Received: 22 March 2005 / Accepted: 10 June 2005 / Published online: 12 August 2005

Abstract Cyanobacterial symbionts in the sponge *Diacarnus erythraenus* from the Red Sea were identified in both adult sponges and their larvae by 16S rDNA sequencing. A single cyanobacterial type was found in all samples. This cyanobacterial type is closely related to other sponge cyanobacterial symbionts. The cyanobacterial rDNA, together with the morphological analysis by electron and fluorescence microscopy, provided evidence for vertical transmission of the symbionts in this sponge. In addition, we show phenotypic plasticity of the symbionts inside the sponge, probably as a result of variability in light availability inside the sponge tissue. Finally, the reproduction of *Diacarnus erythraenus* is also described.

Introduction

Marine sponges have been found to harbor a wide variety of bacteria that can occupy up to 40% of their body volume (Wilkinson 1983). Bacteria are also a major component of the sponge diet. The mechanism by which the sponge distinguishes between organisms to be digested and its own symbionts has not been discovered yet. The presence of cyanobacteria in sponges has been frequently reported (*e.g.* Wilkinson 1981; Sarà et al. 1998). It has been proposed that the establishment of symbiosis between cyanobacteria and sponges dates back to the Precambrian era (Wilkinson 1983). Sponges are thought to benefit in several ways from this symbiosis. For example, cyanobacteria can contribute part of the host's nutritional requirements by transferring to it some of their photosynthetic products (Wilkinson 1979), and possibly also phosphate and fixed nitrogen (Wilkinson and Fay 1979). Symbiotic cyanobacteria in sponges have also been found to produce secondary metabolites that could have a role in the deterrence of predators and competitors (Unson and Faulkner 1993). It has also been suggested that cyanobacterial symbionts function as a light screen for the underlying sponge tissue (Sarà and Liaci 1964).

Microorganisms associated with sponges may be acquired from the surrounding water, or they may be transmitted vertically (i.e., from a parent sponge to its offspring via sponge reproductive elements). Bacterial acquisition, mainly via mature oocytes, has been documented for several sponge species using transmission electron microscopy (TEM) (e.g., Levi and Levi 1976; Gaino et al. 1987; Sciscioli et al. 1994). Although there are several studies showing vertical transmission of heterotrophic bacterial symbionts in sponges, only a few studies report on this mechanism for sponge-associated cyanobacteria. Scalera-Liaci et al. (1971, 1973) observed, by fluorescence microscopy, a close association of cyanobacteria with the eggs of three sponge species. Using TEM, a cyanobacterial species resembling *Aphanocapsa feldmanni* was observed by Usher et al. (2001) in the sponge *Chondrilla australiensis* and in its eggs. Recently, cyanobacteria were also identified within sponge sperm (Usher et al. 2005).

In the present study, we determined that the sponge *Diacarnus erythraenus* transmits its single type cyanobacterial symbiont vertically. This is the first study in which the vertical transmission of cyanobacterial symbionts in sponges is confirmed by molecular means. We also describe here the reproduction of *D. erythraenus*, as well as the distribution pattern and the morphology of the cyanobacteria in the adult sponge and its larvae.

Communicated by O. Kinne, Oldendorf/Luhe.

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Methods

Sponge collection and preservation

In order to find reproductive specimens of the sponge *Diacarnus erythraenus*, 5–7 individuals were sampled monthly from April 2003 to March 2004. The samples were collected using SCUBA from a shallow (3–6 m) reef in Elat (Northern Red Sea, 31°35′ N, 34°54′ E). Fragments of reproducing individuals were transferred to a tank containing filtered (0.2 μm) aerated seawater in the laboratory. On two occasions, free-swimming larvae were spontaneously released from the sponge fragments into the tank. The free-swimming larvae were collected and their lengths were measured. Six larvae from reproducing sponge individuals, as well as samples of sponge body parts cut out perpendicular to their surfaces, were fixed using 2.5% glutaraldehyde (GA) in filtered (0.2 μm) seawater for subsequent microscopic examinations (light and fluorescence microscopy and TEM). Incubated larvae (not fully developed) from the interior section of the sponge body (the mesohyl) were collected and fixed as well. For DNA extraction, specimens were preserved in 100% ethanol and stored at 4°C. The specimens included two larvae that were released from an adult reproducing sponge and ca. 0.5 cm^3 samples from the cortex of the latter adult sponge and from the cortex of two non-reproductive sponge individuals.

