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What are the benefits in the ascidian-*Prochloron* symbiosis?

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Abstracts

Prochloron, a prokaryotic alga, is an obligate symbiont of some tropical ascidian colonies belonging to the family Didemnidae. In this symbiosis, the host (ascidian) is thought to acquire photosynthetic metabolites provided from the symbionts (*Prochloron*), while the host provides a habitat for the symbionts. Radio-labeling studies suggested that *Prochloron* cells transfer photosynthetically fixed carbon to the host as soluble compounds of low molecular weight (Pardy and Lewin 1981; Griffith and Thinh 1983). In the most host species, *Prochloron* cells are extracellularly distributed in the common cloacal cavity of the host and the water current there runs directly toward the outside of the host colony. Therefore, it is unlikely that the metabolites are effectively translocated to the host by means of diffusion, especially since there are no structures that are adapted for nutrient translocation in the cloacal cavity. The intracellular symbiosis of *Prochloron* cells is known only in *Lissoclinum punctatum,* but the host cells are free cells distributed in the integument of colony (tunic) and they do not have direct connection with the host zooids. Therefore, the occurrence of the metabolite transfer is questionable since previous studies possibly underestimated the amount of the symbionts remaining in the host tissue when they determined the amount of labeled carbon supposedly incorporated into the host.

In *Diplosoma virens,* the parasitic copepods (notodelphyids) that inhabit in the cloacal cavity are surrounded by the numerous amounts of *Prochloron* cells. The copepods seem to feed on the tunic matrices but rarely ingest *Prochloron* cells. This may indicate that *Prochloron* cells contain toxic compounds to make themselves unsuitable as a food for the copepod. The host colony maybe protected from predation by harbouring the toxic symbionts,

and this would be a considerable benefit to the ascidians in the symbiotic system.

Introduction

It is generally accepted that chloroplasts are originated from photosynthetic endosymbionts endocytized and retained by heterotrophic host cells and that all chloroplasts share a common ancestor of cyanobacteria. In some algae (e.g., red algae and green algae) and higher plants, the chloroplasts are thought to be directly originated from photosynthetic prokaryotes (primary symbiosis), and, in the other algae (e.g., brown algae, diatoms, and dinoflagellates), the chloroplasts are originated from photosynthetic eukaryotes where chloroplasts should have originated from prokaryotes (secondary symbiosis). Therefore, symbiosis of prokaryotic algae is of interest in relation to the evolution of chloroplasts.

Prochloron, a prokaryotic alga, is an obligate symbiont of some tropical ascidian colonies belonging to the family Didemnidae (Fig. 1 and 2). This alga is originally described as a unicellular cyanophyte inhabiting the colony surface of *Didemnum candidum*, and is characterized by containing chlorophyll *a* and *b* and lacking phycobilins (Lewin, 1975). Because this pigment composition is so unique among photosynthetic prokaryotes, the division Prochlorophyta was proposed for this alga, *Prochloron didemni* (Lewin, 1977). Since the pigment composition of *Prochloron* is the same as that of the chloroplasts of green algae and higher plants (i.e., Chlorophyta), *Prochloron* may be closely related to the direct ancestor of the chloroplast in chlorophytes (Lewin 1976). *Prochloron* has been found in some other tropical didemnid ascidians, and the symbionts mostly inhabit inside the colony, i.e., common cloacal cavity of the colony. Subsequently, two genera of free-living algae were subsequently found as the member of Prochlorophyta: *Prochlorothrix* that is a freshwater filamentous alga and *Prochlorococcus* that is a tiny unicellular alga. The monophyly of the tree genera is, however, not supported by molecular phylogeny; they are unlikely the specific ancestor of the chloroplasts and each of them is a diverged member of the cyanobacteria (Palenik and Haselkorn, 1992; Urbach et al., 1992; Palenik and Swift, 1996). Therefore, the prochlorophytes are sometimes included in the division Cyanobacteria. In contrast, phylogenetic analyses of chlorophyll *b* synthesis genes suggest the genes of chlorophytes, *Prochloron*, *Prochlorothrix, and Prochlorococcus* share a common evolutionary origin (Tomitani et al., 1999).

