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Still alive? Fine structure of the barrels made by *Phronima* (Crustacea: Amphipoda)

Euichi Hirose*[∫], Masakazu N. Aoki[†] and Jun Nishikawa[‡]

*Department of Chemistry, Biology, and Marine Science, Faculty of Science, University of the Ryukyus, Okinawa 903-0213, Japan. [†]Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka 415-0025, Japan. [‡]Ocean Research Institute, The University of Tokyo, Nakano, Tokyo 164-8639, Japan.
[∫]Corresponding author, e-mail: euichi@sci.u-ryukyu.ac.jp

Amphipods of the genus *Phronima* are known to make a barrel-shaped house from the gelatinous matrix of pelagic tunicates or siphonophores. Among the seven barrels examined here, one barrel of *Phronima curvipes* was supposed to be made from a swimming bell of a siphonophore based on its morphology, while the other six barrels made by *P. sedentaria* were immunochemically and/or morphologically identified as tunicates (i.e. *Thetys vagina*, other salps and pyrosomas). Histological observation showed that the phronimids had completely eaten the animal tissues other than the gelatinous matrix (i.e. tunic or mesoglea). Tunic cells were found in the tunicate barrel and some were probably tunic phagocytes that appeared to be alive and functional. In the tunicate barrels, cuticular layers of the tunic were found on both the outer and inner side of the barrel wall. Tunic cuticle would be regenerated on the inner side after the epidermis was grazed by the phronimids. The cuticular layers would protect the tunic matrix from the invasion of microorganisms. In the barrel supposed to originate from *Thetys vagina*, there are minute protrusions on the tunic cuticle as found in the intact tunic of this species. In the barrel from a siphonophore, neither cells nor cuticle regeneration were found. No bacteria were observed in the barrel, suggesting that the barrel has some antibiotic system.

INTRODUCTION

Phronima spp. are hyperiid amphipods that make a barrel-shaped house from the gelatinous matrix of their prey (pelagic tunicates or siphonophores) (Figure 1A). Since the barrel is used as a nursery by the phronimids (Figure 1B), the lifetime of the barrel should be long enough for this purpose. It is difficult to identify the animals from which the barrel was made, because the phronimids always eat up the internal tissue of the prey and often excise the protuberances on the surface of the barrel. Whereas several attempts were made for the identification of the origin of the barrels based on their morphology (Pagenstecher, 1861; Dudich, 1926; Harbison et al., 1977; Laval, 1978, 1980), these results are in some cases still conjectural. Recently, Nishikawa et al. (2005) showed that immunochemical methods could be applicable to identify the barrels.

Presence of a cellulosic tissue outside the epidermis is a synapomorph of tunicates (Kimura et al., 2001): the tissue forms a feeding apparatus called 'house' in appendicularians and forms a connective tissue (i.e. tunic) overlaying the epidermis in ascidians and thaliaceans. As an outermost tissue totally covering an animal body, tunic has various functions to protect the body. In ascidian tunic, there are several types of free cells (tunic cells) that are involved in many functions, such as, pigmentation, phagocytosis, ultraviolet light protection, acid storage and so on. In thaliaceans, tunic cells are not abundant in salps and doliolids (Hirose et al., 1999), while many tunic cells of various types are distributed in pyrosoma tunic (Hirose et al., 2001). Although tunic matrix consists of fibrous materials, tunic surface is always overlaid by a cuticular layer that has dense structures enough to prevent the invasion of microorganisms. The fine structures of the tunic cuticle show some inter-specific differences, such as thickness and the presence or absence of minute protrusions, in thaliaceans (Hirose et al., 1999), as well as in ascidians (cf. Hirose et al., 1997a). These features may provide some keys for identification of the origin of the barrel. This report describes the microscopic morphology of the immunochemically-identified barrels and suggests that the innate immune system might extend the lifetime of the barrel.

MATERIALS AND METHODS

Phronimids and barrels were collected in Sagami Bay (35°N 139°20′E) using the Isaacs–Kidd midwater trawl (IKMT) during the research cruises of RV 'Tansei Maru'. Part of each barrel was fixed in 2.5% glutaraldehyde–0.1 M cacodylate–0.45 M sucrose (pH 7.4) onboard for morphological investigation and the other parts were frozen at −80°C for immunochemical identification. A barrel made from a pyrosoma was collected in the South Pacific (39°34′S 171°25′W) with the IKMT and fixed in 5% formalin–seawater onboard during the research cruise of RV 'Hakuho Maru'. Table 1 lists six barrels (Specimens I–VI) collected in Sagami Bay and one (VII) collected in the South Pacific. All of the barrels examined here were associated with the phronimiids,

