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Intra- and Extra-Cellular Distribution of Photosynthetic Prokaryotes, *Prochloron* sp., in a Colonial Ascidian: Ultrastructural and Quantitative Studies

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Abstract

Prochlorons are prokaryotic algae (or photosynthetic bacteria) found as specific symbionts of some colonial ascidians in the family Didemnidae, usually on tropical or subtropical marine coasts. In *Lissoclinum punctatum*, they occur both inside as well as outside the tunic. In histological sections of the ascidian colony, the area occupied by *Prochloron* cells (PrC) is about 4.5%. Since more than 45% of these are present inside the tunic, mostly enclosed within certain free mesenchymal cells (tunic phycocytes), nearly half of the photosynthesis in the ascidian colony is estimated to be carried out by such intracellular PrC. Our observations indicate that tunic phycocytes originate from tunic phagocytes that have ingested the symbionts by endocytosis and retained them intact (Hirose et al., 1996). Apparently some tunic phagocytes can ingest two or three PrC at a time, while others can later ingest another. Furthermore, some PrC can possibly survive and divide within symbiosomes. When phagocytic hemocytes of another symbiotic ascidian, *L. patella*,

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were incubated with their symbionts *in vitro*, they become phycocyte-like by endocytosis of PrC. This observation further supports the origin of tunic phycocytes in *L. punctatum*.

Keywords: endosymbiosis, tunic cell, endocytosis, *Prochloron*, Prochlorophyta, Urochordata

1. Introduction

Prochloron cells (PrC) are photosynthetic prokaryotes characterized by containing chlorophylls *a* and *b*. They are symbionts of colonial ascidians belonging to the family Didemnidae (Tunicata, Chordata), where hitherto they have been reported to occur on the surface or in peribranchial/cloacal cavities of the ascidian colony, outside host tissues.

Recently, we found intracellular symbiosis of *Prochloron* sp. in a tropical ascidian, *Lissoclinum punctatum*, where PrC are found not only in peribranchial and cloacal cavities but also in the tunic, an integumentary matrix covering the epidermis (Hirose et al., 1996). While several types of free mesenchymal cells are found in the tunic, most of the PrC are contained in one particular type, designated as phycocytes. Since such phycocytes can still exhibit phagocytic activity, as may be demonstrated when they are incubated with latex beads (Hirose et al., 1996), we conclude that they can ingest PrC by endocytosis and retain them as endosymbionts. We found no morphological differences between intracellular and extracellular PrC, and since intracellular PrC show no evidence for rejection or degeneration, this association between tunic cells of *L. punctatum* and PrC seems to constitute a stable symbiosis. It could be regarded as a model representing an early stage in the evolution of intracellular photosynthetic prokaryotes that ultimately became chloroplasts, and thus could provide clues to the processes of intracellular symbiogenesis.

In this study, the distribution of PrC in colonies of *L. punctatum* was examined qualitatively and quantitatively. We also followed the sequence of intracellular integration of PrC in *L. punctatum*, and compared it with that in another symbiotic ascidian, *L. patella*, from a nearby habitat.

2. Materials and Methods

Animals

We found colonies of *Lissoclinum punctatum* attached to macroalgae in a marine lake (Urukthapel Island: 134°–25'E, 7°–18'N) and *L. patella* growing on coral rubble (Koror Island: 134 °–30'E, 7°–20'N) , both at a depth of 1–3 m in the

Republic of Belau, Western Caroline Islands. The animals, collected by snorkeling, were transported in plastic containers and maintained in tanks with running seawater on board the R/V "Sohgen Maru".

Microscopy

Small pieces of *L. punctatum* were anesthetized in seawater saturated with *l*-menthol, pre-fixed with a 2.5% glutaraldehyde solution containing 0.45 M sucrose and 0.1 M sodium cacodylate (pH 7.4) for at least 2 hr at 4°C, and then incubated in 5% ethylenediamine tetraacetic acid (EDTA) buffered with 0.1 M sodium cacodylate (pH 7.4) for 2 hr or more to dissolve the calcareous spicules in the tunic. After a brief rinse in 0.1 M sodium cacodylate, they were post-fixed with 1% osmium tetroxide buffered with 0.1 M sodium cacodylate for 1.5 hr, dehydrated through an ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in low-viscosity epoxy resin. For light microscopy, specimens were sectioned semi-serially (2 μm thick) and stained with 1% toluidine blue. For transmission electron microscopy, thin sections (ca. 0.1 μm thick) were double-stained with lead citrate and uranyl acetate and examined in a Hitachi HS-9 transmission electron microscope at 75 kV.

