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Symbiont Transmission onto the Cell Surface of Early Oocytes in the Deep-sea Clam *Phreagena okutanii*

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Symbiotic associations with beneficial microorganisms endow a variety of host animals with adaptability to the environment. Stable transmission of symbionts across host generations is a key event in the maintenance of symbiotic associations through evolutionary time. However, our understanding of the mechanisms of symbiont transmission remains fragmentary. The deep-sea clam *Phreagena okutanii* harbors chemoautotrophic intracellular symbiotic bacteria in gill epithelial cells, and depends on these symbionts for nutrition. In this study, we focused on the association of these maternally transmitted symbionts with ovarian germ cells in juvenile female clams. First, we established a sex identification method for small *P. okutanii* individuals, and morphologically classified female germ cells observed in the ovary. Then, we investigated the association of the endosymbiotic bacteria with germ cells. We found that the symbionts were localized on the outer surface of the cell membrane of primary oocytes and not within the cluster of oogonia. Based on our findings, we discuss the processes and mechanisms of symbiont vertical transmission in *P. okutanii*.

Key words: deep-sea clam, chemosynthetic symbiosis, endosymbiotic bacteria, vertical transmission, gametogenesis, oocyte

INTRODUCTION

Deep-sea hydrothermal vent and seep sites often harbor high densities of a variety of invertebrates, such as annelids, arthropods, and mollusks. These invertebrates survive in the deep sea, where little or no sunlight reaches, by establishing symbiotic relationships with chemoautotrophic bacteria. These symbiotic bacteria use the chemical energy contained in reduced compounds such as sulfide, methane, or hydrogen for chemo-biosynthetic processes (Dubilier et al., 2008; Petersen et al., 2011). Deep-sea vesicomyid clams, including those of the genus *Phreagena* (formerly *Calyptogena*), are endemic and dominant members of deep-sea chemosynthesis-based communities (Fisher, 1990). They harbor sulfur-oxidizing symbiotic bacteria (Gram-negative Gammaproteobacteria) in their gill epithelial cells (Kuwahara et al., 2007). The clams depend on these symbionts for nutrition as their digestive tracts are not functional (Le Pennec et al., 1995). Furthermore, the genome of *Phreagena* symbionts lacks several genes essential for life in free-living Gram-negative bacteria (Kuwahara et al., 2007). The maintenance of such obligate host-symbiont associations is contingent on the transmission of the symbionts across host generations. In general, symbiotic microbes can be transmitted horizontally (between contemporary hosts or through reinfection by free-living forms of symbionts), vertically (directly from parent to offspring, often via gametes), or through a combination of these two transmission mechanisms (Bright and Bulgheresi, 2010). Recently, Ikuta et al. (2016) reported that the endosymbiotic bacteria of *Phreagena okutanii* are associated with the host's eggs and transmitted vertically, but they are attached extracellularly on the outer surface of the plasma membrane of spawned eggs, exclusively at the vegetal pole, forming an oval-shaped cluster. *Phreagena okutanii* is a gonochoristic species with a male or female gonad in separate adult individuals, and no evidence for hermaphroditism has been found (Ikuta et al., 2016). The symbiont resides in the gills of

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both males and females, but it is not detected in the male testis. The female ovary consists of a cluster of acini, also called follicles, where the symbionts are localized in a small part of the periphery of the oocyte and in the acinar wall cells (Ikuta et al., 2016). These observations suggest that behavior of the symbiont during the host development may be dynamic, exhibiting transmission of symbiotic bacteria among host cells. However, the processes and mechanisms of how and when the symbionts enter gill epithelial cells, enter gonadal acinar wall cells in females but not in males, and attach to the next generation of eggs, are totally unknown.

In this study, we focused on whether the symbiotic bacteria of *P. okutanii* attached on the vegetal pole of the eggs are continuously associated with the germline across host generations or whether there is cell-to-cell transmission including somatic cells. We also investigated when any transmission from cells other than germ cells may occur during host development. To address these issues, we first established a method to identify females in small *P. okutanii* individuals, and morphologically classified female germ cells observed in the ovary of the small and adult individuals. Focusing on the ovary of small individuals, in which we observed comparatively many germ cells in the early maturation stages, we investigated the association of the endosymbiotic bacteria with germ cells. Based on our findings, we discuss the processes and mechanisms of vertical transmission of the symbiont in *P. okutanii*.