To date, *Prochloron didemni* is the only described species of the genus *Prochloron*. *Pro-*

chloron cells are actually found in several didemnid species that are widely distributed in tropical waters, and thus *Prochloron* possibly consists of several species. There are, however, few differences in morphology among them. Moreover, there are no reliable reports on the *in vitro* culture of *Prochloron* cells, and thus there are no cultured strains even for the type species *P. didemni*. This is the reason why this symbiotic alga is referred as *Prochloron* or *Prochloron* sp. in several reports. Although there have been some attempts to discriminate several groups of *Prochloron* based on cell size (Kott, 1982) and cell structures (Cox, 1986), molecular phylogeny showed that *Prochloron* isolated from several host species and from geographically different sites are phylogenetically too close to be considered different species (Stam et al., 1985; Holton et al., 1990; Palenik and Swift, 1996). Diversity of *Prochloron* is one of the unsolved problems that are essential to discuss its evolution and ecology.

The biology of *Prochloron* has been studied from many aspects, e.g., physiology, biochemistry, ultrastructure and phylogeny, which have been covered in a comprehensive review (Lewin and Cheng, 1989). On the other hand, the host ascidians have been also studied for better understanding of this symbiosis system, but the actual condition of the symbiosis still remains to be disclosed. Here, we attempt to discuss the mutualism in the ascidian-*Prochloron* symbiosis.

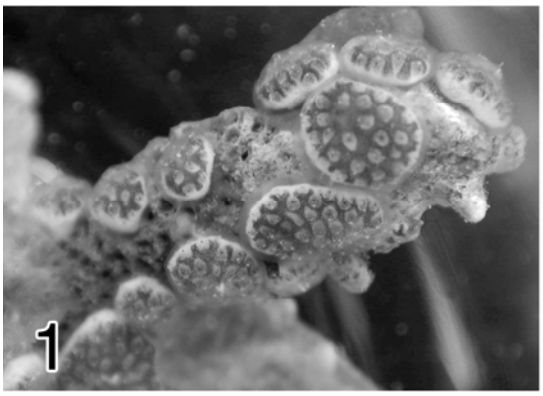

Figure 1: Colonies of *Prochloron*-bearing ascidian, *Tridemnum paracyclops*, attaching on a dead coral branch.

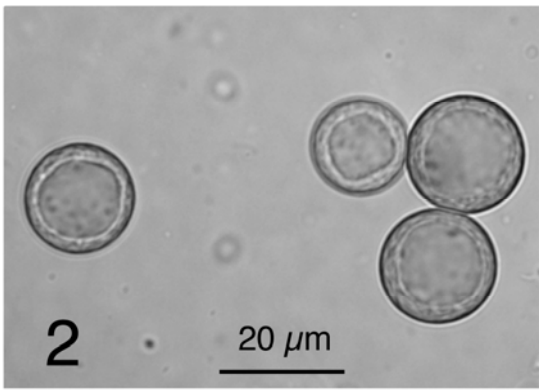

Figure 2: *Prochloron* isolated from *Diplosoma virens.*

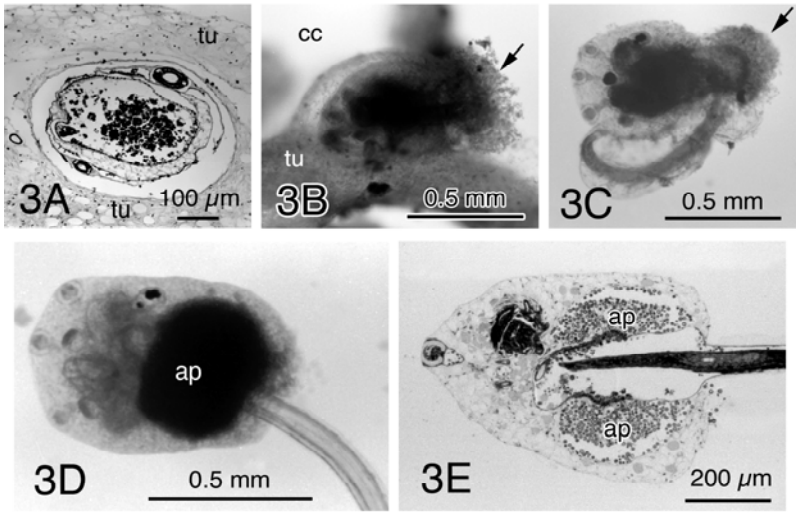

Figure 3: Vertical transmission of the *Prochloron* in *Diplosoma similis.* **A**, Embryo in the tunic does not associate with the symbionts. **B**, Immature larva protruding the plant rake in the lateral view of the cross-section of the colony. **C**, Immature larva dug out from the tunic. **D**, Spawned larva. **E**, Semi-frontal section of the spawned larva (resin section stained with toluidine blue). Arrows indicate plant rake; ap, algal pouch; cc, cloacal cavity; tu, tunic.

