

— Report —

Monoclonal antibodies that recognize symbiotic bacteria and hemocytes in the deep-sea vesicomyid clam *Phreagena okutanii*

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Vesicomyid clams, including the genus *Phreagena*, are dominant members of various deep-sea chemosynthesis-based animal communities. They harbor symbiotic sulfur-oxidizing bacteria in the epithelial cells of their gill tissue, and the bacteria are transmitted to the next generations via eggs. We created a monoclonal antibody (mAb) library against the gill of *P. okutanii* and observed mAb CokG-D1D3 to react to the symbiotic bacteria and three mAbs (CokG-Y1F7, CokG-J10D2, and CokG-J3G4) to bind to hemocytes of *P. okutanii*. The signals of mAb CokG-D1D3 were localized in the epithelial gill cells called bacteriocytes. We also observed a small number of clear signals of the antibody in the epithelial follicular cells of the ovary. The signals of mAb CokG-D1D3 almost exactly overlapped those of the anti-*E. coli* GroEL polyclonal antibody, while, in the gill, the signal areas of the latter seemed to be slightly wider than those of the former. Among the three mAbs against the hemocytes, mAbs CokG-Y1F7 and CokG-J10D2 reacted to a large fraction of the hemocyte populations, but mAb CokG-J3G4 reacted to a smaller fraction. mAb CokG-Y1F7 was observed to bind to the hemocytes distributed widely in the interstitial spaces of various tissues. These monoclonal antibodies are expected to be useful for studying the interactions between symbiotic bacteria and host cells and the distribution and functions of hemocytes in deep-sea vesicomyid clams.

Keywords : monoclonal antibody, *Phreagena*, deep sea, symbiotic bacteria, hemocyte

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1. Introduction

Vesicomid bivalves are one of the most abundant animals that inhabit deep-sea chemosynthesis-based ecosystems (Cavanaugh et al., 1981; Felbeck et al., 1981). They possess large gills with highly packed sulfur-oxidizing bacteria inside the epithelial cells; however, they have reduced gill ciliary groove, labial palps, and digestive tube (Boss and Turner, 1980; Cavanaugh, 1983; Fiala-Medioni and Metivier, 1986). The presence of many symbiotic bacteria in the bacteriocytes in the gill filament has been confirmed using *in situ* hybridization (Nakamura et al., 2013). The symbiotic bacteria are transmitted vertically to the offspring via eggs (Cary and Giovannoni, 1993; Ikuta et al., 2016). In the ovary, symbiotic bacterial cells have been observed in follicle cells and on egg cells by using transmission electron microscopy and *in situ* hybridization (Ikuta et al., 2016). Genome analyses of the symbiotic bacteria in vesicomid clams such as *Phreagena okutanii* Kojima and Ohta 1997 and *Calyptogena magnifica* Boss & Turner, 1980 have shown that the symbionts are composed of a single species: *Candidatus* (*Ca.*) *Vesicomysocius okutanii* strain HA (Vok) and *Ca.* *Ruthia magnifica* strain Cm (Rma) (Kuwahara et al., 2007; Newton et al., 2007). They have reduced genomes and lack some genes that are essential for survival; however, they possess genes for sulfur-oxidizing metabolism, carbon fixation, and synthesis of amino acids and organic compounds (Kuwahara et al., 2007; Newton et al., 2007). The findings are consistent with those of a previous stable isotope analysis that showed that the hosts nutritionally depend on the organic compounds synthesized by the chemosynthetic symbionts (Le Pennec et al., 1995). Thus, vesicomid clams and their symbiotic bacteria form an obligatory symbiotic system.

The clams are known to possess a unique oxygen transport system. Hemoglobin is contained in the red hemocyte, which is thought to circulate in the body and transfer oxygen to tissues (Suzuki et al., 2000). In general, the hemocytes function in immunological defense against microbial invasion to maintain homeostasis (Bayne, 1983; Hine, 1999; Muroga and Takahashi, 2007; Takahashi and Muroga, 2008; Donaghy et al., 2009; Tame et al., 2015). The symbiotic bacteria must escape from the host defense system, including defense by the hemocytes. However, the mechanism underlying the escape is still unclear.