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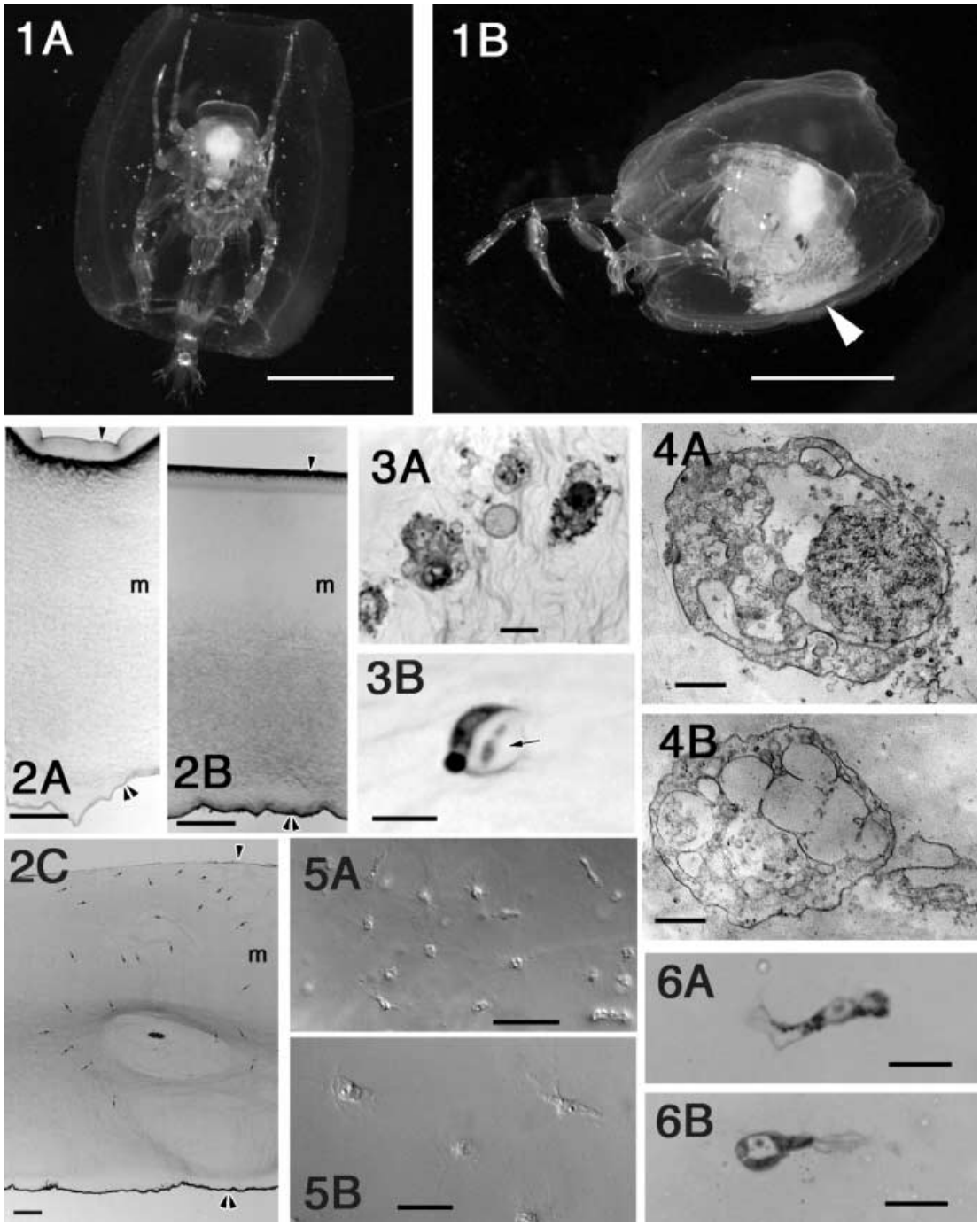

Figure 1. *Phronima sedentaria* within the barrel. (A) Anterior view and (B) lateral view. Arrowhead indicates the juveniles brooded in the barrel. Scale bar: 1 cm.

Figure 2. Histological sections of the barrel wall. (A) Specimen I (non-tunicate, resin section); (B) Specimen III (tunicate, resin section) and (C) Specimen VII (pyrosoma, paraffin section). Arrowheads indicate the outer surface of the barrel; double arrowheads indicate the inner (luminal) surface. Small arrows in (C) indicate tunic cells. m, matrix of the barrel wall. Scale bars: A, B, 10 μm; C, 100 μm.