Microscopy

Histological preparations were made from samples fixed in the GA. For scanning electron microscopy (SEM), the fixed larvae were dehydrated through increasing ethanol concentrations, and then critical point dried with liquid CO_2. The samples were coated with gold and examined with a JEOL JSM 840A scanning electron microscope at 25 kV. Samples for TEM, fluorescence and light microscopy were prepared as follows: GA-fixed sponge fragments were rinsed in sterile seawater, transferred into 4% fluoric acid for 24 h for desilicification, and then rinsed with 70% ethanol to wash out all acid remains. GA-fixed larvae were transferred directly to 70% ethanol. For light microscopy, samples were embedded in paraffin, serially cross-sectioned (6 μm thick sections) and hematoxylin- and eosin-stained. For TEM, samples were stained with 1% OsO_4, dehydrated through increasing ethanol concentrations, and embedded in Epon. Sections were cut with a glass knife, stained with uranyl acetate and lead citrate, and viewed with a JEOL 1200 EX TEM.

The abundance and distribution patterns of the cyanobacterial symbionts were analyzed using a Zeiss Axioscope fluorescence microscope. Slides of the parental sponge and its larvae were illuminated with UV light using Zeiss filter 05 (excitation: 395–440 μm). The cyanobacterial symbionts were detected by their orange–yellow auto-fluorescence. The abundance of the cyanobacterial symbionts in the larvae ($n = 3$) was determined in three serial cross-sections from the center of each larva. Each cross-section was divided into two zones, A and B, according to the distance from the larval surface. Zone A, in the inner part of the larva, contained cyanobacterial symbionts. On the other hand, zone B, which surrounds zone A, was almost devoid of cyanobacteria. Zone B widths were 75–150 μm along the lateral sides and 450–600 μm along the rear and the front of the larvae. In each zone, five equal squares, 125×125 μm in size, were sampled randomly. All visible cyanobacterial symbionts within each square were counted. The same counting method was also applied for the incubated larvae ($n = 3$).

Molecular identification of cyanobacterial symbionts

Sponge and larval samples were preserved in 100% EtOH, then frozen and lyophilized, followed by DNA extraction using the procedure of Bernatzky and Tanksley (1986). Part of the cyanobacterial 16S rRNA gene was amplified with the specific primers 361F (5′-GAATTTTCCGCAATGGGC-3′) and 1459R (5′-GGTAAYGACTTCGGGCRT-3′) (Diaz and Ward 1997). Amplification reactions contained 50 ng of DNA, 1.5 mM $MgCl_2$, 10 mM TRIS pH 8.8, 50 mM KCl, 0.08% NONIDET P_{40}, 50 μM dNTPs, and 1 U *Taq* Polymerase (New England Biolabs). The amplification conditions were 94°C for 5 min followed by 36 cycles of 94°C for 45 s, 64°C for 45 s, 72°C for 90 s, and a final extension at 72°C for 5 min. Amplified fragments (of 1060-bp) were cloned in the PTZ57R/T vector (Fermentas) according to the manufacturer's instructions. Nineteen individual positive clones were selected randomly and amplified by PCR using M13 forward and reverse universal primers. The PCR products were digested with restriction enzymes *Apa*I, *Hae*III and *Sau3A*I. This was done to recognize possible different restriction patterns in order to determine the number of different cyanobacterial types. Two clones were sequenced for each individual adult sponge and larva. Sequencing was performed by the dideoxy chain termination method (Sanger 1981) on an ABI PRISM 3100 Genetic Analyzer, using M13 vector primers and a newly designed primer: 2000R (5′-TGCGGGACTTAACCCA ACATC-3′). The 16S rDNA sequences for all specimens (adult sponges and larvae) were found to be identical, therefore, in this study, a single sequence was obtained and deposited in the database. In order to identify the cyanobacteria found in the adult sponges and larvae, the 16S rDNA sequence obtained was blasted on the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). This enabled us to recognize the sequences of the most closely related cyanobacteria present in the database to the one identified

in the present study and determine their percentage of identity.