Mutualism

The Ascidian-*Prochloron* symbiosis is undoubtedly a mutual symbiosis. *Prochloron* is an obligate symbiont of some didemnid ascidians inhabiting tropical waters. In rare cases, *Prochloron* is found on other invertebrates as epizoic film (reviewed in Lewin and Cheng, 1989). Free-living *Prochloron* has never been found so far. On the other hand, the *Prochloron*-bearing ascidian species always contain the symbionts, except for the host species in which the symbionts inhabit on the colony surface but not in the cloacal cavity (e.g., *Didemnum candidum*). This means that *Prochloron* cannot survive without host ascidians and *Prochloron* provides some benefits to the host to survive. Exceptionally, it is uncertain whether the epizoic *Prochloron* has specific association with the host (Kott, 1977). The host ascidian positively maintains the symbiosis. Vertical transmission of the symbionts usually occurs; the host larvae inherit *Prochloron* cells from their mother colony. The colony has the symbionts at the beginning of the life history, though the larvae need to have a tissue to catch and carry *Prochloron* cells. The occurrence of the vertical transmission suggests that the symbionts are important to the host for survival. Diversified adaptations for the vertical transmission are found in the host larvae (see Kott, 1982; 2001). Since the adaptations are different in each genus, there would be multiple origins of the symbiosis in

the phylogeny of didemnid ascidians (Kott, 1980; 1982). For instance, the larva of *Diplosoma* species possesses specialized organs to collect *Prochloron* cell (plant-rake or rastrum) and to carry them (algal pouch) (Eldredg, 1967; Kott, 1981; Hirose, 2000a): the embryo brooded in the tunic of the mother colony is free of *Prochloron* cells, a tassel-like structure (plant-rake) protrudes from the postero-dorsal part of the trunk of the immature larva and it is extended into the cloacal cavity to collect the *Prochloron* cells distributed there, subsequently the posterior-half of the trunk extends posterior forming a large pocket (algal pouch) that packs the plant-rake, the spawned larva carries *Prochloron* cell in the algal pouch, and then the algal pouch becomes the cloacal cavity in the metamorphosed larva or young colony (Fig. 3). As shown above, the cost of vertical transmission of the symbionts would not be so small for the host, and thus, the benefits from the symbionts should be enough large to cancel the cost.

Benefit for *Prochloron*

The cloacal cavity of the didemnid colony, habitat for *Prochloron*, is a highly protected space against predators. In didemnid ascidians, the cloacal cavity is usually sandwiched between the upper and lower layers of tunic (Fig. 4), and the tunic contains acidic fluid (Cf. Hirose, 2001) and/or calcareous spicules (Cf. Kott, 2001). Other organisms are rarely found in the cavity, except for notodelphyid copepods (see below), indicating that *Prochloron* appears to be protected from competitors and predators.

Ultraviolet radiation (UVR) is a harmful environmental factor for survival particularly in tropics, because of the severe irradiation and clear water there. In contrast, appropriate amount of photosynthetically active radiation (PAR) is essential for photosynthetic organisms. Photosynthesis of isolated *Prochloron* cells is affected by light-intensity, pH, temperature and UV irradiation (Dionisio-Sese et al., 2001). In a *Prochloron*-bearing didemnid *Lissoclinum patella*, the tunic contains UV-absorbing substance identified as mycosporine-like amino acids (MAAs), and the *Prochloron* cells isolated from the host are much more susceptible to UVR than those in the host (Dionisio-Sese et al., 1997; 2001). *Prochloron* cells are susceptible to UVR, and they are protected in the host colony by a tunic wall transparent to PAR but not UVR. Our preliminary survey showed that the tunic of some other *Prochloron*-bearing species also contains UV-absorbing substances, whereas the tunic is almost transparent to UVR in some non-*Prochloron*-bearing ascidians that inhabit in

similar environment (Hirose and Ohtsuka, unpublished) (Fig. 5). The ascidian-*Prochloron* symbiosis might relate to the presence of UV-absorbing substances in the tunic.