Figure 3. Tunic cells in the barrels made from tunicates (A, Specimen III; B, Specimen IV). Arrow indicates a possible phagosome. Scale bars: 5 μm.

Figure 4. Electron micrographs of tunic cells in tunic matrix of the barrel (Specimen II). Scale bars: 1 μm.

Figure 5. A hand slice of the fixed barrel (Specimen VII) observed (A) with a differential interference contrast optics and (B) an enlargement of some tunic cells. Many tunic cells were distributed beneath the outer surface. Scale bar: A, 50 μm; B, 20 μm.

Figure 6. Tunic cells in the barrel made from a pyrosoma (resin section). Scale bars: 10 μm.

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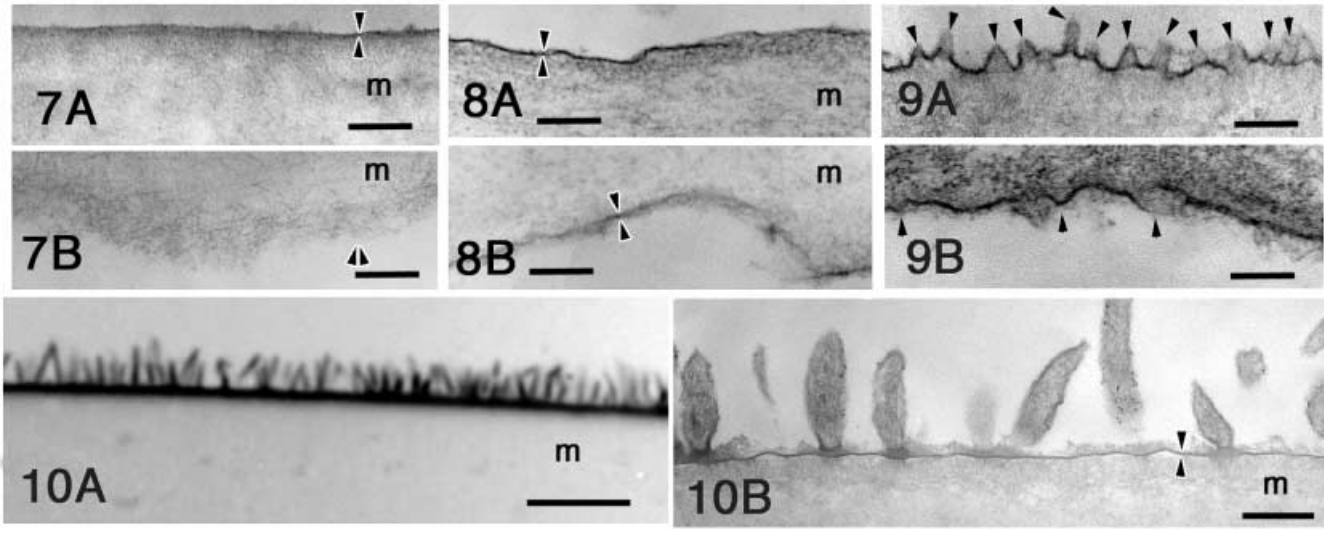

Figure 7. Electron micrographs of (A) the outer surface and (B) inner (luminal) surface of the barrel made from a non-tunicate organism (Specimen I). Facing arrowheads indicate a cuticular layer; double arrowheads indicate the matrix of the barrel wall (m) directly exposed to the lumen. Scale bars: 0.2 μm.

Figure 8. Electron micrographs of (A) the outer surface and (B) inner (luminal) surface of the barrel made from a tunicate (Specimen V). Facing arrowheads indicate a cuticular layer. m, matrix of the barrel wall. Scale bars: 0.2 μm.

Figure 9. Minute protrusions (arrowheads) of the cuticle on (A) outer and (B) inner (luminal) surface of the barrel made from *Thetys vagina* (Specimen VI). Scale bars: 0.2 μm.

Figure 10. (A) Histological and (B) electron micrographs of rod-shaped bacteria attaching to the cuticular surface of the barrel (Specimen IV). Facing arrowheads indicate a cuticular layer. m, matrix of the barrel wall. Scale bars: A, 5 μm; B, 0.5 μm.

when they were collected: the barrel Specimen I was made by a female of *Phronima curvipes* Vosseler, and the other Specimens (II–VII) were made by females of *Phronima sedentaria* (Forskål).