To observe structures of internal tissues by scanning electron microscopy (SEM), specimens were fixed with 2.5% glutaraldehyde (as above), dehydrated through a butanol series, embedded in paraffin and then cut with a microtome blade to expose the inner structures. The paraffin blocks were then washed in xylene (1 hour, three changes, to remove the paraffin), cleared with absolute ethanol, dried in a critical-point dryer, sputter-coated with gold-palladium, and examined with a Hitachi S-570 scanning electron microscope at 20 kV.

Quantitative estimation of PrC in L. punctatum

The areas occupied by intra- and extra-cellular PrC in colonies, zooids and cavities were estimated in four semi-serial sections, cut 50 μm apart, by digital imagery (NIH-Image 1.60).

Experimental endocytosis

A small piece of a *L. patella* colony wrapped in nylon cloth (84 μm mesh) was gently squeezed by hand into seawater buffered with 50 mM Tris at pH 8.4 (to neutralize acid liberated from bruised ascidian tissue). Some of the nucleated host hemocytes and/or free mesenchymal cells expressed along with

the PrC were periodically observed under a light microscope with Nomarski differential-interference contrast (DIC).

3. Results

Distribution of PrC in the ascidian colony

In colonies of *Lissoclinum punctatum*, PrC were found in the peribranchial/cloacal cavities and tunic (Fig. 1) but not in other parts of the colony or on the colony surface. The cloacal cavity wall usually has a complex surface through which PrC could enter the tunic (Hirose et al., 1996), since many PrC can be seen adhering to the wall in this area, some almost embedded in the tunic (Fig. 2). Within the tunic, the PrC are usually contained in free mesenchyme cells, which we call tunic phycocytes (Hirose et al., 1996). TEM observations revealed no significant ultrastructural differences between intracellular and extracellular PrC (Fig. 3); the thylakoid membranes are concentrically arranged around a central vacuole, and the cell surface is covered with fuzzy material through which papillate cytoplasm occasionally protrudes (arrowheads in Fig. 3B and D).

In the peribranchial cavities, some PrC are evidently caught and ingested by free nucleated cells (Fig. 4). Such nucleated cells have characteristics typical of phagocytes among ascidian hemocytes, being ameboid and often containing phagosomes (arrowhead in Fig. 4). A very few were also found in the lumen of the zooids' esophagus or intestine (Fig. 5), together with other materials common in their digestive tracts (Fig. 6).

Tunic phycocytes

Tunic phycocytes contain one, two, or occasionally three PrC, generally enclosed in a single large vacuole (symbiosome) and surrounded by only a thin layer of cytoplasm containing phagosomes (asterisk in Fig. 7A), as is consistent with their phagocytic activity (Hirose et al., 1996). Those containing two or three PrC can probably not divide since the bulk of the cytoplasm is occupied by a symbiosome, and since they have few or no pseudopodia they are probably not motile. Many PrC in symbiosomes are in stages of cell division (Fig. 7C), and give rise to paired cells within a common symbiosome. However, in other cells two PrC are separated by a thin layer of cytoplasm (arrowhead in Fig. 7C), indicating that they occupy separate symbiosomes and were probably ingested separately.