In the coral-zooxanthellae endosymbiosis, the host coral provides nitrogen sources to the endosymbiotic microalgae that are not capable of N_2 fixation. In ascidian-*Prochloron* symbiosis, it is uncertain whether the host provides nitrogen sources to the symbionts. In contrast, natural N^{15}/N^{14} abundance suggested that *Prochloron* seems to be a nitrogen fixer and possibly provides a source of fixed nitrogen to the host (Kline and Lewin, 1999).

Benefit for ascidians

Translocation of the metabolites In ascidian-*Prochloron* symbiosis, the benefit of the host ascidians has been thought to be photosynthetic metabolites provided from the symbionts. Fisher and Trench (1980) showed that *Prochloron* cells release maximum of 7% of the fixed carbon mainly as glycolate *in vitro*. This indicates the symbionts have the potential to provide nutrient material to the host by secretion of soluble metabolite. There are some reports of radio-labeling studies showing that *Prochloron* cells transfer photosynthetically fixed carbon to the host as soluble compounds of low molecular weight (Pardy and Lewin, 1981; Griffiths and Thinh, 1983). In these studies, *Prochloron* cells were isolated from the host after the incubation with $^{14}CO_2$ for appropriate period in light or dark control, and then, the radioactivities of the isolated symbionts and residual host colony were measured to estimate the amount of fixed carbon by photosynthesis and the amount of the transferred carbon to the host. Because it is almost impossible to separate *Prochloron* cells completely from the ascidian colony, many *Prochloron* cells still remain in the ascidian tissue after harvesting the *Prochloron* cells. In order to subtract the *Prochloron* contaminants in the colony, they determined the amount of chlorophyll (Pardy and Lewin) or phaeophytin (Griffiths and Thinh, 1983) in the residual colony by means of spectrophotometry. The ascidian host usually contains strong acid in the tunic (Cf. Hirose, 2001). In intact colonies, the acid is stored in vacuoles of the tunic cells, but it is leaked during the isolation of the symbionts from the host colony. The acidic environment would denature the chlorophyll pigments and other molecules and may cause the miscalculation of the amount of *Prochloron* cells and metabolites. This indicates that the amount of *Prochloron* contaminants might be underestimated in the radio-labeling studies described above.

In most host species, *Prochloron* cells are extracellularly distributed in the common cloacal

cavity of the host (Fig. 6). The ascidians are usually filter-feeders and they produce a continuous water current with the cilia of their branchial basket for filtration of food particles in seawater. The water current in the cloacal cavity runs directly toward the outside of the host colony (Fig. 7). It is unlikely that the photosynthetic metabolites are efficiently translocated to the host by means of diffusion. There are no structures that are adapted for nutrient uptake in the cloacal cavity. Although metabolite transfer is doubtful in the cloacal cavity, its possible existence cannot be ruled out. Furthermore, the histological study of the host colony demonstrated that the alimentary tract of the ascidian rarely contains *Prochloron* cells, indicating that *Prochloron* cells would not be usual diet of the host ascidian (Hirose et al., 1998). Accordingly, it is possible that the host ascidian hardly receives nutritive benefits, and definitive evidence for nutrient exchange in this symbiosis system remains to be established.

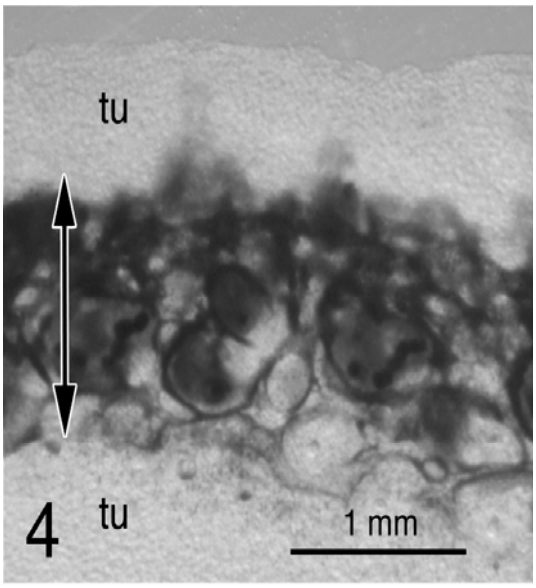

Figure 4: A cross section of the colony of *Diplosoma virens*. Cloacal cavity (double-pointed arrow) containing *Prochloron* is sandwiched in the two layers of tunic (tu).