The barrels from Sagami Bay were immunochemically identified by the method described in Nishikawa et al. (2005), using the rabbit antibodies (IgG) respectively raised against three cnidarians (*Periphylla periphylla* (Péron & Lesueur), *Atolla wyvillei* Haeckel, *Aequorea coerulescens* (Brandt)) and three thaliaceans (*Pyrosoma atlanticum* Péron, *Thetys vagina* Tilesius, *Thalia democratica* (Forskål)). Specification of each antibody was delineated in Nishikawa et al. (2005). Briefly, the antibodies against *Periphylla periphylla* and *Atolla wyvillei* cross-react with the antigens of each other; the antibody against *Aequorea coerulescens* specifically reacts with the antigen; the antibodies against thaliaceans cross-reacted with all the eight pelagic tunicates tested but did not react with any other animals so far examined. Moreover, there was a specific band in the immunoblot of *Thetys vagina* treated with anti-*T. vagina* antibody that enables this species to be identified from other thaliaceans. Immunoblot analysis was not applied for the barrel collected in the South Pacific.

The glutaraldehyde-fixed specimens were briefly rinsed with 0.1 M cacodylate–0.45 M sucrose, and postfixed in 1% osmium tetroxide–0.1 M cacodylate for 1.5 h. The specimens were dehydrated through an acetone series, cleared with *n*-butyl glycidyl ether, and embedded in low viscosity epoxy resin. Sections of 1 μm thick were stained with 1% toluidine blue for light microscopy. Sections of about 0.1 μm thick were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Hitachi HS-9) at 75 kV.

The formalin-fixed specimen was embedded in paraffin and its sections, about 8 μm thick, were stained with haematoxylin and eosin for light microscopy. Some pieces of the specimens were post fixed with 1% osmium

Table 1. *List of barrel specimens, immunochemical identification and inhabiting phronimids.*

	Barrel							Phronimids	
	Immunoblotting[#]								
Sample no.	PP	AW	AC	PA	TV	TD	Estimated origin	Species	Body length (mm)
I	–	–	–	–	–	–	non-tunicate[§]	*P. curvipes*	12
II	–	–	–	+	+	+	tunicate	*P. sedentaria*	35
III	–	–	–	+	+	+	tunicate	*P. sedentaria*	28
IV	–	–	–	+	+	+	tunicate	*P. sedentaria*	30
V	–	–	–	+	+	+	tunicate	*P. sedentaria*	25
VI	–	–	–	+	+*	+	*Thetys vagina*	*P. sedentaria*	35
VII[†]							pyrosoma	*P. sedentaria*	29

[#], Antibodies raised against three cnidarians (*Periphylla periphylla* [PP], *Atolla wyvillei* [AW] and *Aequorea coerulescens* [AW]) and three thaliaceans (*Pyrosoma atlanticum* [PA], *Thetys vagin*a [TV] and *Thalia democratica* [TD]). [§], Supposed to be a siphonophore, of a species other than those used for raising antibodies. *, *Thetys vagina* specific band was detected. [†], Immunoblot analysis was not carried out.

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tetroxide and embedded in epoxy resin for light microscopy as described above.

RESULTS AND DISCUSSION

The barrel of *Phronima curvipes* (I) originated from a non-tunicate organism, since no antibodies reacted with this specimen. The form of the barrel strongly suggested that Specimen I was made from a swimming bell of a siphonophore. Thus, Barrel I was supposed to be made from a siphonophore species, other than the species used to raise the antibodies. Immunoblot analysis demonstrated that Specimens II, III, IV, V and VI all originated from tunicates. Moreover, the specific band in the immunoblot indicated that Specimen V originated from a salp *Thetys vagina*. Specimen VII obviously originated from a pyrosoma due to the presence of test protrusions that were characteristic of *Pyrosoma* and *Pyrostremma* and supposed to correspond to the type 'C' barrel in Laval (1978). These results were consistent with Laval's descriptions: *Phronima curvipes* made barrels from the siphonophore (Laval, 1968) and *P. sedentaria* made barrels exclusively from salps and pyrosomes (Laval, 1978).