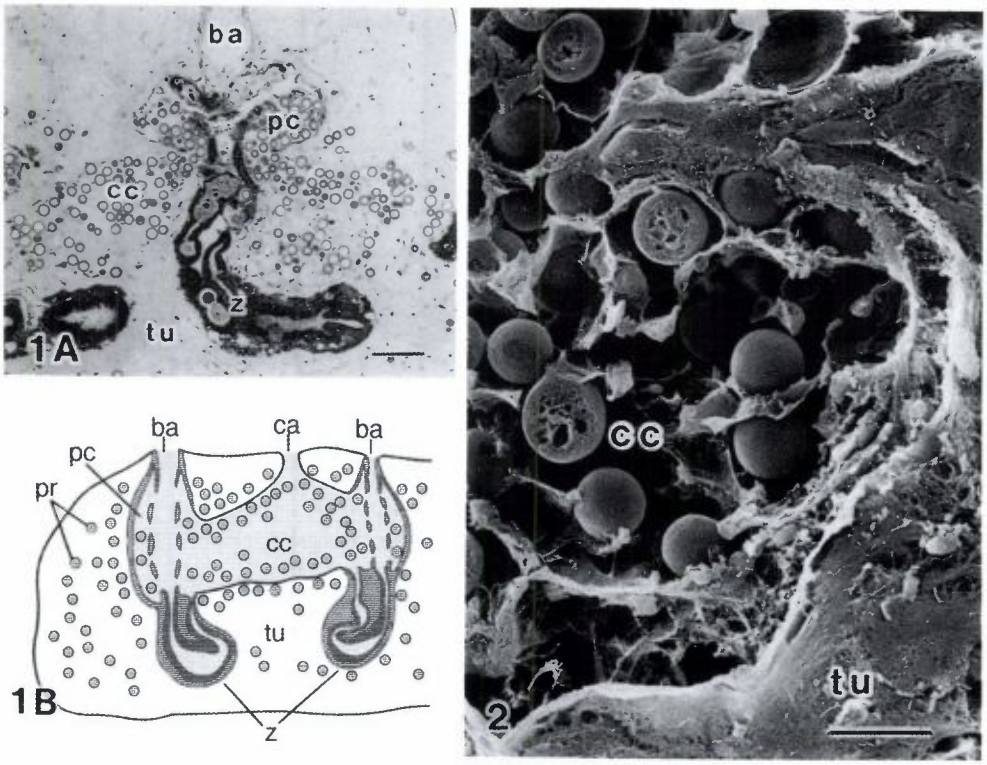

Figure 1. Light micrograph (A) and schematic drawing (B) of the cross section of a colony of *Lissoclinum punctatum*. *Prochloron* cells are distributed in the cavity and tunic. ba = branchial aperture; ca = cloacal aperture; cc = cloacal cavity; pc = peribranchial cavity; pr = *Prochloron*; tu = tunic; z = zooid. Scale bar in A = 0.1 mm.

Figure 2. Some *Prochloron* cells in cloacal cavity (cc) embedded in the complex lining of a tunic (tu). Scale bar = 20 μm.

Quantitative estimates of PrC in colonies

In histological sections (e.g., Fig. 1A), PrC occupied about 4.5% of the total area of the ascidian colony, about 45% being found in the tunic (where they occupy only about 2.5% of the area) and the rest in the cavity (where they occupy about 30% of the area) (Table 1). Almost all PrC in the tunic are intracellular. (These quantitative estimates may be somewhat inaccurate because of errors due to shrinkage of tissues during preparation of the specimens.)

