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Microdistribution of two endosymbionts in gill tissue from a hadal thyasirid clam *Maorithyas hadalis*

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Introduction

A thyasirid clam, *Maorithyas hadalis* Okutani, Fujikura & Kojima, 1999, is a conspicuous species at the deepest yet known chemosynthetic environment (7326 m depth, in the Japan Trench; Fujikura et al., 1999). TEM observations confirmed that the symbiosis in *M. hadalis* was intracellular unlike the extracellular symbiosis in other thyasirid clams (Fujikura et al., 1999, Fujiwara et al., 2001). Two distinct phylotypes of endosymbiotic bacteria were discovered within the gill tissues by molecular phylogenetic analysis and in situ hybridization (Fujiwara et al., 2001). Symbiont I was affiliated with thioautotrophic symbionts of vesicomyid clams and deep-sea mussels from hydrothermal vents and cold seeps. Symbiont II was not related to previously reported-bacterial symbionts, and was distantly related to the free-living chemoautotrophic bacteria (genera *Thiomicrospira* and *Hydrogenovibrio*). In situ hybridization experiments indicated spatial partitioning between the two *M. hadalis* symbionts with symbiont I occurring mainly in the outer regions of bacteriocyte zones and symbiont II situated predominantly within the inner regions of bacteriocyte zones (Fujiwara et al., 2001).

In order to elucidate the microdistribution patterns of two types of endosymbiotic bacteria, we tested an in situ hybridization technique on the TEM sections of the gill tissues of *M. hadalis*. This preliminary result, along with a discussion of the technical problems, is presented here.

Material and methods

Specimen collection
Maorithyas hadalis specimens were collected from the Japan Trench during ROV *Kaiko* dives in 2000 using a sample rake and placed into a thermally insulated watertight sampling box. Upon recovery, the clams were immediately transferred to fresh, chilled (~4 °C) seawater.

In situ hybridization on transmission electron microscopic sections
Two ribosomal RNA-targeted oligonucleotide probes were designed for detection of two types of bacteria as described by Fujiwara et al. (2001). Probe I (5'-TCGCCACTAAGAGGTAAATCCT-3') was used for detection of bacterial 16S ribosomal RNA genes (16S rDNA) of "Symbiont I." Probe II (5'-TCGACACTAAAATCTCTAAGGAT-3') was used for detection of bacterial 16S rDNA of "Symbiont II." Both probes corresponded to positions 839-859 in *Escherichia coli* 16S rDNA. EUB338 (Aman et al., 1990) was also used to label members of the domain Bacteria as a positive control.

For in situ hybridization, small pieces of gill tissue were fixed with 0.5% glutaraldehyde and 1.5% formaldehyde in filtered seawater at 4 °C for 12 hours. Blocks were rinsed 3 times with filtered and sterilized artificial seawater for 10 minutes at room temperature. Then, tissues were dehydrated and embedded in the LR White Resin (London Resin Company, Reading, UK). The hybridization was conducted on the sequential ultra-thin sections using 3 different probes,

probe I, probe II and EUB338 (10 μg ml^{-1}) with tRNA (0.1 mg ml^{-1}) extracted from yeast in 5x SSC for 15 hours. All three probes were labeled at the 5' -end with digoxigenin (DIG) and purified by HPLC (Amersham Pharmacia Biotech). The hybridization temperature was 15 °C lower than the melting temperature of each probe. The sections were rinsed 2 times with PBST (0.1% Tween 20 in PBS) for 5 minutes, following the rinsing 3 times with 2x SSC for 5 minutes at room temperature. Blocking was performed with PBST and BG (1% bovine serum albumin and 0.1% gelatin from cold water fish) for 15 minutes at room temperature. Anti-digoxigenin-gold (4 μg ml^{-1} in PBST+BG, Roche, Mannheim, Germany) antibodies were applied for one hour at room temperature. After rinsing 3 times with PBST for 5 minutes at room temperature, silver deposition on colloidal gold particles was conducted for clear visualization, for 10 minutes at room temperature. The sections were again rinsed 6 times with deionized sterile water for 5 minutes at room temperature, then, stained with uranyl acetate and Reynolds lead. Finally, the sections were coated with carbon and were observed with a JEOL JEM-1210 transmission electron microscope at an accelerating voltage of 120 kV.

Results

In situ hybridization was conducted on the sequential TEM sections of gill tissue of *M. hadalis* using 3 different probes (probe I, probe II, and EUB338). All 3 probes hybridized with the sections of gill tissue. The hybridization reactions of the 3 probes were localized within the bacteriocytes in the ctenidial epithelium. Each probe hybridized to gram-negative bacterial symbiont nucleic acids in the bacteriocytes. The hybridization reactions were primarily limited to the ribosomal regions (Fig. 1). No morphological differences between the bacteria hybridized by probe I and probe II were observed (Fig. 1). The hybridization patterns of probe I and probe II were different (data not shown). The hybridization reaction of probe I was concentrated in epithelial cells close to the surface of the gill tissue (Fig. 2). The intensity of the reaction gradually decreased towards the proximal region. The hybridization reaction of probe II was concentrated in epithelial cells at the center (far from the surface) of the gill tissue. The hybridization reaction of EUB338 was observed in most epithelial cells of the gill filaments (data not shown).