Results

Reproduction

The sponge *Diacarnus erythraenus* was found to be viviparous hermaphrodite. Reproduction products of the sponge were found in several individuals throughout the year, showing that there is no restricted reproductive season for this species. The release of larvae was recorded twice (November 2003 and February 2004), each time in different individuals. Embryos, oocytes and sperm cysts were found together in the sponge body, indicating asynchronous reproduction. Embryos and incubated larvae in a sponge fragment were visible to the naked eye as shiny yellow droplets located in collagenic niches inside the sponge mesohyl. The incubated larvae in their early developmental stages have a ball-like shape with folded ciliated surface (Fig. 1a). As the larvae develop, they stretch (Fig. 1b) until they open completely and get their final elongated shape (Fig. 1c). The free-swimming, fully ciliated larvae that were released from *D. erythraenus* are of the parenchymella type. Their color was dark yellow, and their size was 5075 ± 592 μm long and 1250 ± 236 μm in diameter ($n = 5$).

The presence of cyanobacteria in the sponge

Diacarnus erythraenus was found to contain unicellular cyanobacterial symbionts. The cyanobacteria that were observed by TEM ($n = 23$) were ovoid in shape, 1.1–3.2 μm in length and 0.8–2.5 μm in diameter, and had 2–9 thylakoid spirals. A gradual increase in cyanobacterial size and number of thylakoid spirals was observed from the sponge cortex towards its interior (Fig. 2). The cortex area (ca. 1 mm wide) was found to contain small cyanobacteria, 1.1–1.5 μm long and 0.8–1.3 μm in diameter with only two spiral thylakoids ($n = 9$, Fig. 3a). The abundance of these cyanobacteria was of over 10,000 cells per mm^2 . At a depth of ca. 2 mm inside the sponge tissue we observed larger cyanobacteria, 1.2–2.6 μm long and 1.1–2.4 μm in diameter with 3–7 spiral thylakoids ($n = 6$, Fig. 3b), scattered in a lower abundance. The interior section of the sponge (ca. 1 cm deep) was found to contain the largest cyanobacteria, 1.9–3.2 μm in length and 1.5–2.2 μm in diameter with 5–9 spiral thylakoids ($n = 8$).