To understand the molecular mechanisms

underlying the maintenance of the symbiosis, tools for marking the protein components are necessary. We constructed a monoclonal antibody (mAb) library against the gill component of *P. okutanii* and reported an mAb that reacts against the mucus component secreted from epithelial cells in the gill (Nakamura et al., 2013). The mAb and hybridoma are available at the JAMSTEC Marine Biological Sample Database [<http://www.godac.jamstec.go.jp/bio-sample/showDetail.do?menuId=6&sampleId=1090053148>, IDs, 1090053149 and 1090053150; Accessed on 2017-11-04]. For studying the interaction between the symbiont and host, markings of the symbionts and hemocytes are mandatory. Here, we have reported four new mAbs: one mAb that reacts to the symbiotic bacteria and three mAbs that bind to the hemocytes.

2. Materials and Methods

An mAb library against the gills of *P. okutanii* was generated as described previously (Nakamura et al., 2013). Briefly, homogenized gills from *P. okutanii* collected during the R/V Natsushima cruise (NT09-06 Leg1: April 24 to May 6, 2009) by using the ROV *Hyper-Dolphin* (Dive#973) were used as antigens for inoculating BALB/c mice. The mAb library was constructed using a conventional polyethylene glycol method (Galfre and Milstein, 1981). Cloning of the hybridoma was performed three times using the limited dilution method.

The binding activity of the mAbs in the supernatant of the hybridoma culture was examined using indirect immunofluorescence microscopy with 4- μ m-thin sections of the gills or ovaries from adult clams or whole bodies of very small immature clams. These clams were collected using the ROV *Hyper-Dolphin* during cruises of the R/V Natsushima (NT09-06 Leg1, Dive#973: NT10-08, Dive#1125; NT11-09, Dive#1291 and 1293; NT13-07, Dive#1508 and 1511) and ROV *KAIKO* during the R/V Kairei cruise (KR12-05, Dive#545) (Inoue, 2010, 2011; Oguri, 2012; Yoshida, 2013). *P. okutanii* was identified and distinguished from the sibling species *Phreagena soyoe* by partial sequencing of the cytochrome c oxidase subunit 1 (*COI*) gene (Harada et al., 2009). The adult tissues and small clams were fixed with 4% paraformaldehyde in seawater for 18 h. The shells of immature clams were removed using decalcifying buffer (Wako Co., Tokyo), and three very small clams

(length of the shell long axis, 16.0, 17.7, and 22.6 mm) were used to prepare the frozen sections. The adult tissues and the three small clam bodies were frozen-embedded in optimal-cutting-temperature compound in liquid nitrogen and sliced using a cryomicrotome (Microm HM550; Thermo Fisher Scientific Inc., Tewksbury, MA). They reacted to the supernatant of the hybridoma culture at 4°C for 2 h. After washing with PBS (135 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 8.0), they were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies Co. Ltd., Wyman, MA) diluted at 1:2000 at 4°C for 2 h. Simultaneously, the sections reacted with anti-*E. coli* GroEL rabbit polyclonal antibody (Sigma Aldrich Co. Ltd., St. Louis, MO) diluted at 1:1000, followed by incubation with Alexa Fluor 647-conjugated anti-rabbit IgG antibodies (Life Technologies Co. Ltd., Wyman, MA) diluted at 1:2000 at 4°C for 2 h. The sections were also stained with 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich Co. Ltd., St. Louis, MO) at a concentration of 10 µg/ml at room temperature for 15 min. For morphological observation, 4-µm-thin sections were obtained from paraformaldehyde-fixed and paraffin-embedded adult tissues or immature clam bodies. Conventional hematoxylin and eosin (HE) staining was performed, and a microscope equipped with

fluorescence optics (BZ-900; Keyence Co. Ltd., Osaka, Japan) was used for observation.

3. Results

3.1 mAb CokG-D1D3 reacted to the symbiotic bacteria in the gills and ovaries of *P. okutanii*

Newly established mAbs from the mAb library against *P. okutanii* gills are listed in Table 1. In a transverse section, the gill filament is composed of three parts: the inner zone containing many symbiont cells and two symbiont-lacking zones, the frontal zone and the junction area between the inner and the frontal zones (Ohishi et al., 2016; IZ, FZ, and J in Fig. 1a). Black particles indicating elemental sulfur were observed in the inner zone of a frozen section (Fig. 1b) (Ohishi et al., 2016). Strong signals for mAb CokG-D1D3 were detected in only the inner zone (Fig. 1c). No signal was detected in a negative control using PBS instead of primary antibodies. We concomitantly immunostained the symbionts with the anti-*E. coli* GroEL antibody, which has been used to detect them (Fig. 1d and e; Nakamura et al., 2013; Ohishi et al., 2016). Signals of both the mAb and anti-*E. coli* GroEL antibody were detected in almost the same area, indicating that this mAb specifically