MATERIALS AND METHODS

Sampling

Phreagena okutanii were collected from the Off Hatsushima Island seep sites in Sagami Bay, Japan, at depths of 855 m (Dive #1291), 856 m (Dive#1293), 912 m (Dive #1512), 857 m (Dive #1641), and 860 m (Dive #1644) during the cruises NT11-09 (15–26 June, 2011), NT13-07 (2–10 April, 2013), and NT14-05 (2–8 April, 2014) with the ROV *Hyper Dolphin* operated by the R/V *Natsushima* of the Japan Agency of Marine-Earth Science and Technology (JAMSTEC). For collected clams that were larger than approximately 80 mm in shell length, the gonad and foot were immediately excised onboard with disposable scalpels. For in situ hybridization (ISH) analysis (including sequential observation with fluorescence and electron microscopy), and hematoxylin-eosin (HE) and lectin staining, the gonad was fixed in 4% paraformaldehyde in 1 × phosphate-buffered saline (PBS) for 16 h at 4°C, followed by stepwise dehydration in an ethanol series, and stored at −30°C. For RNA extraction, the gonad was cut into small pieces and placed into RNAlater (Qiagen, Hilden, Germany), incubated for 16 h at 4°C, and stored at −80°C. For DNA extraction, the foot was frozen in liquid nitrogen and stored at −80°C. For the clams smaller than 50 mm in shell length, the shell was removed, the excised mantle was frozen in liquid nitrogen for DNA extraction, and the remaining body part was fixed in 4% paraformaldehyde as described above for histological observations. The species of clam was identified by a previously described multiplex-PCR method (Watanabe et al., 2013) using template DNA extracted from the foot or mantle with the DNeasy Blood and Tissue Kit (Qiagen).

Transcriptome analysis

Total RNA was extracted from the ovary of three adult *P. okutanii* individuals using the RNeasy mini kit (Qiagen). cDNA libraries were constructed with a TruSeq RNA Sample Preparation v2 kit following the Low-Sample protocol (Illumina, San Diego, CA, USA), and paired-end sequencing was performed with an Illumina HiSeq 2000 system, yielding 179.1 M paired-end reads (250 bp). After removing adapter and low-quality sequences, filtered sequences were then assembled into 173,494 transcripts using Trinity r2012-06-08 (Grabherr et al., 2011) with the '--min_kmer_cov 2' command option and under default settings for all other options. The length of the transcripts ranged from 201 to 33,024 bp, with an average length of 982 bp and N50 value of 1912. Raw sequence data thus obtained in this study were deposited into the DDBJ database under the accession number DRA010469.

The *vasa* and *piwi* genes of *P. okutanii* were isolated by tblastn screening of the *P. okutanii* ovary transcriptome data using the amino acid sequences of *Mytilus galloprovincialis* vasa (BAJ15435) and *Crassostrea gigas* piwi (EKC35279) as queries. cDNA sequences of *P. okutanii vasa* and *piwi* genes were submitted to DDBJ under accession numbers LC571761 and LC571762, respectively.

Histological staining

The samples fixed and stored for histological staining as described above were embedded in paraffin and cut into 8 μm thick sections. HE (Muto Pure Chemicals, Tokyo, Japan) staining was performed as described previously (Ikuta et al., 2016). For lectin staining, approximately 20 μl of Lectin PNA from *Arachis hypogaea* (peanut), Alexa Fluor 594 Conjugate (Thermo Fisher Scientific, Waltham, MA, USA) diluted 10-fold with pure water was applied to the deparaffinized section, covered with parafilm, and placed for 1 h at 4°C in a container humidified with pure water. The samples were washed twice in PBS at room temperature (RT) for 5 minutes and mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken using a BZ-9000 microscope (Keyence, Osaka, Japan) or an IX73 microscope equipped with a DP73 camera (Olympus, Tokyo, Japan). Micrographs were processed using Adobe Photoshop CS 6 (Adobe Systems, San Jose, CA, USA).

In situ hybridization

For the preparation of ISH probes, total RNA was extracted from the adult ovary using an RNeasy Mini kit (Qiagen) with on-column DNase digestion according to the manufacturer's instructions. Synthesis of cDNA was performed as described previously (Takishita et al., 2017). Specific PCR primer sets were designed for the 16S rRNA gene from the endosymbiont of *P. okutanii* (Kuwahara et al., 2007), and for the *vasa* and *piwi* genes from *P. okutanii*. The PCR primers used in this study were as follows: 16S-F, 5′-ACGGAAACGATACTAGCTTGC-3′; 16S-R, 5′-AACCCAACATCTCACGACAC-3′; vasa-F, 5′-GTAGTGGCGATGGAAAATGCT-3′; vasa-R, 5′-GATACGTCTGTGTTCGCTCC-3′; piwi-F, 5′-GGAGTTAAACGATGACAGAAATGA-3′; and piwi-R, 5′-CGAATCACTTGCATTACGAA-3′. The *16S rRNA* gene was PCR-amplified from endosymbiont genomic DNA extracted from the gill of *P. okutanii* as previously described (Kuwahara et al., 2007). The two host genes, *vasa* and *piwi*, were amplified from the ovary cDNA using Easy-A High-Fidelity PCR Cloning Enzyme (Agilent Technologies, Santa Clara, CA, USA). The PCR fragments were cloned into the pBluescript II KS+ vector (Agilent Technologies), and digoxigenin (DIG)- or fluorescein-labeled RNA probes were synthesized as described previously (Takishita et al., 2017).