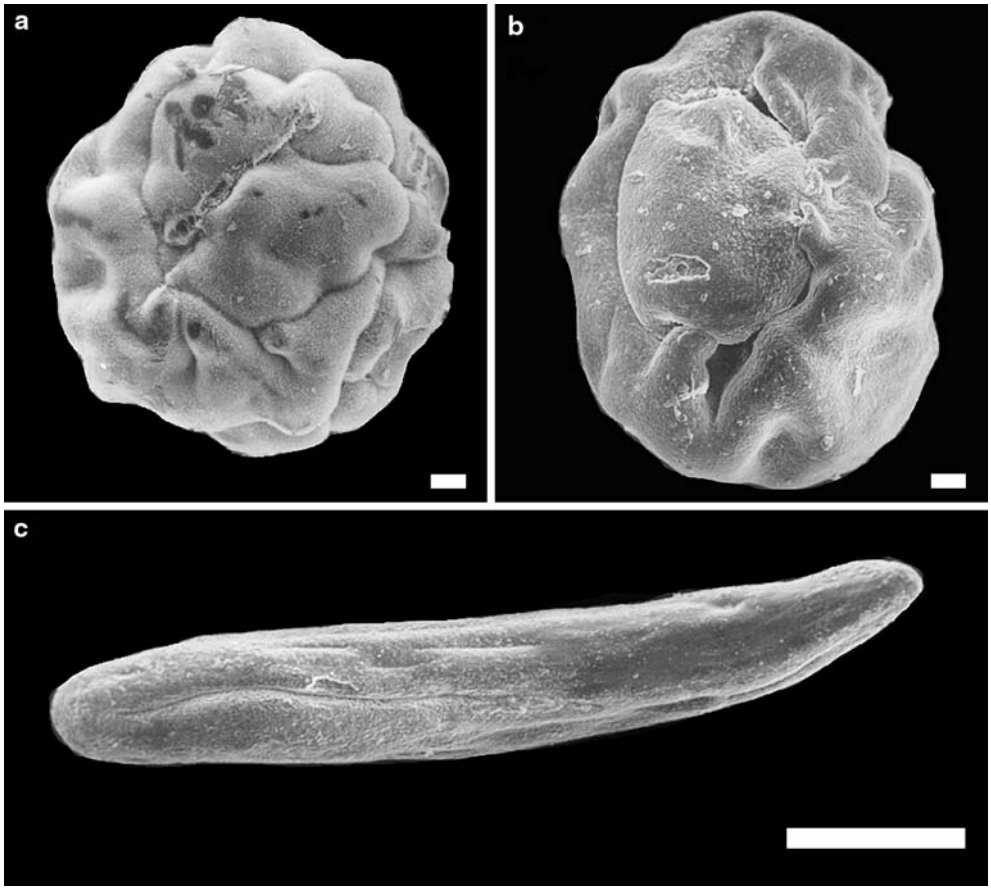

Fig. 1 *Diacarnus erythraenus* incubated larvae (SEM) (**a**) Folded ball-shaped incubated larva. Scale bar = 100 μm. (**b**) Incubated larva in the process of unfolding. Scale bar = 100 μm. (**c**) Fully unfolded larva just before release. Scale bar = 1 mm

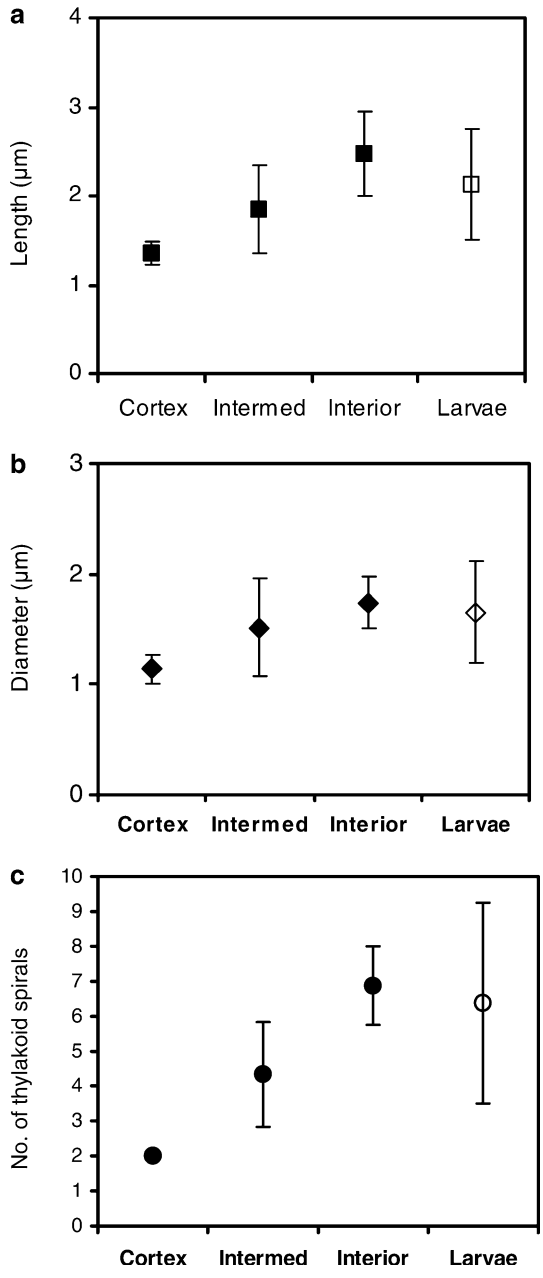

Fig. 2 Morphological characteristics (mean ±s.d.) of cyanobacteria from three different areas in an adult *Diacarnus erythraenus* sponge (Cortex: ca. 0–1 mm from the surface; Intermediate: ca. 2 mm from the surface; Interior: ca. 1 cm from the surface) and from the larvae. **(a)** Cyanobacterial length. **(b)** Cyanobacterial diameter. **(c)** Number of thylakoid spirals

