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Fine Structures of Tunic Cells and Distribution of Bacteria in the Tunic of the Luminescent Ascidian *Clavelina miniata* (Ascidiacea, Urochordata)

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ABSTRACT—In a colonial ascidian *Clavelina miniata*, physical stimulations induce strong luminescence in the tunic. We described here the tunic cell morphology and bacterial distribution in the tunic that is a luminous tissue of this species. Three types of tunic cells are morphologically discriminated as morula-like tunic cells, tunic phagocytes, and tunic granulocytes, and they correspond, respectively, to the Type I, Type II, and Type III cells described by Aoki *et al.* (1989). Morula-like tunic cells are similar in morphology to morula cells that are hemocytes commonly found in ascidians. Tunic phagocytes contain round granules, clear vacuoles, and occasionally phagosomes. Tunic granulocytes characterized by a number of elliptical granules and they occasionally contain phagosomes and round granules that are similar in structure to tunic phagocytes. According to the description by Aoki *et al.* (1989), tunic phagocytes are supposed to be luminous cells. Elongated bacteria of unique forms are found in tunic phagocytes. However, these bacteria are probably not luminous ones, since they also are distributed in tunic granulocytes and outside of the tunic cells. Because other bacteria-like inclusions are not present in tunic phagocytes, we found no structural evidence to support the bacterial origin of bioluminescence in *C. miniata*. The clear vacuoles of tunic phagocytes may be a possible candidate for the subcellular site producing bioluminescence.

INTRODUCTION

Various marine animals exhibit bioluminescence (Nicol, 1958, 1960). In tunicates (urochordates), bioluminescence has been well documented in some pelagic tunicates—pyrosomids (Pyrosomata, Thaliacea) and oikopleurids (Appendiculata) (cf. Harvey, 1952). Pyrosomids have light organs that consist of a group of cells containing bacteria-like bodies (bacteroids), and these inclusions are thought to be luminous symbionts. Furthermore, Leisman *et al.* (1980) showed that the light organ extract from *Pyrosoma* has bacterial luciferase activity and the decay kinetics indicate that the luciferase is similar to that of the luminous bacterium *Photobacterium*. On the other hand, oikopleurids do not have bacterial luciferase (Hastings, 1983), and the source of their bioluminescence is thought to be membrane-rich cell fragments produced by the oral gland cells (Fredriksson and Olsson, 1981) or fluorescent granular inclusions (Galt and Sykes, 1983) in the house (an external filtering apparatus secreted by the animal).

In sessile tunicates (ascidians), there are a few old reports on bioluminescence (Landsborough, 1842; Will, 1844), but they show rare reproducibility (see Harvey, 1952). Except for these doubtful descriptions, *Clavelina miniata* Watanabe et Tokioka is the only known ascidian that definitely shows bioluminescence. Although this colonial ascidian does not have a particular light organ, physical stimulations induce strong luminescence in the tunic (an integumentary, extracellular layer that typically covers the epidermis) (Aoki *et al.*, 1989). The authors reported that free mesenchymal cells distributed in the tunic (tunic cells) are the source of bioluminescence in *C. miniata*. There are three types of tunic cells (Type I, Type II, and Type III), and the Type II cells seem to luminesce when stimulated appropriately. In *C. miniata*, it is not known whether the luminous tunic cells contain luminous bacteria/bacteroids or whether these tunic cells luminesce intrinsically. The present study is intended to provide information about the structural basis of the tunic, a luminous tissue in *C. miniata*. Here, we describe three types of tunic cells and their fine structures, especially the cellular inclusions and the intra- and extracellular bacteria.

MATERIALS AND METHODS

Animals

Colonies of *Clavelina miniata* were collected from the Noroshi Point of Nabeta Bay (Shimoda, Shizuoka Prefecture, Japan) by skin diving. The colonies were attached to glass slides with cotton thread, and they were reared in a culture box immersed in Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba.

Microscopy for live specimens

Tunic specimens were cut from the colony with a razor blade;

they were gelatinous, transparent sheets. The specimens were mounted in seawater without any fixation and observed under a light microscope equipped with Nomarski differential interference contrast or phase contrast optics.

Microscopy for fixed specimens

The specimens were fixed in 2.5% glutaraldehyde solution containing 0.45 M sucrose, buffered with 0.1 M cacodylate at pH 7.4, on ice for 2 hr. They were rinsed in the same buffer and then postfixed with 1% osmium tetroxide in the same buffer for 1 to 2 hr. After dehydration through an ethanol series, these fixed specimens were cleared with *n*-butyl glycidyl ether and embedded in epoxy resins. Thick sections were stained with 1% toluidine blue for light microscopy. Thin sections were double-stained with uranyl acetate and lead citrate, and they were examined in a Hitachi HS-9 transmission electron microscope at 75 kV.