ISH of 8 μm ovary sections was performed as described previously (Takishita et al., 2017; Ikuta et al., 2019). Endosymbiont 16S rRNA was detected using fluorescein tyramide (PerkinElmer, Waltham, MA, USA), and both *piwi* and *vasa* were detected using Cyanine 5 (Cy5) tyramide (PerkinElmer). When required, peanut agglutinin (PNA) lectin staining was performed as described above. The samples were mounted in Vectashield with DAPI (Vector Laboratories), and images were taken using a BZ-9000 microscope (Keyence) or an IX73 microscope equipped with a DP73 camera (Olympus). Micrographs were processed using Adobe Photoshop CS 6 (Adobe Systems). For ISH of tissue blocks, pieces of the ovary

approximately 5 × 5 × 1 mm were cut out and rehydrated in 1.5 ml sample tubes. ISH was carried out as above with the following modifications. The samples were treated with 2 μg/ml proteinase K (Takara, Shiga, Japan) in 1 × PBS with 0.1% Tween 20 (PBST) for 30 min at 37°C, then refixed with 4% paraformaldehyde in PBST at RT for 1 h, and washed with PBST. Hybridization was carried out at 62°C for 3 days. After antibody reactions, the samples were incubated in 1/100 fluorophore (Cy5 or fluorescein) tyramide (PerkinElmer) in TNT (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween 20) for 30 min, and then in 1/50 volume of fluorophore tyramide in 1× Plus Amplification Diluent (PerkinElmer) for 1 h. After washing in TNT, the samples were incubated in 10 μg/ml DAPI in PBST at RT for 1 h, washed in PBST, and then incubated in FocusClear (CelExplorer, Hsinchu, Taiwan) at RT for 1 h. Z-stack images were captured using an A1RMP confocal scanning system (Nikon Instech, Tokyo, Japan) at 1 μm intervals, and processed using the Nikon NIS-Elements software (Nikon Instech).

Sequential observation of juvenile ovary sections with fluorescence microscopy and scanning electron microscopy (SEM)

Pieces of the ovary fixed and stored as described above were embedded in Technovit 8100 resin (Heraeus Kulzer, Hanau, Germany) at 4°C. Sections (2 μm in thickness) were prepared, and ISH for endosymbiont 16S rRNA was performed as described above, but without refixation in 4% paraformaldehyde. After coloring, the sections were embedded in 0.1% *p*-phenylenediamine (Merck, Kenilworth, NJ, USA) in 50% sucrose, and observed using an Olympus IX73 microscope (Olympus) to detect fluorescent signals and bright field images. After washing in PBS, the sections were stained with 2.0% uranyl acetate solution for 10 min at RT and 2.0% lead aqueous solution for 10 min at RT. The sections were coated with osmium using a POC-3 osmium coater (Meiwafosis, Tokyo, Japan). The areas examined using fluorescence microscopy were observed using a JSM-6700F scanning electron microscope (JEOL, Tokyo, Japan) at 3 kV. Micrographs were processed with Adobe Photoshop CS 6 (Adobe Systems).

RESULTS

Sex identification of *P. okutanii*

The gonad of *P. okutanii* occupied a major portion of the visceral mass at the dorsal side of the foot (Fig. 1A). The sex of adult *P. okutanii* (> approximately 80 mm in shell length) could be predicted from the external appearance of the excised gonad. The gonads with many whitish spot-shaped acini were inferred to be female (Fig. 1B), while the gonads lacking such whitish spots were inferred to be male (Fig. 1C). Observation of HE-stained sections of these gonads revealed oocytes with the germinal vesicle in the acini of the former (Fig. 1D), and many spermatids and spermatozoa with condensed nuclei in the latter (Fig. 1E). Thus, the sex of adult clams could be determined by observations of external morphologies and sections of gonads. In contrast, the sex of some small clam individuals (< approximately 50 mm in shell length) was difficult to determine not only from the external gonad appearance but also from observation of gonad sections (Table 1). In the small clams, the acini were mostly unclear from the external appearance of the gonad. When HE-stained sections of these gonads were observed, oocytes with the germinal vesicles were visible in the acini of some remaining individuals (Fig. 1F, G), and spermatids or spermatozoa were present in others (Fig. 1H, I). However, the sex of some individuals was difficult to determine by observation of the gonad sections because neither germinal vesicles nor condensed nuclei were discernable in the cells in the acini (Fig. 1J).