Since no epithelial tissues were found in the histological sections of any barrels, the *Phronima* probably ate the animal tissues other than the gelatinous matrix in the process of the barrel making (Figure 2) as described by Laval (1978). Although many zooids are embedded in the tunic of intact pyrosomas, we could not find any cavities where the zooids were present (Figure 2C). Laval (1978) reported a similar observation on the pyrosoma barrels and he supposed that the cavities were re-filled with the material secreted by tunic cells. Since the barrel wall was made of homogeneous matrix of tunic in the histological sections, natural inflation of the matrix might fill the cavities after the removal of zooids. In the barrels made from tunicates (Specimens II–VII), tunic cells were found in the tunic matrix, but no cellular components were found in the barrel from a non-tunicate organism (Specimen I). In Specimens II–VI, tunic cells were sparsely distributed in the tunic matrix of the barrels as those in an intact tunic of salps (Figure 3), as primarily described by Pagenstecher (1861). The tunic cells in the barrels were usually spherical or irregular in shape and some cells appeared to be phagocytes with phagosomes (Figure 3B). The cells still retain their shapes but their sub-cellular structures of the tunic cells were not well preserved (Figure 4). In the barrel made from a pyrosoma (Specimen VII), many tunic cells were found particularly beneath the outer surface of the barrel (Figures 2C, 5 & 6). Although tunic net cells are densely distributed beneath the inner (cloacal) surface of the tunic in pyrosoma colonies (Hirose et al., 2001), they were not found in the barrel of Specimen VII, suggesting that the phronimid scraped the cloacal wall of the pyrosoma. According to the distribution of tunic cells, much more concentrated in pyrosomas than salps and doliolids (Hirose et al., 1999, 2001), the barrel made from pyrosoma contained many more tunic cells than the barrels from salps. The amount of tunic cells in the barrels potentially distinguishes the pyrosoma barrels from salp barrels. Many of the tunic cells in the barrels had extended filopodia and were supposed to be alive and functional. Moreover, some had phagosome-like vacuole(s). It is possible that these tunic cells are involved in innate immunity in the phronimid barrels. This is not a surprise, because the tunic cells in a tunic slice of a colonial ascidian survived for several days or more (Ishii & Hirose, 2003).

The outer surface of the barrels always had a cuticle, an electron-dense layer overlaying the gelatinous matrix that appeared to be loosely packed fibres in electron micrographs. On the inner (luminal) surface of the barrels, a cuticular layer was also found in the barrels from tunicates (II–VI) but not in the barrel from a non-tunicate organism (I) (Figures 7 & 8). In intact salps, the inner surface of the barrel-shaped tunic is totally lined with a mantle epithelium and a cuticular layer is never present there. Therefore, the cuticle on the inner surface of the barrels should be regenerated after the *Phronima* eats out the mantle. Alternatively, phronimids might daub their secretion on the inner surface of the barrel, but this idea is not supported, given the absence of such a layer in Specimen I. In some ascidians, the regeneration of the tunic cuticle proceeds from aggregation of fibrous materials of tunic matrix, while cellular components are not directly involved in this process (Hirose et al., 1995, 1997b). The cuticle on the luminal surface of the barrels could be formed through a similar process. While tunic cuticles are flat in many thaliacean species, the presence of cuticular protrusions were described in *Thalia democratica*, *T. orientalis* (Tokioka) and *Thetys vagina* (Hirose et al., 1999). The cuticle had minute protrusions about 50 nm in height in Specimen VI that were immunochemically identified as *T. vagina* (Figure 9A). The presence of the cuticular protrusion confirms the identification based on the species-specific band in the immunoblot. In Specimen VI, minute protrusions were also found on the luminal side of the barrel wall, although they were small and sparse (Figure 9B). This also supports that the electron-dense layer overlaid on the luminal surface is the tunic cuticle regenerated after the predation of internal tissues by phronimids.

Bacteria were often found on the cuticular surface of the barrel made from tunicates. For instance, rod-shaped bacteria densely adhered to the surface in Specimen IV (Figure 10). On the other hand, bacteria were rarely found in the matrix of the barrel wall, suggesting that the cuticular layer protected the fibrous matrix from the invasion of the microorganisms. In the siphonophoran barrel (I), bacteria were rarely found both on the surface and in the matrix of the barrel, although the inner surface of the barrel wall was not covered with a cuticular layer. It is possible that there are some antibiotic systems in the matrix of the siphonophoran barrel.

The present observations were consistent with the immunochemical identification of the barrels and supported the validity of this method. Combination of immunochemical and microscopic methods should be more informative. In future, DNA markers are also considered to be potentially effective for the identification of the barrel origin. However, DNA extraction from phronimid barrels may be difficult, because the barrels contain only small amounts of cellular components sparsely distributed in the acellular matrix. On the other hand, the presence of tunic cells and the occurrence of cuticle

regeneration suggest that phronimids' barrels appear to be still alive at tissue level. For phronimids, the barrels should be durable enough to incubate eggs and care for their juveniles. Innate immune systems of the prey organisms (i.e. tunicates and siphonophores) may function and extend the lifetime of the barrel.

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