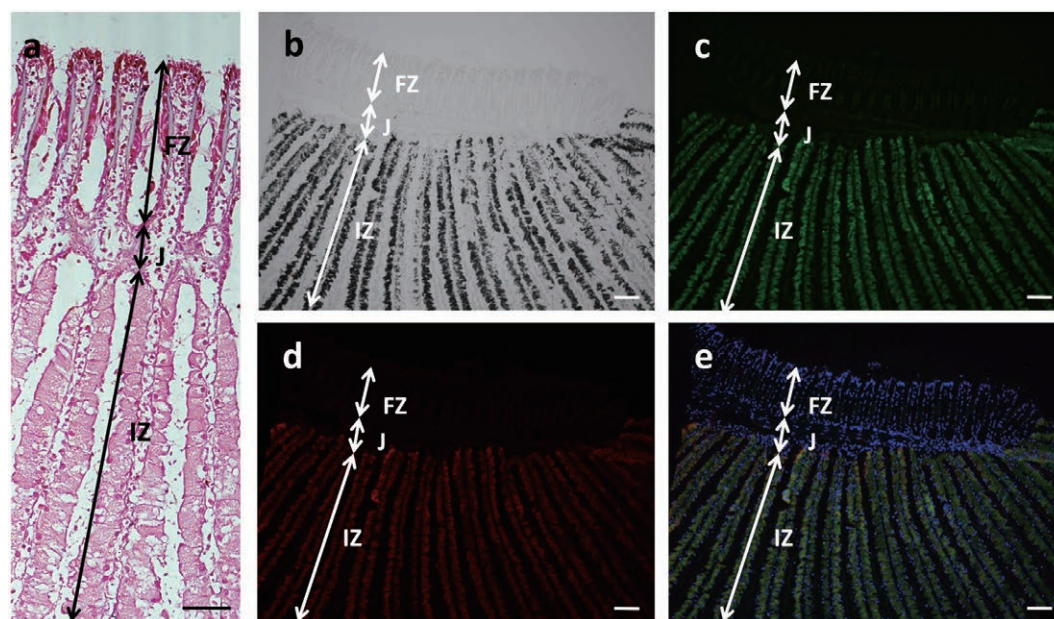


Fig. 1. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-D1D3 binding to the gill filament of *Phreagena okutanii*. **a**, HE-stained paraffin-embedded transverse section of the gill. **b**, Bright-field micrographs of a frozen transverse section. **c** and **d**, Fluorescent images with mAb CokG-D1D3 (green) and anti-*E. coli* GroEL antibody (red). **e**, Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-*E. coli* GroEL antibody (red), and DAPI (blue). Scale bars indicate 50 µm (**a**) and 20 µm (**b–d**). IZ, inner zone; FZ, frontal zone; J, junction area between IZ and FZ.

reacts to the symbiont cells. However, their appearances were different. At higher magnification, the mAb signals appeared as clear dots in the cytoplasm of the epithelial cells, called bacteriocytes, of the inner zone (Fig. 2). However, the signals from the anti-*E. coli* GroEL antibody were not clear but blurred (Fig. 2b and d). In addition, it is noteworthy that the signals of the anti-*E. coli* GroEL antibody were distributed a little wider than those of the mAb: red color protruding slightly out of the area containing green dot (white arrows in Fig. 2d).

Because the symbiotic bacteria are vertically transmitted via the female gonad, the reactivity of mAb CokG-D1D3 to the ovary tissue was examined using frozen sections from adult female clams. The follicle cells were observed as the epithelium lining the ovarian cavity (yellow triangles in Fig. 3a), and various maturation stages of the

oocytes were in the cavities (white triangles in Fig. 3a). The dotted signals of the mAb were clearly observed in the follicle cells, although the number of the dots in a cell (yellow triangles in Fig. 3b and c) was much less than those in the gill (Fig. 2a and d). The difference in signal appearances between the mAb and anti-*E. coli* GroEL antibody detected in the gill was less conspicuous in the gonad than in the gill. We could detect no signal from either mAb or anti-*E. coli* GroEL antibody in the oocytes (white triangles in Fig. 3b–d). In some immature clams, we observed many ring-like or ellipsoidal structures in the gonad tissue area at the base of the foot in the HE-stained paraffin-embedded whole body (black triangles and a dotted ellipse in Fig. 4a), which seemed to be immature follicle cells in the ovaries. The frozen sections from the three very small clam bodies were also immunologically stained with mAb