Next, we performed lectin staining on the sections of these gonads. PNA lectin, which recognizes Galβ1-3GalNAc, has not only been widely used as an animal sperm (acrosome)-specific marker, but also is known to be positive in spermatogonia and spermatocytes (Arenas et al., 1998; Bakst et al., 2007; Kekalainen et al., 2015; Nakata et al., 2016). The PNA lectin signal was not detected in the gonads of adult female clams (Fig. 1K) but was detected in the gonadal acini of adult male clams (Fig. 1L), and was consistent with the morphological observations. Similarly, in the small individuals, the PNA lectin signal was not detected in those with oocytes (Fig. 1M), but was detected in those with spermatids or sperm cells (Fig. 1N). In the individuals whose sex was difficult to determine from the morphological observations described above, the PNA lectin signal was examined in the germ cells in which the ISH signals of the germ cell marker genes, *vasa* and *piwi* (Fabioux et al., 2004a; Ma et al., 2017), were detected (Fig. 1O–Q). Thus, in the present study, small *P. okutanii* individuals (< approximately 50 mm in shell length) in which germinal vesicles were observed and no PNA lectin signal was detected in the gonadal acini were identified as females. Conversely, the small individuals in which spermatids or sperm cells were observed were identified as males. The individuals whose sex was difficult to determine morphologically and that showed positive PNA lectin signals in the gonadal acini were designated as males (Table 1). However, we could not completely exclude the possibility of PNA lectin binding to immature gonads irrespective of the sex, since interaction of PNA with other types of cell has been reported in mammals. (Rose et al., 1980; Merant et al., 2005; Amemiya et al., 2008).

Morphological classification of female germ cells

The morphology of female germ cells in the ovary of the small and adult individuals of *P. okutanii* was classified into four stages according to the observations in previous studies (Pipe, 1987; Najmudeen, 2008; Parra et al., 2009). Oogonia and oocytes were discriminated based on whether a germinal vesicle with conspicuous nucleolus was observed. Oogonia (OG) could be identified as cells having the following characteristics. The cell shape was generally round with thin cytoplasm expressing the germ cell markers (Fig. 1R, S). The nucleus was approximately 10 μm in diameter, DAPI staining was very clear compared with the surrounding cell nuclei, and nucleoli were not observed or not conspicuous (Fig. 1R, S). Oocytes could be classified into the following three stages. (1) Primary oocyte (POC): The cell shape was roughly polygonal, with cytoplasm expressing the germ cell markers (Fig. 1R–U). The nucleus was enlarged to approximately 20 μm diameter forming the germinal vesicle, and the nucleolus was large and conspicuous (Fig. 1R, S). DAPI staining showed faint or spotty signals in the nucleus (Fig. 1R, S). In the adult ovary, POCs were often distributed in the epithelial acinar wall (Fig. 1T, U). (2) Previtellogenic oocyte (PVO): The cell shape was roughly oval, with increased cytoplasm, enlarged nucleus, and often a nucleolus (Fig. 1T, U). DAPI staining was barely visible (Fig. 1T, U). In the adult ovary, PVOs abutted the basement membrane of the acinar wall (Fig. 1T, U). (3) Vitellogenic oocyte (VOC): Very few structures were observed in the nucleus,