Vertical transmission of cyanobacteria

Like for the adult sponge specimens, all larvae were also found to contain cyanobacteria. These cyanobacteria were 1.3–3.2 μm in length and 1.1–2.5 μm in diameter with 2–10 spiral thylakoids ($n = 8$, Fig. 3d). Upon comparing the morphology of cyanobacteria from mature sponges versus those from within larvae, the latter resembled the cyanobacteria found in the interior section of the mature sponge rather than those close to the surface (Fig. 2). Within-larvae cyanobacteria were found almost exclusively in the interior part (Fig. 4, zone A) with an average abundance of 1152 mm^{-2} in the free larvae and 1728 mm^{-2} in the premature folded incubated larvae. In contrast, the external part of the sponge larvae (Fig. 4, zone B) was found to be almost devoid of cyanobacteria, with an average abundance of less then 25 mm^{-2} . The cyanobacteria observed in the adult sponge and in the larvae were extra-cellular, except for several cases in which cyanobacteria were surrounded with sponge cell extensions, indicating phagocytosis (Fig. 3c), or in more advanced stages of digestion (Fig. 5). Some cyanobacteria were observed dividing by binary fission in which they were stretched and pinched at the center (Fig. 5). Bacteria of different morphological types were present in both sponge and larval samples. These bacteria were also extra-cellular; bacteriocytes were not observed in any sponge or larva sample.

Molecular analyses

Molecular analysis enabled us to identify more specifically the cyanobacteria and investigate whether different sized cyanobacteria are different species or different developmental stages of the same species. Restriction fragment length polymorphism analysis (with three different restriction enzymes) showed a single cyanobacterial type found in both mature sponges as well as in the larvae. The sequence (partial 16 rRNA gene) has been deposited in the database under the accession number AY882559. The closest related sequences found on the database were also from sponge-associated cyanobacteria, 99% identity with the cyanobacterial symbiont of *Chondrilla nucula*, accession number AY190186 and 98% with the cyanobacterial symbiont of *Aplysina aerophoba*, accession number AY190186, with both host sponge species collected in the Mediterranean.

Discussion

In marine sponges, the acquisition and transmission of cyanobacterial symbionts has been poorly studied. The absence of records of a free-living stage of sponge-associated cyanobacterial symbionts (Usher et al. 2004a, 2004b) suggests that vertical transmission could be the main (or even the only) source for acquiring these symbionts to a new sponge generation. In addition, there are reports of sponges losing their cyanobacterial symbionts after a period of darkening (e.g. after being transplanted to caves, Regoli et al. 2000), but the mechanism by which the cyanobacteria were expelled was not described, and the aposymbiotic sponges did not re-acquire their symbionts when transferred back to an illuminated environment. Vertical transmission of cyanobacterial symbionts in sponges could explain their specificity to the host like the specificity found for sponges of the genus *Dysidea* (Thacker and Starnes