RESULTS

Three types of tunic cells are recognized in unfixed tunic specimens (Fig. 1). Based on their morphology, they are called as morula-like tunic cells, tunic phagocytes, and tunic granulocytes, and they correspond, respectively, to the Type I, Type II, and Type III cells described by Aoki *et al.* (1989). Morula-like tunic cells (Fig. 1A) have a roundish cell shape with few pseudopodia and most of their cytoplasm is occupied by vacuoles. Tunic phagocytes (Fig. 1B) are flattened cells with long, protruding filopodia; they also have several vacuoles and occasionally phagosomes. Tunic granulocytes (Fig. 1C) are irregularly shaped cells with some filopodia, and their cytoplasm is filled with elliptical granules. All three types of tunic cells are distributed in the transparent, gelatinous matrix of the tunic that is an integumentary tissue covering the epidermis. The three types are also distinguishable in histological sections by the morphological characteristics of each cell type (Fig. 1D). Elongated bacteria are usually found in the tunic (arrowheads in Fig. 1C) and they are motile in the tunic matrix. On the inner side of the epidermis, there is a mesenchymal space where various kinds of hemocytes are distributed.

In electron microscopic observation, three types of cells are found in the tunic and they correspond to the three types of tunic cells defined in light microscopic observation. In morula-like tunic cells, roundish vacuoles are separated from each other by a partition of thin cytoplasm, and these vacuoles are filled with moderately electron-dense materials (Fig. 2). These cells do not have clear vacuoles or granular inclusions. They are similar in morphology to morula-like tunic cells in *Aplidium yamazii* (Hirose *et al.*, 1994b) and morula cells or vacuolated cells that are hemocytes commonly found in ascidians.

Tunic phagocytes have several clear vacuoles and round granules of variable sizes (Fig. 3). They often contain phagosomes, indicating the presence of phagocytic activity. The elongated bacteria that are distributed in the tunic have a unique cell shape with regular notches (Fig. 3A, arrowheads). This type of bacterium is commonly found in the tunic of *C. miniata*, whereas other types are rarely found there. Sometimes, tunic phagocytes contain these elongated bacteria (Fig. 3B). We occasionally found a cell that looks like a tunic phagocyte in an epithelial cell (Fig. 3C), which indicates that the cell is migrating from the mesenchymal space to the tunic by passing through the epidermis.

Tunic granulocytes have two types of granular inclusions (Fig. 4A). They are elliptical granules of about 1 μm in the long axis and round ones of various sizes; and each granule (both types) is partitioned from the cytosol by a unit membrane (Fig. 4B). The elliptical granules are heavily electron-dense and have unique substructures in which irregularly shaped granular components are arranged to form an ellipsoid. The round granules are similar in structure to tunic phagocytes. They consist of homogeneous materials and the electron density of the granules is variable. Tunic granulocytes sometimes contain phagosomes and the elongated bacteria (Fig. 4C). In the mesenchymal space, there are some