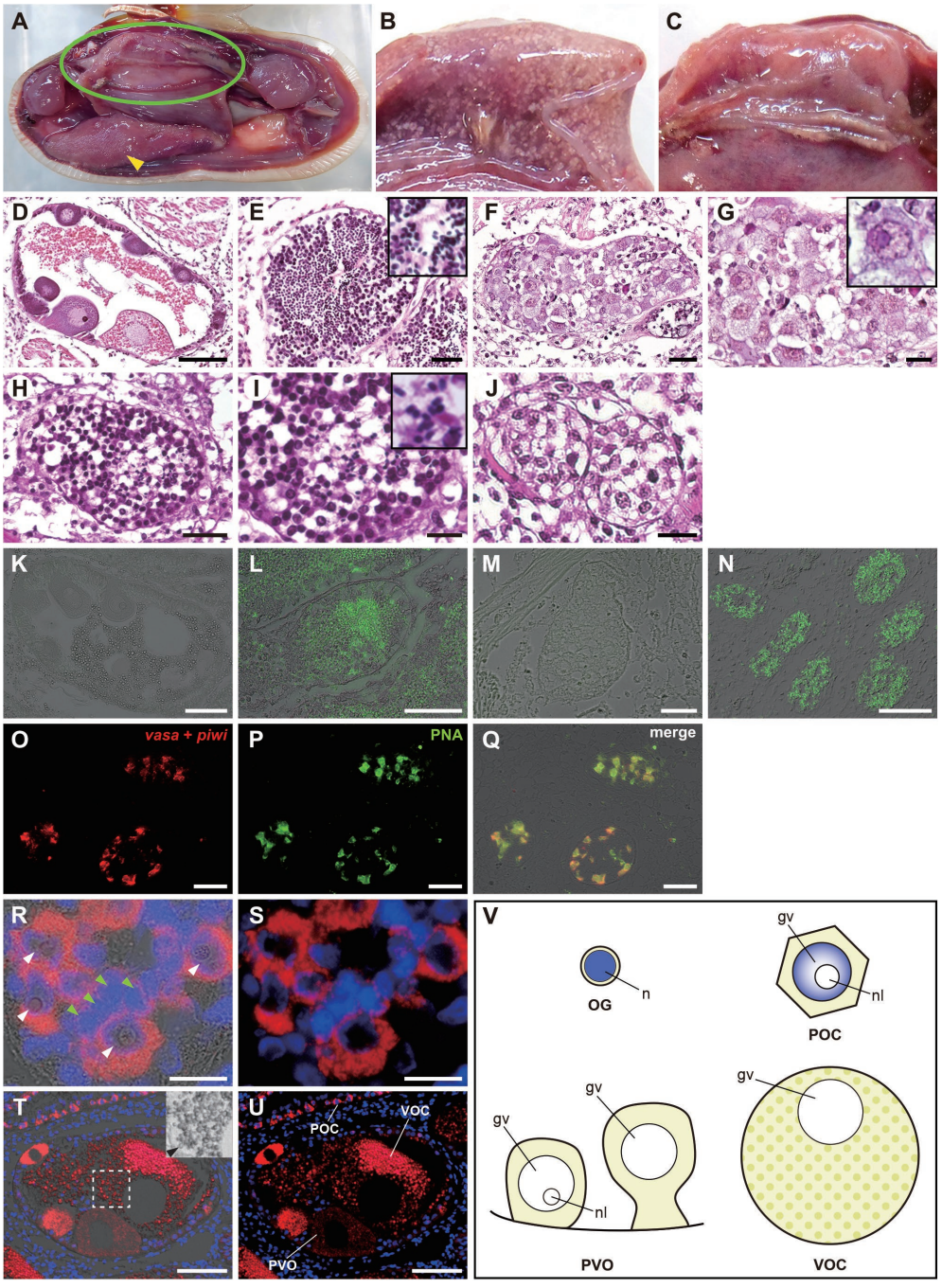

Fig. 1. Sex identification and morphological classification of female germ cells in *Phreagena okutanii.* **(A–C)** Sex prediction from the external gonad appearance: **(A)** the clam with the gills removed; **(B)** female gonad; **(C)** male gonad. The green oval in **(A)** indicates the location of the gonad, and the yellow arrowhead indicates the foot. Dorsal is at the top. **(D–J)** HE-stained gonad sections: **(D)** adult female; **(E)** adult male; **(F, G)** small individual in which oocytes with a germinal vesicle were observed; **(H, I)** small individual in which spermatids or sperm cells were observed; **(J)** individual for which sex determination from observation of the gonad sections was difficult. **(G)** and **(I)** show higher magnification of **(F)** and **(H)**, respectively. Insets in **(E)** and **(I)** show higher magnification of spermatids or sperm cells, and inset in **(G)** shows higher magnification of an oocyte with a germinal vesicle. **(K–Q)** Gonadal sections stained with PNA lectin (pseudo green): **(K)** adult female; **(L)** adult male; **(M)** small individual in which oocytes with a germinal vesicle were observed; **(N)** small individual in which spermatids or sperm cells were observed; **(O–Q)** individual for which sex determination from observation of the gonad sections was difficult, in which fluorescent ISH signals for germ cell markers (*vasa* and *piwi*, pseudo red) are also shown. **(R–U)** Female germ cells in the acinus of a juvenile **(R, S)** and an adult **(T, U)**. In **(R)** and **(T)**, fluorescent ISH signals (*vasa* and *piwi*, pseudo red) were merged with DAPI signals (blue) and DIC images, which are omitted in **(S)** and **(U)**. Green and white arrowheads indicate nuclei of oogonia and germinal vesicles of primary oocytes, respectively. Inset in **(T)** shows higher magnification of DIC image of the area indicated with dotted line. Black arrowhead indicates the globular structure in the VOC cytoplasm. **(V)** Schematic drawings of morphological characteristics and classification of *P. okutanii* female germ cells. OG, oogonium; POC, primary oocyte; PVO, previtellogenic oocyte; VOC, vitellogenic oocyte. n, nucleus; nl, nucleolus; gv, germinal vesicle. Scale bars represent 100 μm in **(D, K, N, T, U)**; 40 μm in **(E, F, H, L, M, O, P, Q)**; 20 μm in **(G, I, J, R, S)**.

Table 1. *Phreagena okutanii* small (< 50 mm in shell length) individuals used in this study. The 'morphology' column indicates whether the clam sex could (Y) or could not (N) be determined from the external appearance of the gonad or from observation of the gonad sections. The 'PNA lectin' column indicates whether the PNA lectin signal was detected (+) or not detected (–) in the gonadal acini. The 'symbiont' column indicates whether the symbiont ISH signal was detected (+) or not detected (–) in the gonadal acini.

individual No.	shell length (mm)	morphology	PNA lectin	sex	symbiont	Figure
1	15.33	N	+	♂*	–	1J, 2D
2	16.88	Y	–	♀	+	1M, R, S; 2A
3	18.17	N	+	♂*	–	1O-Q
4	20.16	N	+	♂*	–	
5	20.52	Y	–	♀	+	1F, G; 2B
6	27.72	N	+	♂*	–	
7	28.70	N	+	♂*	–	
8	30.06	N	+	♂*	–	
9	33.07	N	+	♂*	–	
10	33.15	Y	–	♀	+	2C, F
11	35.80	Y	+	♂	–	1H, I, N; 2E
12	36.62	Y	+	♂	–	
13	42.84	Y	–	♀	+	
14	43.27	Y	–	♀	+	
15	46.03	Y	–	♀	+	2G-L
16	49.98	Y	+	♂	–	