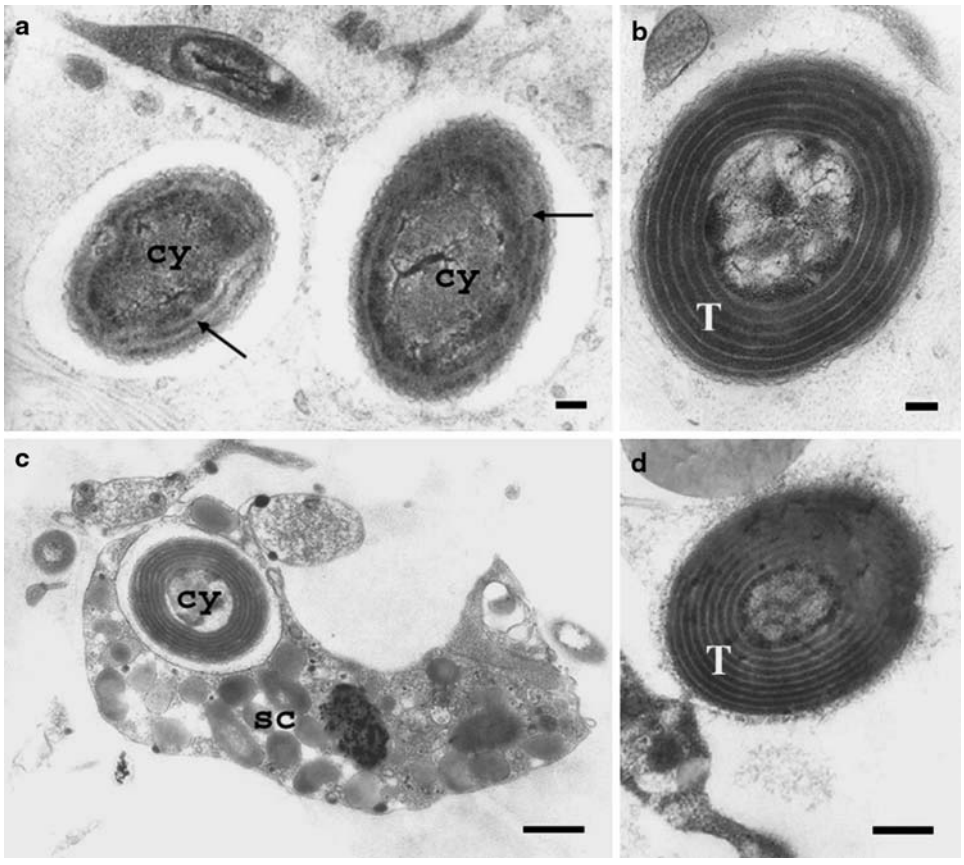

Fig. 3 Cyanobacteria in *Diacarnus erythraenus* larvae and adult sponge (TEM). (**a**) Small cyanobacteria (cy) with two spiral thylakoids (arrows) from the cortex area. Scale bar = 200 nm. (**b**) Cyanobacterium with six spiral thylakoids (T) from the sponge interior. Scale bar = 200 nm. (**c**) Cyanobacterium (cy) within a sponge cell (sc) in the process of phagocytosis. Scale bar = 1 μm. (**d**) Cyanobacterium with eight thylakoid spirals (T) from a larval interior part. Scale bar = 500 nm

2003). Our finding that cyanobacterial symbionts in the sponge *D. erythraenus* from the Red Sea are vertically transmitted, as are those of *Chondrilla australiensis* from Australia (Usher et al. 2001), suggests that vertical transmission indeed is a broad phenomenon since it apparently exists in different sponge genera from distant geographic locations.

The presence of cyanobacterial symbionts in the free non-feeding larvae supports the idea that cyanobacteria are transmitted vertically to the next generation, since the larva is the first developmental stage that is physically independent from the parent. The larva cannot acquire its symbionts from the water column since it is a sealed entity having no openings towards the surrounding environment. The vertical transmission of photosynthetically active symbionts may thus not only be of relevance for their transmission to the next sponge generation, but might also be significant for the survival of the larva in the period before it can actively feed by water filtration. The symbiont may here transfer

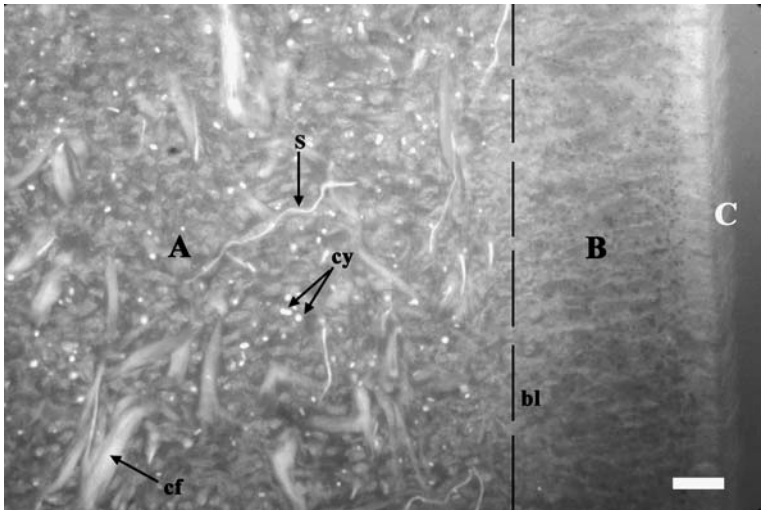

Fig. 4 *Diacarnus erythraenus*, cross-section of a larva viewed by fluorescence microscope. The larval interior zone (A) contains cyanobacteria (cy), whereas the larval external layer (B) is devoid of cyanobacteria. The two defined zones are separated by a clear border line (bl) at a distance of 75–150 μm from the larval surface and 450–600 μm from its anterior or posterior surface. The larva is covered with cilia (C) on its surface, and contains collagen fibers (cf) and premature spicules (s). Scale bar = 30 μm