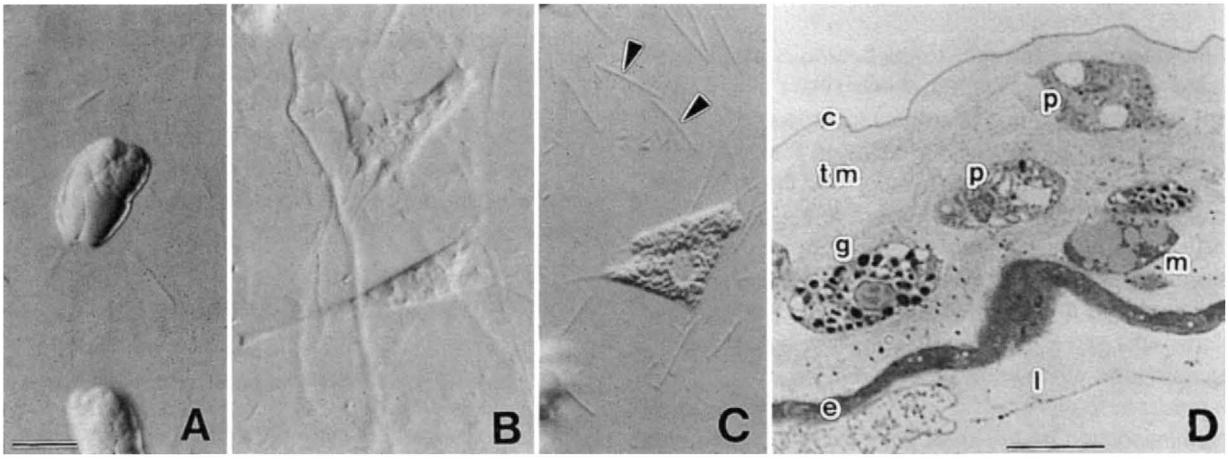

Fig. 1. Three types of tunic cells in light microscopy: live specimens under Nomarski optics (A-C) and histological section stained with toluidine blue (D). The tunic cell types are morula-like tunic cells (A; m in D), tunic phagocytes (B; p in D) and tunic granulocytes (C; g in D). Arrowheads indicate elongated bacteria in the tunic. c, tunic cuticle; e, epidermis; l, lumen of mesenchymal space; tm, tunic matrix. Photographs A-C are the same magnification. Scale bars = 10 μm.