Table 1. List of the mAbs to *Phreagena okutanii*

mAb ID No.	Targets of mAb	JAMSTEC Database ID*	
		Antibody (supernatant)	Hybridoma cell
CokG-D1D3	Symbiotic bacteria	1090053151	1090053152
CokG-Y1F7	Hemocyte	1090053153	1090053154
CokG-J10D2	Hemocyte	1090053155	1090053156
CokG-J3G4	Hemocyte	1090053157	1090053158

*, mAb and hybridoma cell line are available. Please see Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Marine Biological Sample Database (<http://www.godac.jamstec.go.jp/bio-sample/showDetail.do?menuId=6&sampleId=1090053148>, Accessed on 2017-11-04)

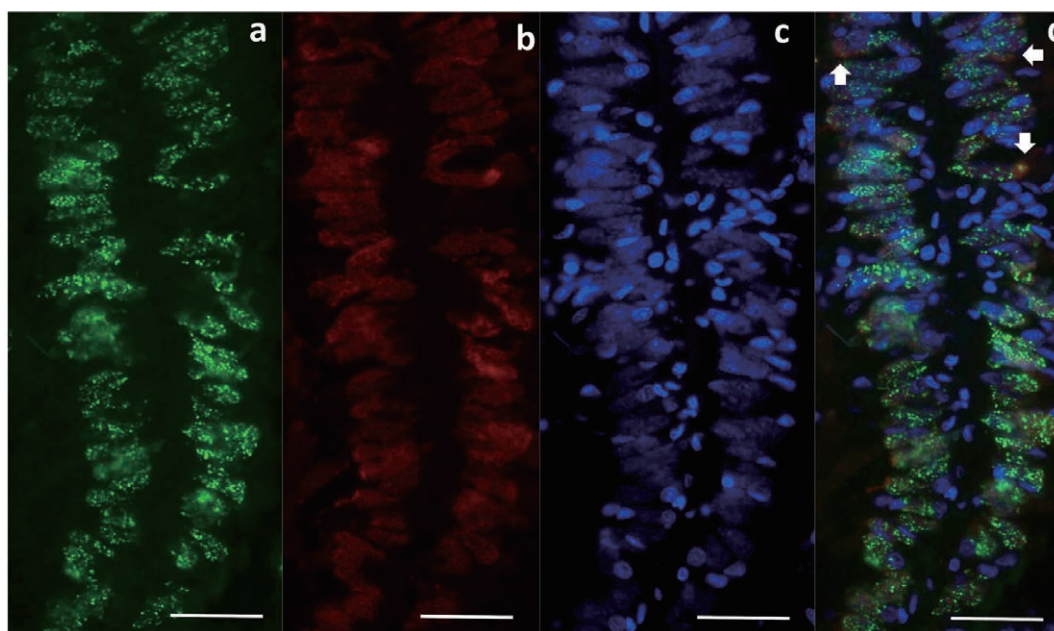


Fig. 2. Higher-magnification immunofluorescence micrograph of the monoclonal antibody (mAb) CokG-D1D3 binding to the gill filament of *Phreagena okutanii*. **a–c**, Fluorescent images with mAb CokG-D1D3 (**a**, green), anti-*E. coli* GroEL antibody (**b**, red), and DAPI (**c**, blue) of the same frozen transverse section of a gill filament. **d**, Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-*E. coli* GroEL antibody (red), and DAPI (blue). White arrows indicate the wider stained area with anti-*E. coli* GroEL antibody than that with mAb CokG-D1D3. Scale bar, 40 μ m.

CokG-D1D3 or anti-*E. coli* GroEL antibody. Both signals were observed in the ring-like or ellipsoidal shapes in the gonad tissue area in the two immature clams (length of the shell long axis, 16.0 mm and 17.7 mm; white arrows and a dotted ellipse in Fig. 4b).

3.2 mAbs CokG-Y1F7, CokG-J10D2, and CokG-J3G4 reacting to hemocyte-like cells in *P. okutanii*

The signals of mAbs CokG-Y1F7, CokG-J10D2, and CokG-J3G4 reacted with hemocyte-like cells inside the gill filament (Table 1). Since these cells were