* Designated from the PNA lectin staining

and the DAPI signal was very unclear (Fig. 1T, U). Many globular structures likely to be egg yolk or oil globules (Fig. 1T, U) were observed in the cytoplasm. VOCs were often localized within the acinus lumen (Fig. 1T, U). VOCs were observed in the adult clam ovaries, but not in those of the small individuals with shell length less than 50 mm. Therefore, in the present study, the small females (< 50 mm in shell length) were designated as juveniles. These morphological characteristics and classification of *P. okutanii* female germ cells are summarized in Fig. 1V.

Association of the endosymbiotic bacteria with ovarian germ cells

Multicolor fluorescent ISH was performed on paraffin sections of the gonads of the small individuals, using a mixed probe for symbiont 16S rRNA (green), and the *vasa* and *piwi* (red) genes. Green signals of the symbiotic bacteria were observed in the ovaries of all six female juveniles, while no symbiont signal was detected in the testes of any of 10 small male individuals (Table 1). The coronal section of the ovarian acini was approximately round or oval along the anterior-posterior axis on the right and left sides of the ovary. Most of the symbiont signals were observed in the cells without *vasa* and *piwi* signals distributed along the periphery of the acini (Fig. 2A), indicating that the symbionts reside in acinar wall cells. The spotty signals of symbiont 16S rRNA were also detected at the periphery of POCs (Fig. 2A–C). OG formed cell clusters in the juvenile acini, and no 16S rRNA signal was observed in these cell clusters (Fig. 2A, B). Additionally, no 16S rRNA signal was observed in the male gonads (Fig. 2D, E). To confirm the symbiont localization pattern in the ovary observed in sections, we conducted two-color ISH assays on the tissue blocks and observed them three-dimensionally using a confocal scanning system. Symbiont 16S rRNA signals were observed in acinar wall cells and at the periphery of POCs, whereas no 16S rRNA signal was detected in the clusters of OG (Fig. 2F). These results were consistent with those obtained from the sections.

Ikuta et al. (2016) reported that the endosymbiotic bacteria of *P. okutanii* are attached extracellularly on the outer plasma membrane surface of spawned eggs. To investigate whether the symbionts are also attached extracellularly on the outer surface of POCs in the juvenile ovaries, we performed sequential observations of resin sections of the juvenile ovaries with fluorescence microscopy and SEM. ISH signals for the symbiont 16S rRNA were detected in a very small part of the periphery of POCs (Fig. 2G, H). SEM of the same areas of the sections revealed bacteria-like structures on the outer surface of POCs in the same location as the symbiont 16S rRNA ISH signals (Fig. 2I–L). These results indicate that the *P. okutanii* symbiotic bacteria are attached extracellularly on the outer surface of POCs in the juvenile ovaries. Despite extensive observation, we did not find any symbionts inside POCs or associating with OG.

DISCUSSION

In this study, we investigated the association of symbiotic bacteria with ovarian germ cells in juvenile *P. okutanii*. We found that the symbiotic bacteria were not distributed in the cluster of oogonia, but were localized on the outer surface of the cell membrane of primary oocytes. Ikuta et al. (2016) reported that the *P. okutanii* symbiont was attached on the outer surface of the vegetal pole of spawned eggs. Based on observations in cell lineages in some mollusk species (Fabioux et al., 2004b; Wanninger and Wollesen, 2015), it was expected that a population of the *P. okutanii* symbiont on the egg vegetal pole would be directly integrated into the germline (Ikuta et al., 2016). However, our results demonstrated that the symbiont does not have a continuous association with the germline throughout development, because it was not associated with oogonia. Instead, the symbionts on eggs come from elsewhere via cell-to-cell transfer. In some insects, symbiotic microorganisms leave bacteriocytes in a symbiotic organ, the bacteriome, and migrate into the ovaries via the hemolymph (Buchner, 1965; Dasch et al., 1984; Koga et al., 2012). In *Phreagena* clams, the symbiotic bacteria have never been detected in the hemolymph, and in this study, they were not detected by ISH analysis in the extracellular space around the ovarian acini of the *P. okutanii* juveniles. Therefore, it is unlikely that the symbiotic bacteria are transported from the gill to the ovary via blood flow. In aphid sexual reproduction, the obligate endosymbiont *Buchnera* is delivered to the posterior pole of the oocyte in