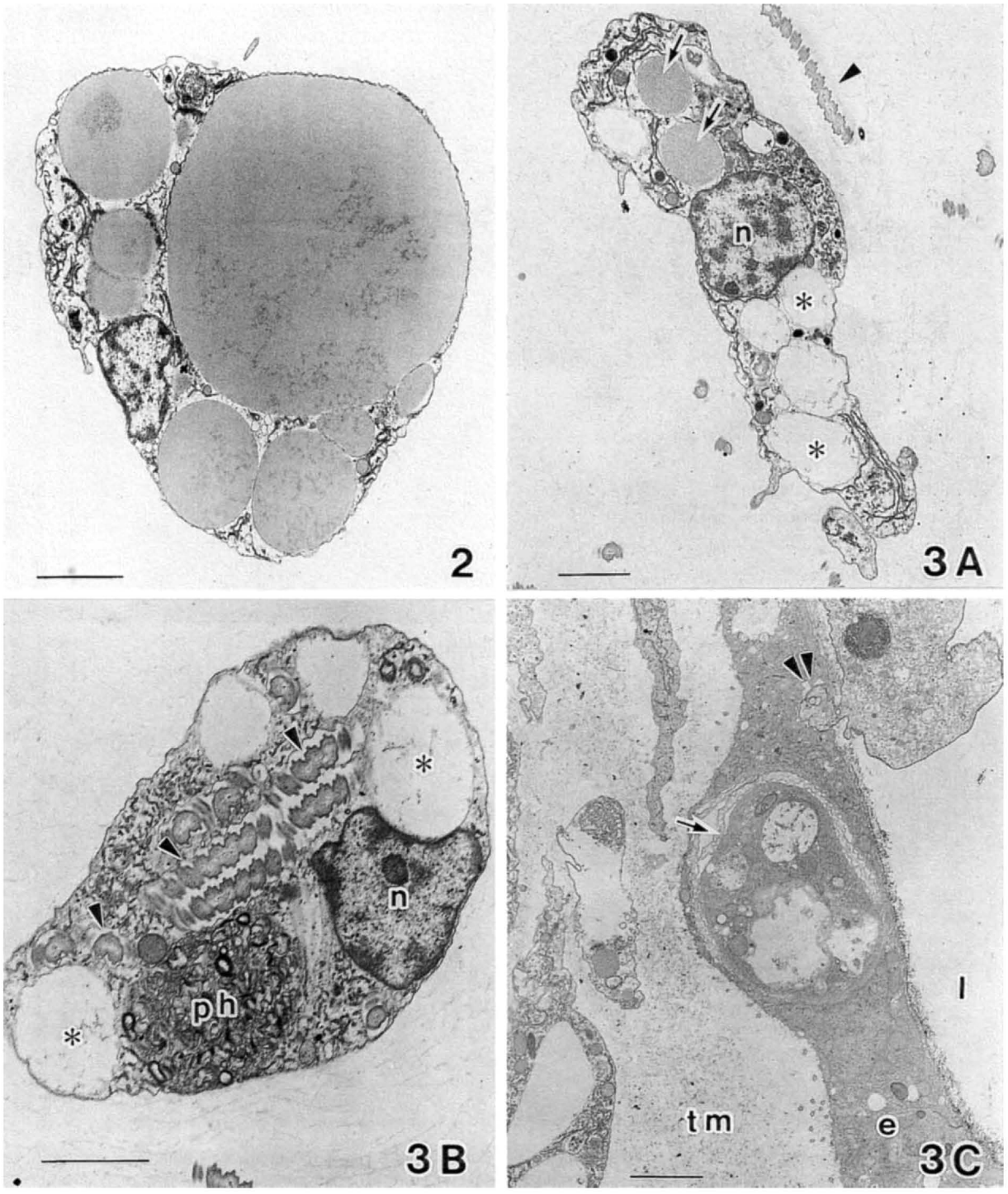

Fig. 2. Transmission electron micrographs of a morula-like tunic cell. Scale bar = 2 μm.

Fig. 3. Transmission electron micrographs of tunic phagocytes. Asterisks indicate some clear vacuoles. e, epidermis; l, lumen of mesenchymal space; n, nucleus; ph, phagosome; tm, tunic matrix. Scale bars = 2 μm. (A) A tunic phagocyte containing clear vacuoles and round granules (arrows). Arrowhead indicates elongated bacterium in the surrounding tunic. (B) A tunic phagocytes containing bacteria (arrowheads). (C) A tunic phagocyte-like cell in an epidermal cell (arrow). Another hemocyte protrudes a pseudopod into the space between epidermal cells (double arrowheads).

hemocytes with morphology similar to that of tunic granulocytes and often they are attached to the epidermis (Fig. 4D).

DISCUSSION

Certain tunic cells are thought to be the light source in the luminescent ascidian, *Clavelina miniata* (Aoki *et al.*, 1989).