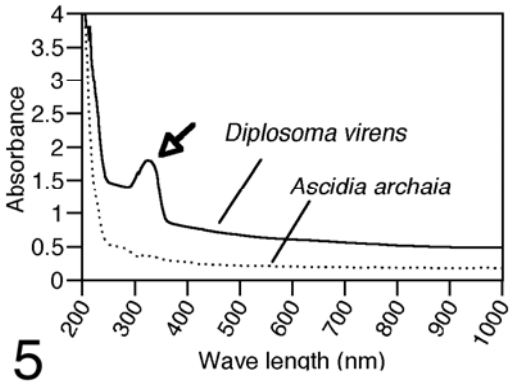

Figure 5: UV-visible light absorption spectra of a hand-sectioned tunic of *Diplosoma virens* (*Prochloron*-bearing species) and *Ascidia archaia* (non-bearing species). Arrow indicates the UV-absorption in *D. virens*.

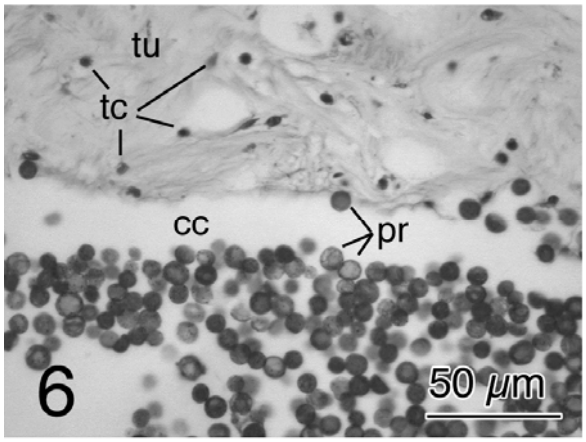

Figure 6: *Prochloron* cells (pr) in the cloacal cavity (cc) in *Diplosoma similis*. The symbionts never enter the tunic (tu).

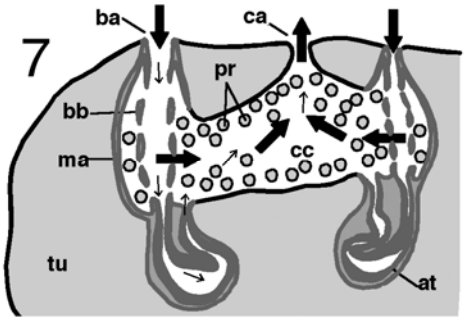

Figure 7: Schematic drawing of the cross section of the colony. Thick arrows indicate the direction of water current, and thin arrows indicate the route of food particles/feces. at, alimentary tract; ba, branchial aperture; bb, branchial bascket; ca, cloacal aperture; cc, cloacal cavity; ma, mantle; pr, *Prochloron* cells; tu, tunic.

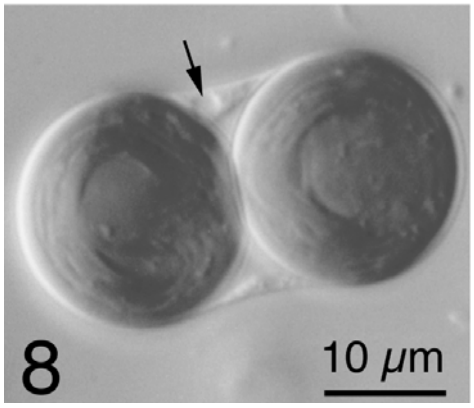

Figure 8: Tunic phycocyte containing two *Prochloron* cells in *Lissoclinum punctatum*. Arrow indicates the nucleus of the host cell.