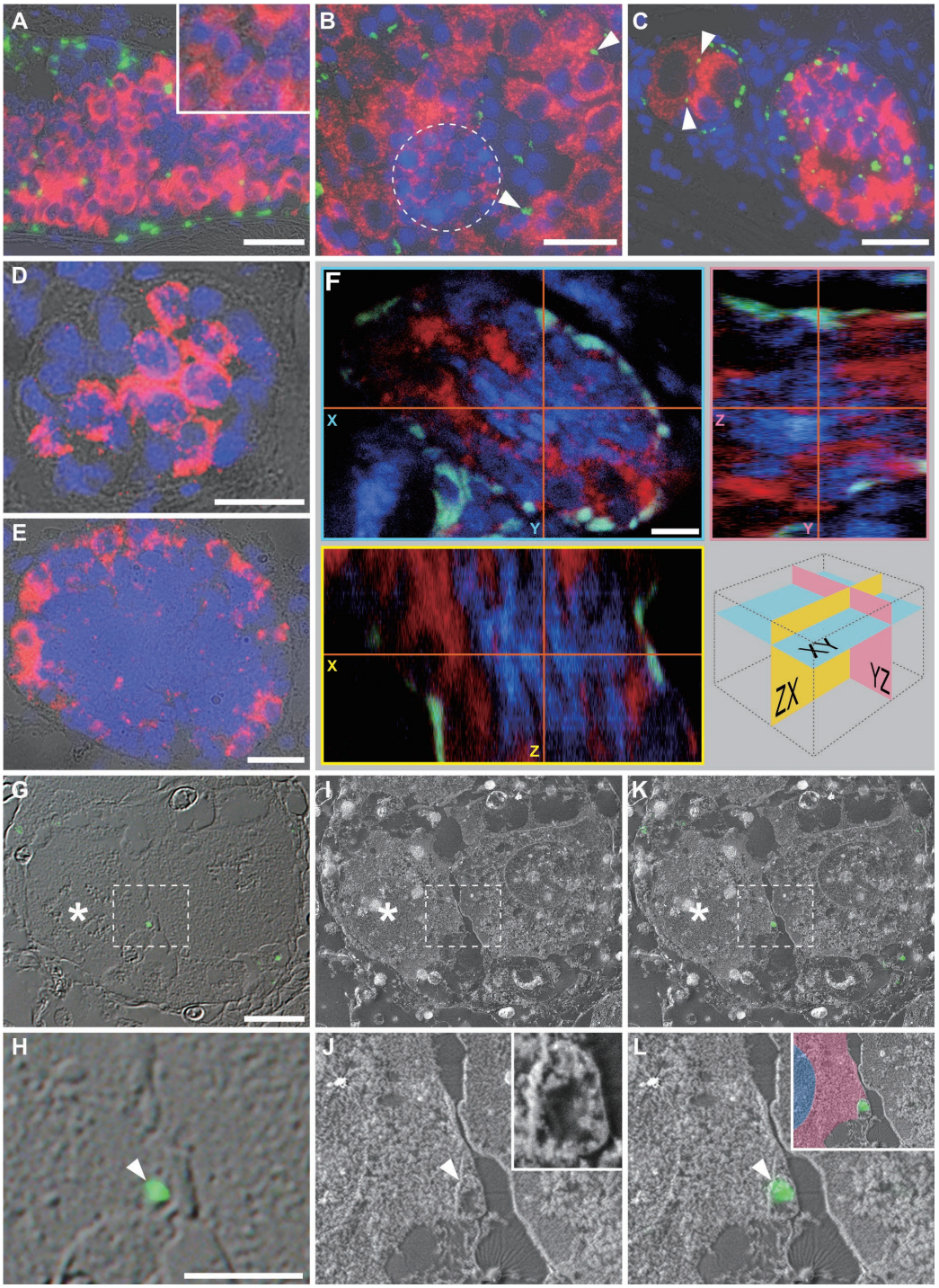

Fig. 2. Association of endosymbiotic bacteria with gonadal germ cells in *Phreagena okutanii*. **(A–E)** Fluorescent ISH signals (green for the symbiont and pseudo red for *vasa* and *piwi*) were merged with DAPI signals (blue) and DIC images: **(A–C)** juvenile females **(D, E)** small males. The inset in **(A)** shows a higher magnification image of oogonia. The dotted circle in **(B)** indicates the cluster of oogonia. Arrowheads indicate symbiont cells associated with primary oocytes. **(F)** Representative 3D reconstruction from confocal z-stack view of fluorescent ISH and DAPI signals in the juvenile ovary. The reconstructed slices are shown in the upper right and lower left frames, and a diagram representing the orientation of the slices is shown in the lower right frame. **(G–L)** Sequential observations of juvenile ovary sections with fluorescence microscopy and SEM. **(H)**, **(J)**, and **(L)** show higher magnification images of the areas indicated by dotted squares in **(G)**, **(I)**, and **(K)**, respectively. **(G, H)** Fluorescent ISH signals (green for the symbiont) were merged with DIC images. **(I)** and **(J)** show SEM images corresponding to the areas examined in **(G)** and **(H)**, respectively. The inset in **(J)** shows a high magnification image of the symbiont attached on the outer surface of the egg plasma membrane of a primary oocyte. **(K, L)** Fluorescent ISH signal (green for the symbiont) merged with SEM image. In the inset in **(L)**, the nucleus and cytoplasm of the primary oocyte are shown in blue and pink, respectively. Asterisks and arrowheads indicate the germinal vesicle and symbiont cell, respectively. Scale bars represent 40 μm in **(A, B, C)**; 20 μm in **(D, E, G)**; 10 μm in **(F, H)**.

the final stages of oogenesis via cell-to-cell transfer from follicle cells (Miura et al., 2003). In the present study, the symbionts were detected in acinar wall cells, which was consistent with the results of Ikuta et al. (2016) in the adult *P. okutanii* ovary. Therefore, the *P. okutanii* symbiont may be transported from acinar cells to the oocyte during oogenesis.