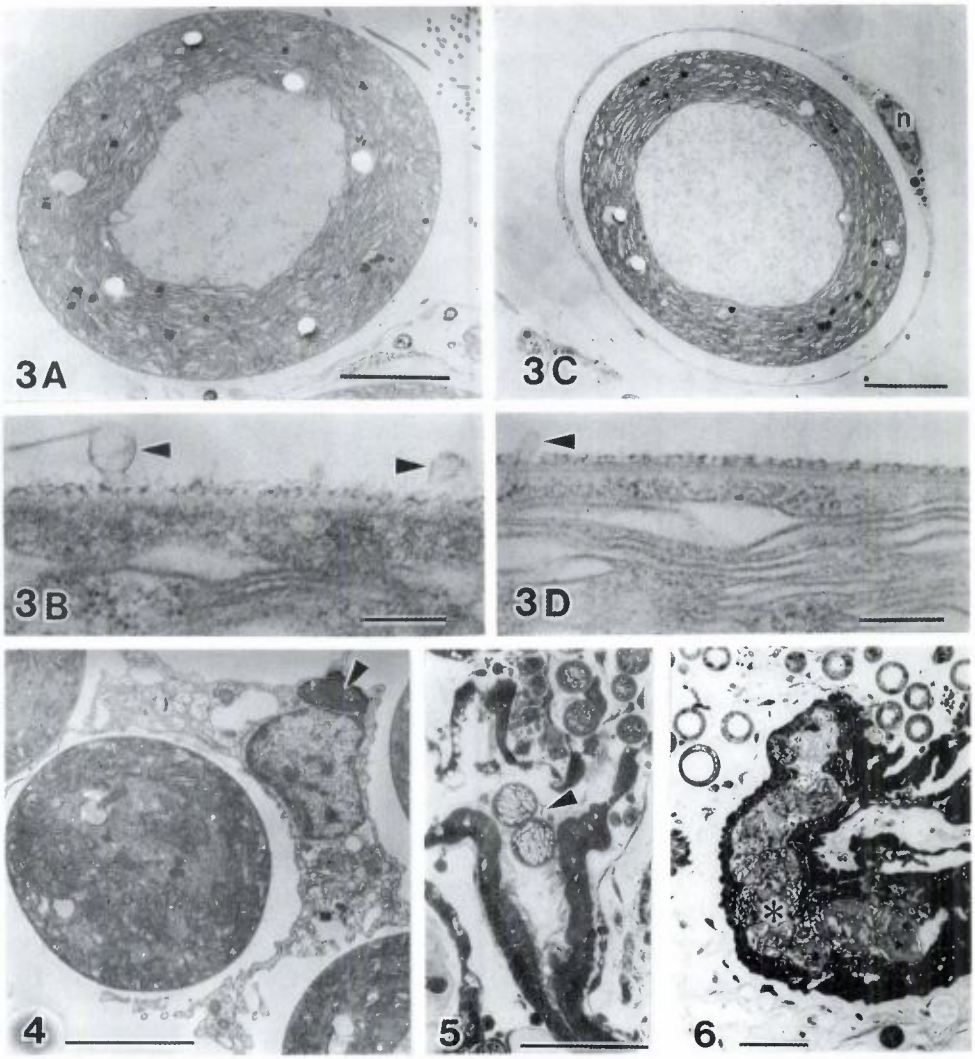

Figure 3. *Prochloron* cells in peribranchial cavity (A) and in a tunic phycocyte (C). B and D are enlargements of surface of the *Prochloron* cells in A and C, respectively. Arrowheads indicate cytoplasmic protrusions. n = nucleus of tunic phycocyte. Scale bars = 5 μm (A, C), 0.2 μm (B, D).

Figure 4. Nucleated cell containing phagosome (arrowhead) engulfing a *Prochloron* cell in the peribranchial cavity. Scale bar = 5 μm.

Figure 5. *Prochloron* cells in esophagus of host (arrowhead). Scale bar = 50 μm.

Figure 6. Transverse section of digestive tract of a zooid, showing lumen filled with ingested material (asterisk). Scale bar = 50 μm.