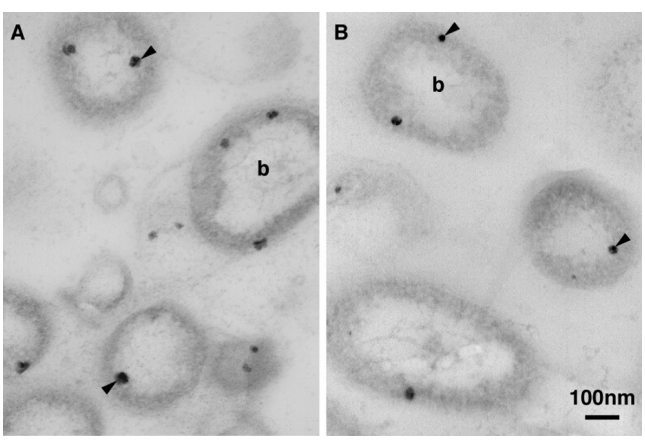

Figure 1. *Maorithyas hadalis*. Transmission electron micrographs of intracellular gram-negative bacterial symbionts in gill tissue. In situ hybridization with probe I in the gill outer region (A) and probe II in the gill inner region (B) are shown in transverse sections of the gill filaments. Arrowheads indicate the hybridization reactions. (*b*) bacteria. Scale bar: 100 nm.

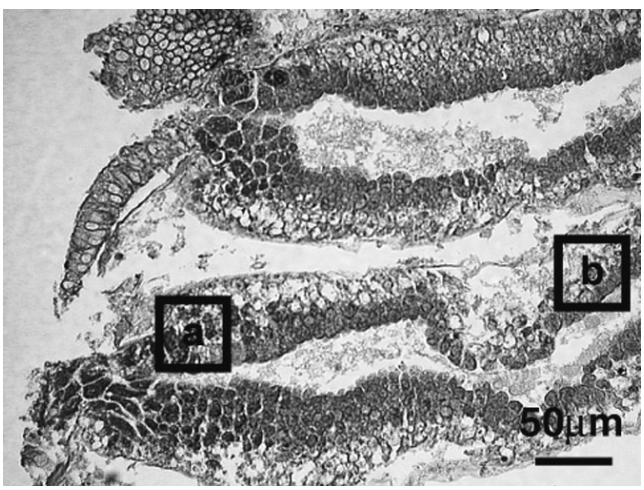

Figure 2. *Maorithyas hadalis*. Photomicrographs of transverse sections of gill filaments (Haematoxylin-eosin staining). The square frames (a) and (b) indicate areas where probes I and II hybridized, respectively. Scale bar: 50 μm.

Discussion

In situ hybridization experiment on the TEM sections confirmed that the hybridization signal was clearly located in the bacterial symbionts. In previous studies, it had been very difficult to directly demonstrate that the putative symbiont-specific sequences were derived from the endosymbiotic bacteria because of the low resolution of in situ hybridization using a light microscope. In situ hybridization using TEM can be an effective solution to this problem. Most hybridization reactions were located within the ribosomal regions instead of the nucleoids, indicating that the symbiont-specific probes targeted not only the genomic DNA but also the ribosomal RNA.

Symbiont I was characterized as a thioautotroph on the basis of its phylogenetic placement, morphological features and habitat (Fujiwara et al., 2001). However, the physiological nature of symbiont II remains unknown, although the branches neighboring symbiont II consisted of members of the free-living chemoautotrophic bacteria (genera *Thiomicrospira* and *Hydrogenovibrio*). However, it was too distantly related to conclusively infer its metabolism from its phylogeny (Fujiwara et al., 2001). Although the phylogenetic positions of symbiont I and

symbiont II were quite different (Fujiwara et al., 2001), no morphological differences were observed between them.

Differences in the population densities were observed between the two symbionts. Symbiont I occurred mainly in the outer regions of gill tissue and symbiont II was situated predominantly within the inner regions. However, the spatial partitioning of the two symbionts was not as clear in this study as in the previous in situ studies using light microscopy, because the hybridization patterns of the two probes, although exhibiting a gradient in intensity, overlapped throughout much of the range. Since a single probe per section was used for the hybridization reaction, it was difficult to align and compare the hybridization patterns. Therefore, an in situ double hybridization experiment using the two symbiont-specific probes simultaneously is expected to clarify the microdistribution of the two symbionts in *M. hadalis*.

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