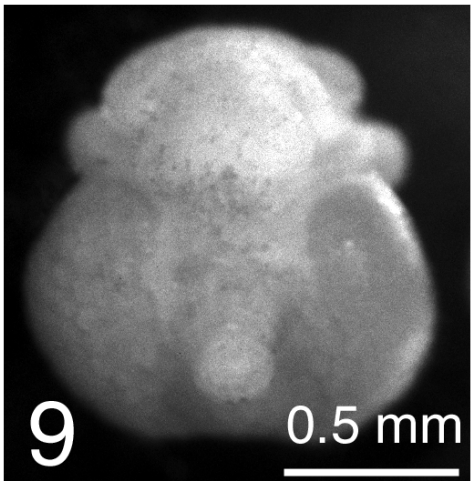

Figure 9: A ventral view of the notodelphyid inhabiting in the cloacal cavity of *Diplosoma virens*.

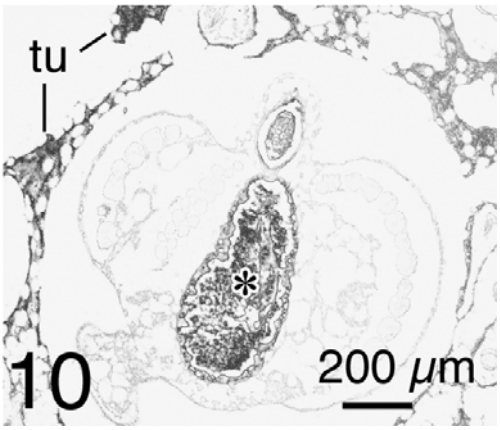

Figure 10: Histological section of the notodelphyid in the cloacal cavity. Alcian blue exclusively stains the tunic (tu) and the gut contents of the parasite (asterisk).

Occurrence of the intracellular symbiosis The intracellular distribution of *Prochloron* cells was first reported in *Lissoclinum voeltskowi*; amoebocytes occasionally found in the cloacal cavity ingest the symbiont cells (Cox, 1983). Similar observations were reported in the cloacal cavity in *Lissoclinum punctatum* and the presumptive cloacal cavity of the larvae of *Diplosoma similis* (Hirose et al., 1998; Hirose, 2000a). They are unlikely an occurrence of endosymbiosis, because the process may involve subsequent digestion of the engulfed *Prochloron* cells. The amoebocytes seem to spill from the ascidian tissue accidentally, and the amoebocytes laden with *Prochloron* cells would probably be too large and immotile to migrate back to the ascidian tissue. Moreover, their relative frequency is too low, and thus the phagocytosis of *Prochloron* cells in the cloacal cavity would provide few benefits to the host ascidians.

The endosymbiosis of *Prochloron* is demonstrated in the tunic of *Lissoclinum punctatum*, where *Prochloron* cells 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 cells (tunic cells) are distributed in the tunic, *Prochloron* cells are almost exclusively contained in one particular cell type, tunic phycocytes (Fig. 8). Since the phycocytes exhibit phagocytic activity, they have probably endocytized the *Prochloron* cells that entered the host tunic. There are no morphological differences between intracellular and extracellular *Prochloron* cells, and the *Prochloron* cells show no evidence for rejection or degeneration in the phycocytes (Hirose et al., 1996; 1998). Hence, the intracellular *Prochloron* cells would be active in photosynthesis as well as extracellular ones, and this association seems to constitute a stable endosymbiosis. On the contrary, Kott (2001) claimed that the intracellular distribution of *Prochloron* cells is artificial; phagocytosis is a response to *Prochloron* cells invading the tunic, when handling disturbs the delicate host colony. This may be irrelevant remark, because our histological survey showed that more than 45% of *Prochloron* cells are distributed in the tunic in *L. punctatum* and almost all *Prochloron* cells in the tunic are intracellularly distributed in the tunic phycocyte (Hirose et al., 1998). Even if some *Prochloron* cells are artificially contaminated into the tunic during the specimen collection, it is unlikely that most of them are phagocytized by tunic phagocytes before fixation of the colonies. The tunic phycocyte are free cells distributed in the tunic and do not have direct connection with the host zooids. Therefore, they may be provided me-

tabolites from their endosymbionts but cannot transport the nutrients to the zooids.

Possible function of the* Prochloron *toxins In comparative survey on epibiotic bacteria on ascidians, abundance and diversity of culturable bacteria are significantly lower in the didemnid species harboring photosymbionts (presumed to be *Prochloron* sp. and/or smaller cyanobacteria) than the species without photosynthetic symbionts (Wahl, 1995). These symbionts, including *Prochloron* sp., may control the fouling and colonization with some compounds, and this would be beneficial to the host ascidians that suffer uncontrolled fouling.