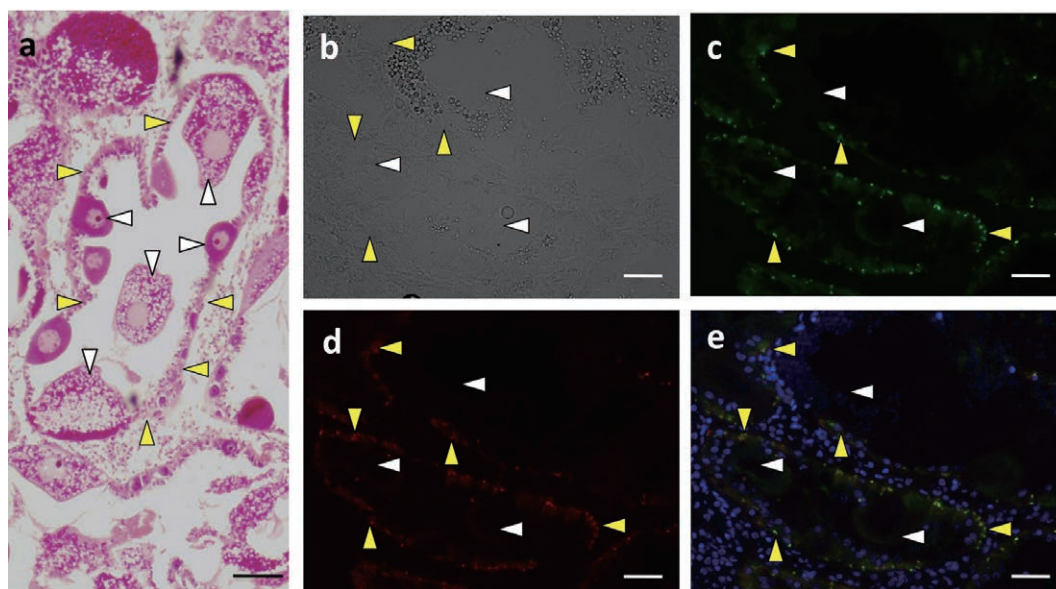


Fig. 3. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-D1D3 bound to the ovary of *Phreagena okutanii*. **a**, HE-stained thin section of the paraffin-embedded ovary. **b**, Bright-field micrographs of a frozen transverse section. **c** and **d**, Fluorescent images with mAb CokG-D1D3 (green) and anti-*E. coli* GroEL antibody (red). **e**, Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-*E. coli* GroEL antibody (red), and DAPI (blue). Scale bars indicate 100 μm (**a**) and 40 μm (**b–e**). Yellow triangles: follicular cells, white triangles: oocytes.

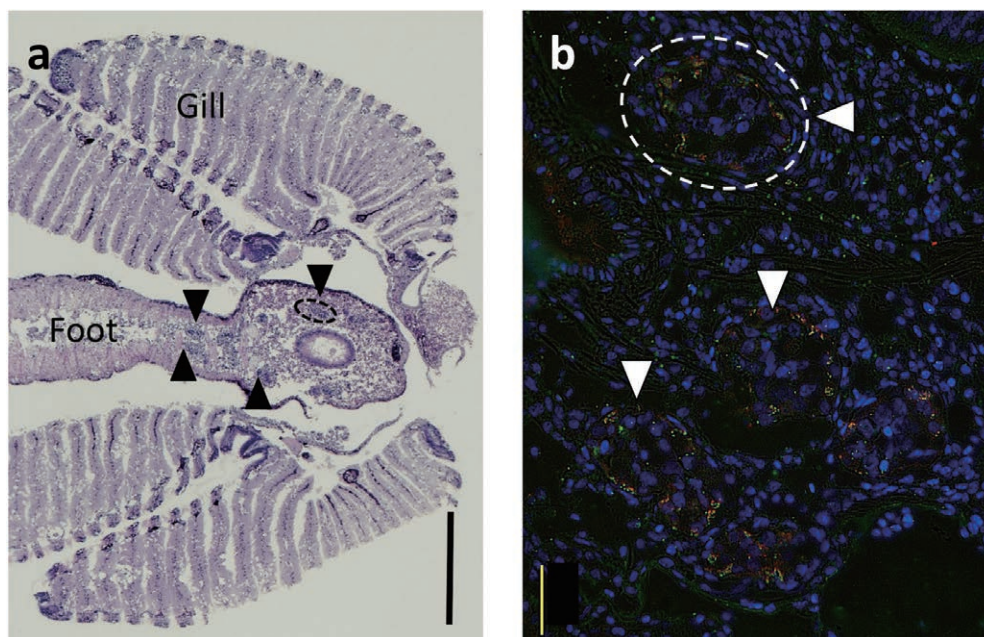


Fig. 4. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-D1D3 bound to an immature *Phreagena okutanii*. **a**, HE-stained cross-section of paraffin-embedded whole immature clams. **b**, Triple merged fluorescent images of mAb CokG-D1D3 (green), anti-*E. coli* GroEL antibody (red), and DAPI (blue) in a frozen section of the whole immature clams. Scale bars indicate 500 μm (**a**) and 40 μm (**b**). Black triangles and white triangles indicate immature ovary-like ellipsoidal structures. A structure was surrounded by black or white dotted ellipses.