In many insects, symbiotic transmission occurs during the late yolk accumulation stage of oogenesis, although in some groups of insects, association of the symbionts with undifferentiated germ cells or early previtellogenic oocytes has been observed (Buchner, 1965; Szklarzewicz and Michalik, 2017; Russell et al., 2019). In addition, although not a symbiotic bacterial system, some scleractinian corals (genus *Montipora*) have been reported to transmit zooxanthellae (genus *Symbiodinium*) several days before spawning (Hirose et al., 2001). In this study, we have shown that the *P. okutanii* symbionts are transmitted to the early oocytes. In bivalves, oocytes are generally enclosed by a polysaccharide membrane called the vitelline membrane late in oogenesis or immediately before egg release, but this membrane is not observed around oocytes early in oogenesis (Eckelbarger and Davis, 1996; Chung, 2007). In *P. okutanii*, the symbiont is directly attached on the cell membrane of the oocyte under the vitelline membrane (Ikuta et al., 2016), which suggests that the symbiont may be transmitted to the early oocyte before the vitelline membrane is formed. Furthermore, in the present study, the symbiotic bacteria were not detected in the cluster of oogonia that can proliferate and undergo somatic cell division. If the symbiont was transmitted to an oogonium, it would need either to be transmitted from an external source each time the oogonium divides, or to proliferate along with the oogonium during each division and be distributed to each daughter oogonium. The easiest and most secure possibility might be the transmission of the symbiont to the oocytes that are not undergoing somatic cell division. Ikuta et al. (2016) reported that an individual egg of *P. okutanii* carries on average 400 symbiont cells. In contrast, in this study a much smaller number of bacteria were observed on the oocyte surface in the juvenile ovary (Fig. 2G–L). At this time, it is not clear whether the symbionts proliferate on the oocyte or are continuously supplied to the oocyte throughout the oocyte maturation. This issue must be studied in the future.

Our results demonstrated that the *P. okutanii* symbiotic bacteria are extracellularly attached not only on the outer surface of spawned eggs as reported previously (Ikuta et al., 2016), but also on that of early oocytes in the juvenile ovary. It is possible that the host maternal immune system prevents the symbiont from entering the host cells during these stages. Mollusk eggs contain several innate immune factors that are transferred from the parent during oogenesis to endow the eggs with effective defense ability against pathogen infection (Wang et al., 2015). However, the contribution of maternal gene products ceases at a certain stage of development when zygotic transcription is initiated. This phenomenon is termed the maternal-to-zygotic transition (MZT) (Vastenhouw et al., 2019). In hydra, the composition of antimicrobial peptides changes before and after the MZT, and the bacterial population size and composition also change (Fraune et al., 2010, 2011). In *P. okutanii*, symbiotic bacteria might be localized outside the egg cells during the oogenesis stage to avoid being eliminated by the maternal immune factors, and they might later migrate into the host cells after the MZT, which is generally around the mid-blastula stage. In addition, there might be an unknown mechanism for the integration of symbiont cells intracellularly into bacteriocytes, symbiotic organs, and germ lines during host development. Improvements in stable rearing methods for *Phreagena* clams in the aquarium (Ikuta et al., 2018) may facilitate further embryological studies to test these hypotheses.

In summary, we have shown that the bacterial endosymbiont of *P. okutanii* is not associated with oogonia but is transmitted on the extracellular surface of early oocytes in the juvenile ovary. This provides insights into the dynamic movements of the symbiont exhibiting cell-to-cell transmission during the host development. The symbionts located outside the vegetal pole of the eggs will be divided into two populations; some symbionts may enter gill epithelial cells, and others enter acinar wall cells exclusively in females, although the developmental origin of gill and acinar cells in *P. okutanii* is unknown. Then, after the female germ cells have begun meiosis, the symbionts may disperse from the acinar wall cells and attach on the outer surface of the oocytes. Extensive analysis of the localization of the symbiont during the host development will be required to test this hypothesis and improve our understanding of these processes and mechanisms.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

KI-U performed the experiments and analyzed data. TI conceived and headed the study, and wrote the manuscript with input from all coauthors. AT contributed to SEM observation. KY, SS, YH, YT, TY contributed to transcriptome analysis. KF, TM, YT conceived and designed the study. All authors have read and approved the manuscript.

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