Many commensal or parasitic small crustaceans are known to inhabit the cloacal cavity, banchial basket, and alimentary tract in both solitary and colonial ascidians (Cf. Monniot, 1990). In *Diplosoma virens*, notodelphyid copepods are often found in the cloacal cavity where *Prochloron* are always distributed (Fig. 9). Although the parasitic notodelphyids are surrounded by numerous *Prochloron* cells, unexpectedly, the *Prochloron*-like structures are rarely found in the alimentary tract of the notodelphyids, suggesting that *Prochloron* cells are unlikely a diet of these parasites (Hirose, 2000b). Histochemistry with alcian blue exclusively stains the gut contents of the parasite and the tunic of the host colony, suggesting that the parasites feed on the tunic matrices of the ascidians (Fig. 10). Why the parasites do not eat *Prochloron*? This may indicate that *Prochloron* cells contain toxic compounds to be unsuitable as a food. Many antibiotic, anti-viral, or cytotoxic compounds have been isolated from *Prochloron*-bearing didemnids, and *Prochloron* cells seem to be involved in the synthesis of some compounds (Biard et al., 1990). It is possible that the host colony can avoid predation by bearing the toxic symbionts, and this would be a considerable benefit of ascidians in this symbiotic system.

Perspectives

There is no doubt that ascidian-*Prochloron* association is a mutual symbiosis, but the exact nature of their relationship has been poorly demonstrated. Although we do not deny the nutrient transfer between the host and symbionts, our histological investigation could not provide evidence of its existence.

There should be several research directions taken to disclose this symbiosis system. *In vitro* culture of *Prochloron* is one of the important problems to be solved for better understanding of physiology of this organism. If the key factor(s) for *in vitro* culture is found, it will

indicate what makes *Prochloron* be an obligate symbiont. Since *Prochloron* cells are probably involved in the production of bioactive metabolites that are isolated from some didemnid ascidians, large-scale culture may be essential for industrial applications. Extensive survey of genomic information in *Prochloron* will answer some unsolved questions, such as, capacity of nitrogen fixation, production of bioactive metabolites, evolutionary history of their unique pigment composition, and geographic and/or host-specific diversity. The last question is closely related with the evolution of this symbiosis, and the possibility of the occurrence of co-evolution with the host needs to be examined by means of molecular phylogeny. As described above, the vertical transmission of *Prochloron* suggests the occurrence of co-evolution, and the diversity of the larval adaptation for the vertical transmission indicates the multiple origin of the symbiosis (Kott, 1980; 1982). However, geographic and/or host specific speciation of *Prochloron* is not supported by molecular phylogeny (Stam et al., 1985; Holton et al., 1990; Palenik and Swift, 1996). While the host ascidians all belong to the family Didemnidae, there are many non-symbiotic didemnid species; each didemnid genus often includes both *Prochloron*-bearing species and non-bearing species. Although the monophyly of this family is generally accepted, the molecular phylogeny in this family has not yet been investigated. A combined survey on both the host and the symbionts should be necessary for better understanding of the phylogenetic relationship between them.

In the last place, we wish to mention the uniqueness of the endosymbiosis in *Lissoclinum punctatum.* This is the only case of the photosynthetic endosymbiosis histologically confirmed in ascidians to our best knowledge. In metazoans, although there are many examples of photosynthetic endosymbiosis (e.g., coral), most of them are secondary endosymbiosis (symbiosis with eukaryotic algae). It is unknown why the occurrence of photosynthetic primary endosymbiosis is rare in metazoans. One possibility is to assume that the epithelium of metazoans is a formidable barrier for cyanophytes and prochlorolophytes to inhabit and make symbiotic interaction. Usually, the epithelium totally covers the surface of metazoan body, but ascidians always have tunic outside the epidermal epithelium. Tunic is a kind of connective tissue in which several types of free cells (tunic cells) are distributed. In the endosymbiosis in the ascidian, the *Prochloron* cells are intracellularly distributed in these free cells, i.e., tunic phycocyte. The endosymbiosis of *Prochloron* would be a unique material to study the mechanism of photosynthetic primary symbiosis.

Acknowledgements

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