

**Department of Environmental Dynamics and Management**

**Phylogenetic characterization and *in situ* localization of endosymbiotic microflora of the Pogonophoran tubeworms**

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Pogonophoras are marine invertebrates containing vestimentiferan and pogonophoran tubeworms. The worms have a unique strategy of life, in which they lack mouth, gut and anus, but harbor chemoautotrophic endosymbiotic bacteria in the body tissue. The worms are completely dependent on endosymbiotic chemosynthesis for nutrition.

The vestimentiferan tubeworms has discovered at bathyal seafloor of hydrothermal vents and methane-seeps. The vestimentiferan endosymbionts, sulfur-oxidizing bacteria, inhabiting sulfide-rich hydrothermal vents are monospecific for a host. In contrast, previous studies suggest that vestimentiferans of methane-seeps may host multispecific symbionts. Bacterial 16S rRNA genes (16S rDNA) were extracted and PCR-amplified from the trophosome of the vestimentiferans, *Lamellibrachia* sp. in methane-seep. Phylogenetic analysis of 16S rDNA and dot-blot hybridization detected the occurrence of 4 operational taxonomic units (OTUs) in the trophosome of the vestimentiferan tubeworm. The OTUs were closely related to 16S rDNA of the species belonging to  $\alpha$ -Proteobacteria (*Sulfitobacter*),  $\beta$ -Proteobacteria (*Junthinobacterium*), and  $\gamma$ -Proteobacteria (*Acinetobacter* and *Pseudomonas*). Localizations of 4 OTUs within the trophosome were confirmed by *in situ* hybridization (ISH). ISI signals of the  $\alpha$ -Proteobacterial OTU were observed in the innermost zone of the trophosome lobules. In contrast, ISH signals of the  $\beta$ - and  $\gamma$ -Proteobacterial OTUs were observed at the periphery of the lobules; however, it remains unclear whether they occur inside or outside of the lobules. These results support the possibility that the studied methane-seep tubeworm has a microflora composed of multispecific endosymbionts. These results support the possibilities that the methane-seep vestimentiferans have a microflora composed of multispecific endosymbionts.

Gutless pogonophoran tubeworms are generally thought to live in symbiosis with methane-oxidizing bacteria (methanotrophs). This study identified a 16S ribosomal RNA gene (16S rDNA) and a ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, E.C.4.1.1.39) gene that encode the form I large subunit (*cbbL*) from symbiont-bearing tissue of the pogonophoran tubeworms collected from shallow seafloor in Tsukumo Bay (20-25 m), Japan Sea, and deep seafloor in Japan Trench (7320-7330 m), northeastern Pacific.

Phylogenetic analysis of the 16S rDNA sequence suggested that the pogonophoran endosymbiont of shallow seafloor belonged to the  $\gamma$ -Proteobacteria. The endosymbiont was most closely related to an uncultured bacterium from a methane-seep, forming a unique clade adjacent to the known methanotrophic 16S rDNA cluster. The RuBisCO gene from the pogonophoran

tissue was closely related to those of the chemoautotrophic genera *Thiobacillus* and *Hydrogenovibrio*. Presence of RuBisCO gene does not rule out a methanotrophic symbiosis because some methanotrophic bacteria are known to be capable of autotrophy via the Calvin cycle. Oppositely, particulate and soluble methane monooxygenase genes (*pmoA* and *mmoX*) and the methanol dehydrogenase gene (*mxoF*), which are indicators for methanotrophs or methylotrophs, were not detected by repeated trial of PCR. For 16S rRNA and RuBisCO genes, endosymbiotic localizations were confirmed by *in situ* hybridization. These results support the possibilities that the pogonophoran host has a novel endosymbiont which belongs to the  $\gamma$ -*Proteobacteria*, and that the endosymbiont has the gene of the autotrophic enzyme RuBisCO.

In contrast, the endosymbiont of an abyssal pogonophorans was shown to belong to the  $\gamma$ -*Proteobacteria* showing the highest matching to a sulfur-oxidizing free-living bacterium, followed by gill symbionts of mussels and endosymbionts of vestimentiferan tubeworms by the phylogenetic analysis of the 16S rDNA sequence. The RuBisCO gene encoding the form I large subunit (*cbbL*) was closely related to those of the chemoautotrophic genera *Thiobacillus* and *Hydrogenovibrio*, and those of the chemoautotrophic endosymbiont of the pogonophorans from shallow seafloor. The RuBisCO gene encoding the form II large subunit (*cbbM*), which is autotrophic enzyme to adaptive to high CO<sub>2</sub> and anaerobic condition, was not detected. Similarly, the methanotrophic gene markers such as particulate and soluble methane monooxygenase genes (*pmoA* and *mmoX*) and methanol dehydrogenase gene (*moxF*) were not detected by repeated trial of PCR. These results support that the possibilities that the symbiont of a deep-sea pogonophorans has common ancestor with sulfur-oxidizing symbionts of bivalves and tubeworms, and that the pogonophorans harbor chemoautotrophic symbiont that has the gene of the autotrophic RuBisCO form I enzyme to adaptive to high O<sub>2</sub> and aerobic condition.

**Key words:** vestimentiferan tubeworm, pogonophoran tubeworm, endosymbiont, 16S rRNA gene, RuBisCO gene, methane monooxygenase genes