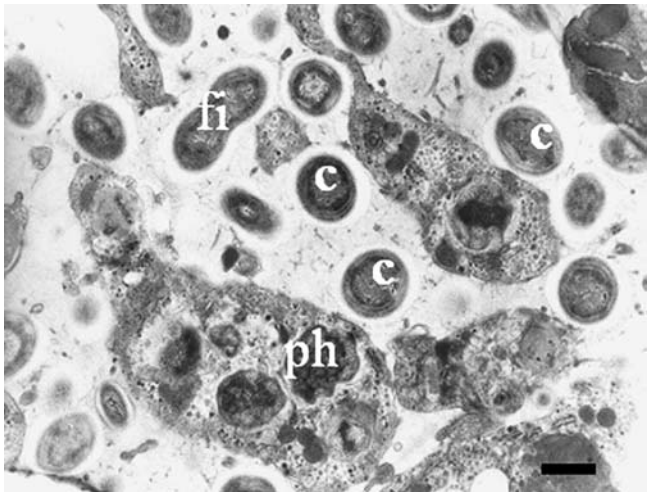

Fig. 5 *Diacarnus erythraenus* cyanobacteria (c) beneath the cortex (TEM). Some cyanobacteria are in the process of fission (fi) while other are being digested by the sponge cells (ph). Scale bar = 1 μm

photosynthetic products to the lecitotrophic (non-feeding) host, thus enhancing its competitive fitness.

In *Diacarnus erythraenus*, as in *C. australiensis* (Usher et al. 2001), some cyanobacterial symbionts inside mature sponges were observed undergoing phagocytosis. The reason for which the sponge host ingests its own symbionts is unknown, and may be related to a balancing mechanism by which the size of the symbiont population is controlled by the host.

The identification of cyanobacteria by morphology only is problematic since they are usually very similar in their phenotypic characteristics. Molecular analysis has already revealed how in the past cyanobacteria were classified wrongly, as morphologically similar cyanobacteria were actually phylogenetically very distantly related (Honda et al. 1999; Robertson et al. 2001). Therefore, for unicellular cyanobacteria a more accurate identification of the species is by analysis of DNA sequences. The 16S rRNA gene was selected here for this purpose because it is now-a-days the most represented one for bacteria in databases. According to the TEM, cyanobacteria of different sizes and numbers of spiral thylakoids were present in both mature sponges and larvae. In the present study, we were able to determine that all the morphologically slightly different cyanobacteria observed were actually molecularly identical. Therefore, *D. erythraenus* contains a single cyanobacterial type, which is transmitted vertically to the next sponge generation. The difference in size and in the number of spiral thylakoids can be attributed to the algal phenotypic plasticity as a consequence of a variable internal environment in the sponge. Cyanobacteria probably adapt to the different light conditions inside the sponge, and, indeed, when located in darker parts (deeper inside the sponge body) were found to have a larger number of spiral thylakoids (which results in an increased cell size) that are likely to increase the efficiency of light capture. The similarity of the large cyanobacteria with the 5–9 thylakoid spirals found within both the sponge internal part and its recently released larvae, gives further support to the direct transfer of cyanobacteria from the parental sponge to its incubated larvae.

The cyanobacterium identified in *D. erythraenus* was found to be most closely related to other cyanobacterial symbionts deriving from sponges collected from the Mediterranean (*Chondrilla nucula* and *Aplysina aerophoba*). Those sponge symbionts have been found to be part of the major cyanobacterial sponge symbiont clade known (Steindler et al. 2005), strengthening their identification as true symbionts and not water-borne contaminations. Usher et al. (2004b) proposed for some members of this clade the name *Synechococcus spongiarum*, as they are closely related to free-living *Synechococcus* species.

More studies are needed in order to determine whether vertical transmission is indeed the main, or even the only, mode by which cyanobacteria are acquired in marine sponges. Such a mode of transmission has been regarded as a parameter intimately linked to the evolution towards mutualistic symbioses that distances symbiosis from parasitism (Yamamura 1993; Herre et al. 1999). This is because in vertical transmission the survival of the symbiont is linked to that of the host, and therefore the exploitation of the host by the symbiont is limited.

Acknowledgements This work was financially supported by Grant 2000-321 from the United States–Israel Binational Science Foundation (BSF) to M. Ilan, S. Beer and J.R. Pawlik. I. Brickner is thanked for histological preparations, and Y. Delarea for assistance with electron microscopy. S. Beer is thanked for his editorial comments of the manuscript. We thank the staff of the Inter-University Institute in Elat for their help during the course of this study. Samples were collected in compliance with the 2003/16729 permit from the Israel Nature and National Parks Protection Authority.

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