morphologically recognized as hemocytes, the mAbs were thought to react to the hemocytes. The mAbs CokG-Y1F7 and CokG-J10D2 were bound to a large proportion of hemocytes in the blood vein-like cavities (Fig. 5a and b), while mAb CokG-J3G4 reacted with a smaller fraction (Fig. 5c). Approximately 80% of the hemocytes in the images reacted positively with mAbs CokG-Y1F7 and CokG-J10D2 and 40% with mAb CokG-J3G4. The distribution of the signal-positive cells in the whole bodies of the immature clams was examined using mAb CokG-Y1F7 because its signal seemed to be the strongest. The signal-positive cells were found in the cavities of various tissues, such as the gill and mantle (Fig. 6); however, the signal was also detected on some parts of the mantle surface facing the shell (white arrow in Fig. 6). In the immature clams, the hemocyte-like cells were observed in a relatively large cavity at the base of the foot, which surrounds the intestine (Fig. 7a), although the cavity and hemocyte-like cells were not well-recognized in the adult clams (data not shown). The mAb CokG-Y1F7 bound to these cells which seemed to be free-moving cells, indicating that the cells in the cavity were hemocytes (Fig. 7b). Thus, mAb

CokG-Y1F7 recognized the hemocytes in the interstitial space of various tissues.

4. Discussion

The dot-like signals of mAb CokG-D1D3 were abundantly found in the bacteriocytes of the gill, while clear but smaller number of signals were detected in the follicular cells of the ovary (Figs. 1–3). The dotted signals were found to be restricted to the symbiosome of the bacteriocyte in the gill filament and follicle cell, where the signal of the anti-GroEL antibody was also co-localized. Thus, the distribution and number of signals by mAb CokG-D1D3 were consistent to those of the symbiotic bacteria. These results indicate that the mAb binds to the symbiotic bacterium of *P. okutanii*, *Vok*, although we failed to identify the target antigen by using western blot analysis. A recent study has shown that approximately 400 cells of the symbiotic bacterium *Vok* were at the vegetal pole of the eggs, but they were not found in the sections of other parts of the large egg cells (Ikuta et al., 2016). This