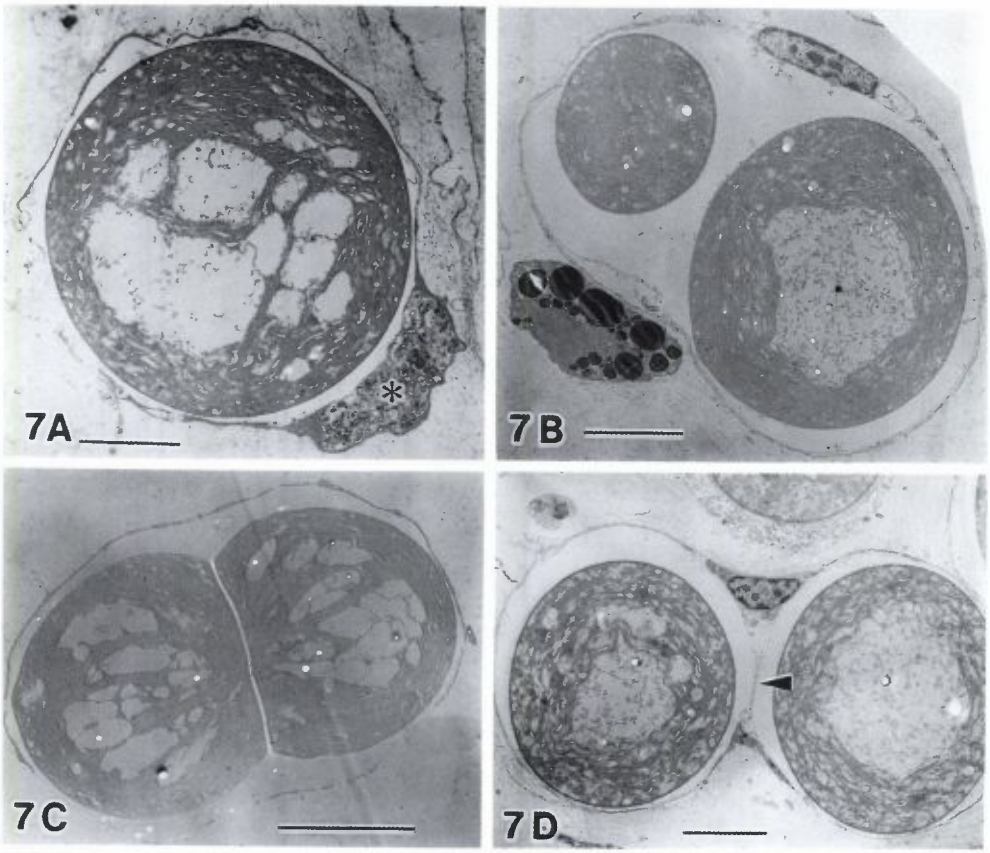

Figure 7. Tunic phycocytes containing one symbiont (A), two symbionts in a symbiosome (B), a dividing symbiont (C) and two symbionts in separate symbiosomes (D). Arrowhead = cytoplasmic wall bordering the two symbionts; asterisk = phagosome. Scale bar = 5 μm.

Experimental integration of PrC in phagocytes of L. patella

In another species, *L. patella*, all the PrC are extracellular, being confined in the peribranchial and cloacal cavities but not in the tunic. In cell suspensions obtained by squeezing a colony, observed immediately after expression, none of the PrC are contained in nucleated cells. However, after several minutes some hemocytes become ameboid, attach to PrC, and within an hour may ingest one, two or three of them (Fig. 8A and B). These hemocytes then appear similar to tunic phycocytes of *L. punctatum* (Fig. 8C), although the ingested PrC are often swollen (which may indicate incipient digestion).

Table 1. Area occupation in histological sections of *L. punctatum* colony

Tissue	Average (%)	SD
Tunic[1]	83.6	3.9
Zooids	7.7	1.4
Cavity[2]	8.7	2.5
Prochloron		
Total	4.6	0.6
In cavity	2.6	0.5
In tunic	2.1	0.3

[1]Area of colony minus areas of zooid and cavity, but including area occupied by PrC.
[2]Area of cavity including area occupied by PrC.

4. Discussion

In *L. punctatum*, cells of the photosynthetic prokaryote *Prochloron* sp. were found distributed densely in peribranchial/cloacal cavities, and more sparsely in the tunic where most are contained within phycocytes.

Prochloron spp. are regarded as obligate symbionts of host ascidians because none are known to be free-living, and because nutrient exchange between alga and host has been demonstrated (Pardy and Lewin, 1981; Griffiths and Thinh, 1983). In all species reported so far, the symbiosis is extracellular. However, in *L. punctatum*, there are also intracellular PrC, which are likewise true symbionts. The following evidence is presented. 1) There are no morphological or ultrastructural differences between intracellular and extracellular PrC. 2) The color and brightness of their autofluorescence when excited by blue light are likewise similar (Hirose et al., 1996). 3) Very few PrC inside symbiosomes show any sign of degeneration by digestion. However, nutrient exchange between PrC and host remains to be confirmed. Since some materials, presumably food, are found in their digestive tracts (Fig. 6), the zooids presumably do not obtain sufficient nutrients from their symbionts and need to supplement this by filter-feeding. Only a few PrC were found in the esophagus of zooids (Fig. 5), having perhaps accidentally entered the lumen through the branchial basket.

We estimate that 1 mm^3 of colony contains 0.045 mm^3 of PrC, and that if each cell is regarded as a sphere 0.02 mm in diameter (ca. 4.2×10^{-6} mm^3) there are about 10,000 PrC per 1 mm^3 of colony. Since about 45% of these PrC are embedded in the tunic, most contained in tunic phycocytes, nearly half of the photosynthesis in the ascidian colony is estimated to be carried out by such intracellular PrC.