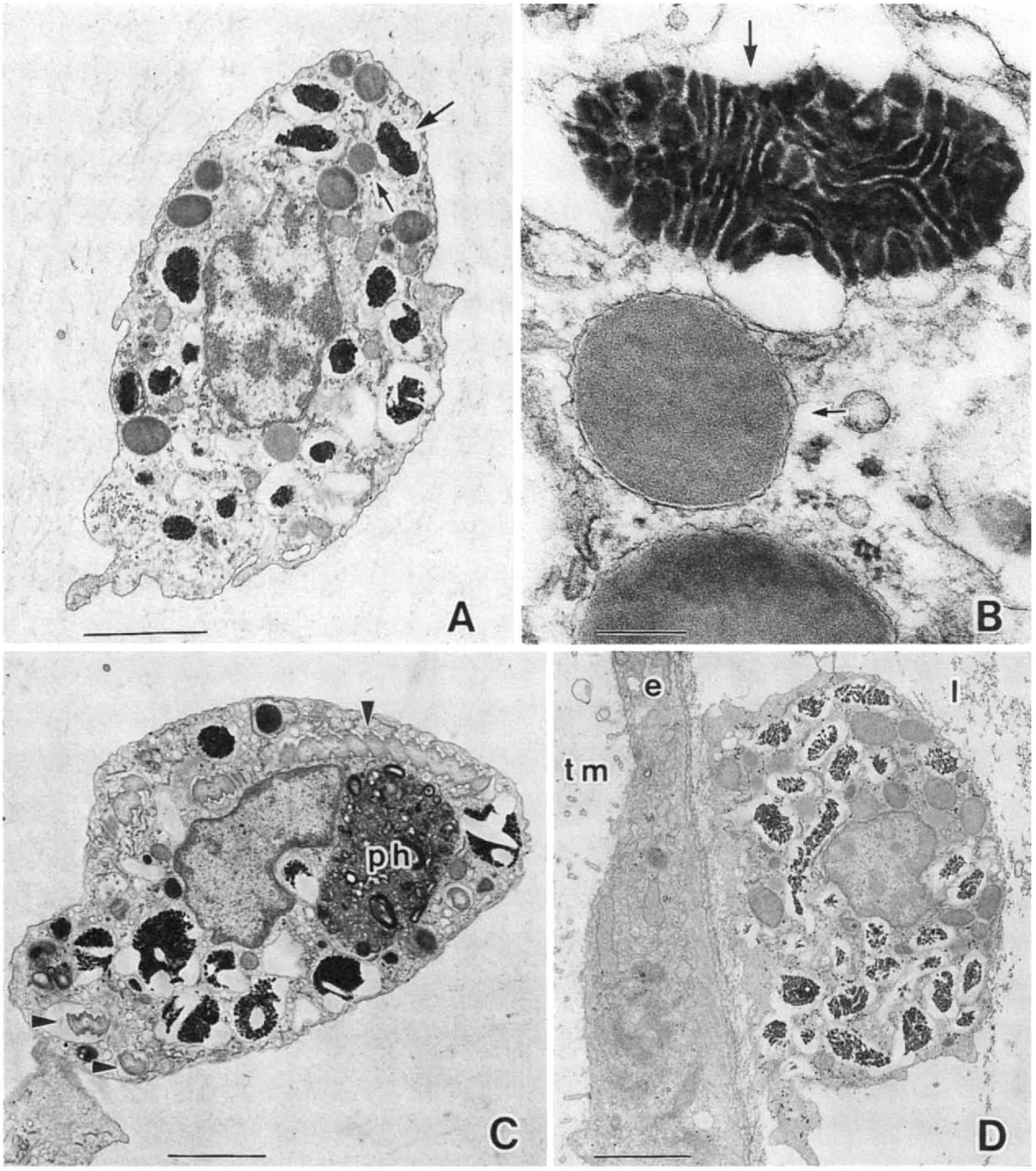

Fig. 4. Transmission electron micrographs of tunic granulocytes. (A) Tunic granulocyte containing heavily electron-dense granules (large arrow) and moderately electron-dense granules (small arrow); scale bar = 2 μm. (B) Enlargement of (A); arrows respectively indicate the same granules that are indicated by arrows in (A); scale bar = 0.2 μm. (C) Tunic granulocyte with phagosome (ph) and bacteria (arrowheads); scale bar = 2 μm. (D) A hemocyte that is similar to a tunic granulocyte; e, epidermis; l, lumen of mesenchymal space; tm, tunic matrix; scale bar = 2 μm.

In this report, three types of tunic cells were classified based on their morphological characteristics. They are morula-like tunic cells, tunic phagocytes, and tunic granulocytes, each of which corresponds, respectively, to Type I, Type II, and Type III cells previously described in this species (Aoki *et al.*, 1989). Of these three types of tunic cells, Aoki *et al.* (1989) proposed that Type II cells (tunic phagocytes) are the only luminous cells in the tunic.

Tunic phagocytes contain round granules of moderate electron density, clear vacuoles, and occasionally phagosomes. If tunic phagocytes are luminous cells, it is possible that the round granules and/or clear vacuoles are the source of luminescence (lumisomes). The round granules are not unique to tunic phagocytes, and similar inclusions are found in tunic granulocytes of *C. miniata* and in tunic phagocytes of *Aplidium yamazii*, a nonluminescent colonial

ascidian (Hirose *et al.*, 1994b). In tunic phagocytes of *A. yamazii*, the round granules are presumed to be derived from the contents of the phagosomes (Hirose *et al.*, 1994a). Because the presence of phagosomes indicates that tunic phagocytes and tunic granulocytes show phagocytic activity in the tunic of *C. miniata*, the round granules in them possibly correspond to those in tunic phagocytes of *A. yamazii*. The clear vacuoles are found in various types of tunic cells in many ascidian species, but the vacuoles in tunic phagocytes are relatively larger in size than those in other amoeboid forms of tunic cells. The clear vacuoles may be a possible candidate for the lumisomes, although there is no prominent evidence to support this view at present.

Figures 3C and 4D indicate that tunic phagocytes and tunic granulocytes originate from some hemocytes. The hemocytes, however, are not luminescent, and this may indicate that some hemocytes acquire luminescence ability after migration into the tunic. If not so, there may be small amount of luminous hemocytes (precursors of luminous tunic cell) in the mesenchymal space, but they are distributed too sparsely to detect their bioluminescence.

Elongated forms of bacteria are found in the tunic, and some are also contained in some tunic cells; however, they are not an independent light source for the following reasons: (1) These bacteria are found not only in tunic phagocytes (candidates for luminous cells) but also in tunic granulocytes. (2) Many of the bacteria are distributed outside of the tunic cells, but the light is probably emitted only from Type II cells (tunic phagocytes) (Aoki *et al.*, 1989). (3) In some nonluminescent ascidian, elongated bacteria are distributed in the tunic and occasionally in the tunic cells, and they are morphologically identical to those in *C. miniata* (Hirose *et al.*, 1991; Hirose and Saito, 1992). Because other bacteria-like inclusions are not found in tunic phagocytes, we suppose that this bioluminescence is attributed to an intrinsic system within the cells. The other possibility, however, still remains; the light is emitted only from the bacteria within the tunic phagocytes and the tunic cells may provide essentials for light production to the bacteria.

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