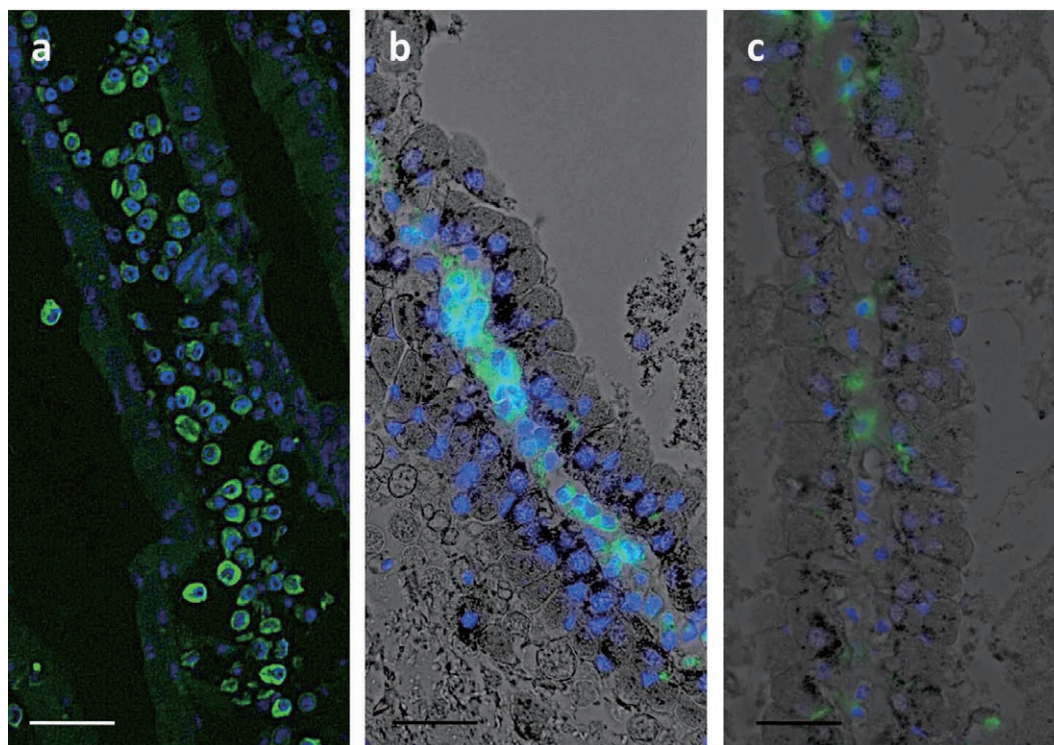


Fig. 5. Immunofluorescence micrographs of the monoclonal antibodies (mAbs) reacting to hemocytes inside the gill filaments of *Phreagena okutanii*. **a**, Merged fluorescent images of mAb CokG-Y1F7 (green) and DAPI (blue). **b**, Merged images of mAb CokG-J10D2 (green), DAPI (blue), and bright-field micrographs. **c**, Merged images of mAb CokG-J3G4 (green), DAPI (blue), and bright-field micrographs. Scale bars indicate 40 μ m (**a**, **b**, and **c**). Haze reduction function (Keyence fluorescence microscope) was used for (**a**).

may explain why we could not find the signal for mAb CokG-D1D3 in the sections of the oocytes. In the basic part of the foot of juvenile clams, the symbiotic bacteria were located in circularly or elliptically arranged cells (Fig. 4). Although further studies are required, this finding suggests that such small-sized clams have ovarian follicle cells that already contain the symbionts. The metabolism of the symbiont in the gill has been suggested to be different from that in the ovary (Ohishi et al., 2016). This difference is understandable because sulfur oxidation metabolism is important for maintaining the lives of both partners in the gill; however, its importance is not clear in the follicle

cells where the symbiont must be securely transmitted to the next generation of the host. Ikuta et al. (2016) raised an important question: How are the symbionts transferred from the outside surface of the egg to the inside of follicle cells, oocytes, and gill epithelial cells during development. Although the target antigen is not known, the symbiont-specific monoclonal antibody, mAb CokG-D1D3, could be useful for tracing symbiotic bacteria during the development of the clam and/or regeneration after the accidental or natural death of bacteriocytes. Studying the fate of symbiotic bacteria during the life cycle of the host clam would be key in understanding how the symbiosis is maintained.

The signals of mAb CokG-D1D3 and anti-*E. coli* GroEL antibody were almost co-localized in the bacteriocytes in the gill epithelium and ovarian follicle cells

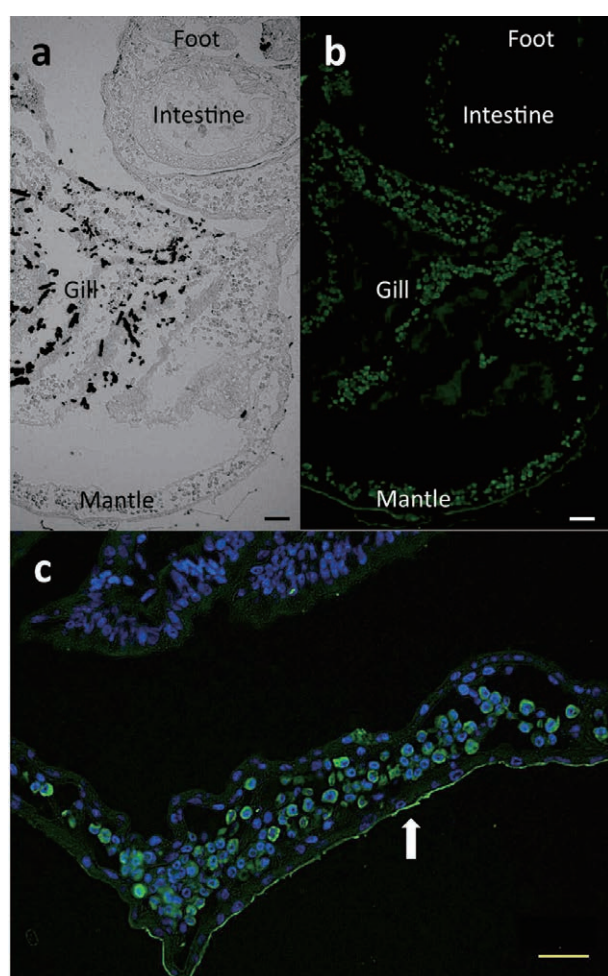


Fig. 6. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-Y1F7 in frozen whole sections from immature *Phreagena okutanii*. **a**, Bright-field micrographs of the frozen cross-section. **b**, Fluorescent image of mAb CokG-Y1F7 (green). **c**, Merged fluorescent images of CokG-Y1F7 (green) and DAPI (blue) in the mantle. White arrow indicates a part of the mantle binding to mAb CokG-Y1F7. Scale bars indicate 50 μm (**a**, **b**) and 30 μm (**c**). Haze reduction function (Keyence fluorescence microscope) was used for (**c**).