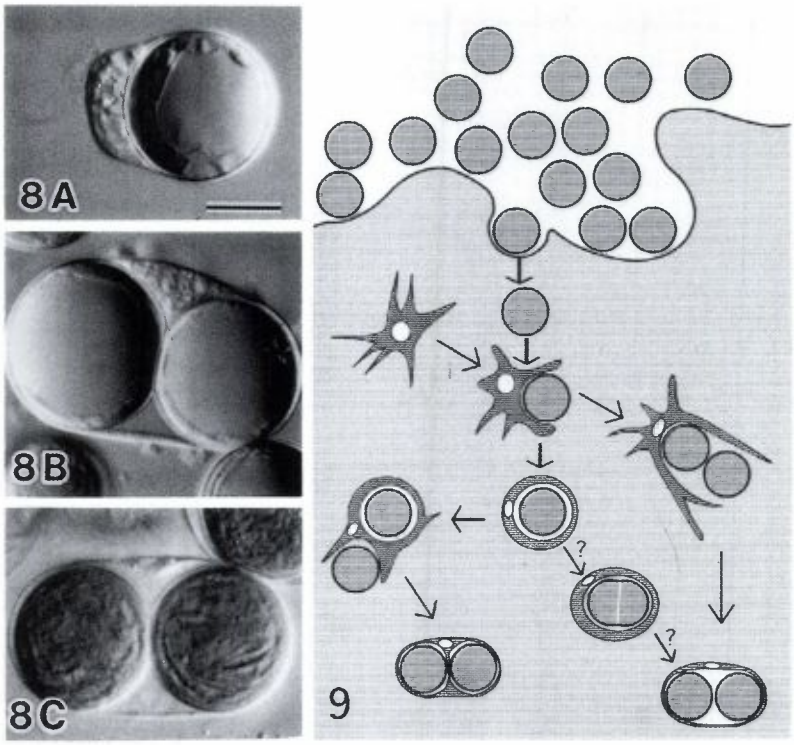

Figure 8. Phagocytic cell of *L. patella* that has ingested *Prochloron* cells (after incubation for one hour with symbionts cells *in vitro*) (A, B), and a tunic phycocyte of *L. punctatum* (C). Note the cell shapes of host cell are almost the same in B and C. All figures are of the same magnification. Scale bar = 10 µm.

Figure 9. Proposed sequence of intracellular symbiosis in the tunic of *L. punctatum* (see discussion in detail). Arrows with question marks indicate possible symbiont proliferation within a symbiosome.

As for the origin of tunic phycocytes, we have postulated that some of the PrC could enter the tunic, where they could be ingested by phagocytes and retained, still viable, in symbiosomes (Hirose et al., 1996). The present ultrastructural observations are consistent with this hypothesis. This is further supported by the observed experimental integration of PrC in phagocytes of *L. patella*, where ameboid cells become phycocyte-like by endocytosis of PrC. The ingested PrC are often swollen in the phagocytes of *L. patella*, possibly indicating digestion in phagosomes.

In peribranchial cavities, PrC are occasionally ingested by free nucleated cells that are probably phagocytic hemocytes released from mesenchymal

spaces (Fig. 4). A similar observation was reported in colonies of *L. voeltskowi*, which have only extracellular PrC in their cavities (Cox, 1983), although we doubt whether they could give rise to tunic phycocytes because their relative frequency is too low. Furthermore, phagocytes laden with PrC would probably be too large and immotile to migrate into the tunic through the peribranchial wall.

Each tunic phycocyte contains at least one PrC. How could it acquire two or three, as frequently observed (Fig 9)? It could ingest two symbionts at a time in a single symbiosome (Fig. 7B). Alternatively, since in some tunic phycocytes two PrC are each individually contained in a separate symbiosome, they were probably ingested independently (Fig. 7D). Some tunic phycocytes contain PrC in stages of cell division (Fig. 7C), suggesting either that the algae can divide within symbiosomes or that some phycocytes ingest PrC already in stages of division. Probably both occur.

This system demonstrates the occurrence of primary symbiosis directly established between photosynthetic prokaryotes and multicellular organisms, and suggests the possibility that intracellular symbiogenesis leading to photosynthetic organelles could still occur in various organisms even today. In the ascidian-*Prochloron* symbiosis system described here, if the intracellular PrC were to be incorporated in a germ line of the host, then one would have achieved photosynthetic organelles containing chlorophyll *a* and *b* like those in green plant chloroplasts.

Acknowledgments

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