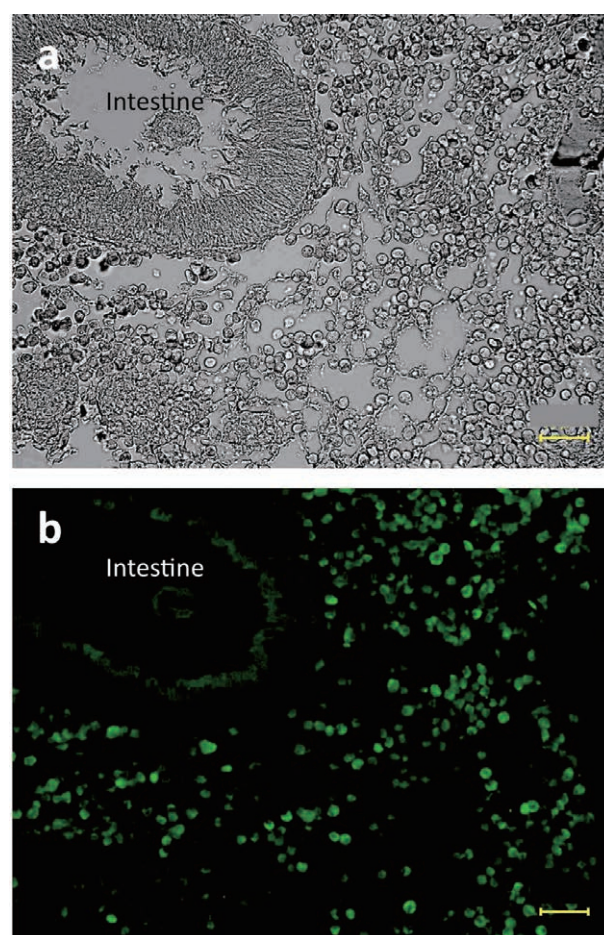


Fig. 7. Immunofluorescence micrographs of the monoclonal antibody (mAb) CokG-Y1F7 bound to hemocyte-like cells in a cavity surrounding the intestine of immature *Phreagena okutanii*. Paraffin-embedded section from an immature clam with a body length of 20 mm was used. **a**, Bright-field micrographs of the section. **b**, Fluorescent image of mAb CokG-Y1F7 (green). Scale bars indicate 30 μm .

(Figs. 1–3). However, the signals of the latter formed blurred dots and seemed to expand slightly more than the former (Figs. 1e and 2d). GroEL, a chaperon protein involved in protein folding, is known to be highly produced in the symbiotic bacterium *Buchnera aphidicola* (Ishikawa, 1984). GroEL in *Vok* is also highly expressed (Yoshida T., personal communication). The slightly expanded signal area detected by the anti-*E. coli* GroEL antibody might be due to the higher production of GroEL in *Vok*. Recently it has been shown that some intracellular pathogenic bacterial GroEL possesses multi-functions and is not always localized in the cytoplasm of the bacteria cells (Garduno et al., 2011). These questions need to be addressed in the future.

We observed that three mAbs (CokG-Y1F7, CokG-J10D2, and CokG-J3G4) reacted with the hemocytes. Two of them, CokG-Y1F7 and CokG-J10D2, were bound to a large fraction of the hemocytes, while the third one, CokG-J3G4, reacted to a smaller fraction. The results may indicate multiple populations in the hemocytes. Alternatively, the hemocytes may be at different developmental stages or immunological activation stages. The mAb CokG-Y1F7 bound to the hemocytes in the cavity at the base of the foot in an immature clam, where premature ovarian follicle cells containing symbiotic bacteria seemed to be localized (Fig. 4). The mAb may be useful for detecting the distribution of hemocytes in the open blood-vascular system at various developmental stages. Because hemocytes of bivalves have been shown to play a crucial role in immunological defense (Bayne, 1983; Hine, 1999; Muroga and Takahashi, 2007; Takahashi and Muroga, 2008; Donaghy et al., 2009; Tame et al., 2015), it would be interesting to investigate whether certain types of hemocytes are specifically localized in the symbiont-containing gill tissue and how the symbiotic bacteria escape from the host defense system. The mAbs are expected to be useful in addressing these immunological queries by recognizing the symbiont cells and different types of hemocytes. It is important to establish additional useful mAbs against various tissue cells or cell components of *P. okutanii* and characterize them for further studies on symbiosis in deep-sea clams.

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