Microbial ecology of endobacteria harbored in Oligobrachia mashikoi

氏　　名	會田　将人
学位の種類	博士（理学）
学位記番号	博甲第１０１７号
学位授与の日付	平成20年3月22日
学位授与の要件	課程博士(学位規則第４条第１項)
学位授与の題目	Microbial ecology of endobacteria harbored in Oligobrachia mashikoi (Oligobrachia mashikoi が細胞内に保持する細菌に関する微生物生態学的研究)
論文審査委員（主査）	福森　義宏（自然科学研究科・教授）
論文審査委員（副査）	笹山　雄一（環日本海域環境研究センター・教授），東　浩（自然科学研究科・准教授），山田　敏弘（自然科学研究科・講師），金森　正明（自然科学研究科・講師）

Abstract

Oligobrachia mashikoi (Siboglinidae, Annelida) is a marine invertebrate inhabiting the reducing sediments of Tsukumo Bay, Japan. The worm lacks a mouth and a digestive tract, and harbors endosymbiotic bacteria in specialized cells called bacteriocytes. In this study, to gain further insight into the nature of the endosymbionts, I investigated the distribution and population of free-living cells related to the major *O. mashikoi* endosymbiont (endosymbiont A) in Tsukumo Bay, and found that the endosymbiont A-related phylotype exists in sediment over the entire this bay with low population density. Furthermore, to elucidate energy metabolism of the *O. mashikoi* endosymbionts, I investigated the morphological features of the *O. mashikoi* endosymbionts using TEM, and compared the sequences of the genes involved in CO_2 fixation (*cbbL*) and sulfur oxidation (*aprA* and *soxB*). These investigations provide the evidences indicating that the *O. mashikoi* endosymbionts are thioautotrophic bacteria.

Introduction

Siboglinid polychaetes are marine invertebrates which lack a mouth and a digestive tract, and generally harbor thioautotrophic or methanotrophic bacteria in special cells called bacteriocytes. The hosts have been thought to rely on their endosymbionts for nutrition [1]. The members of the family Siboglinidae have been split into three lineages, frenulates (beard worms), vestimentiferans (tube worms) and moniliferans [2]. Furthermore, it is generally accepted that siboglinid polychaetes take in the free-living cells of their endosymbionts from the environment in each generation [3]. However, detailed mechanisms of the acquisition of bacterial endosymbionts have not been elucidated due to the difficulties in the isolation and cultivation of both bacterial endosymbionts and their host.

In 1973, one species of frenulates was identified as new species called *Oligobrachia mashikoi* which inhabits the muddy bottom of Tsukumo Bay on Noto Peninsula, Japan [4]. The adult worm lacks a mouth and a digestive tract, and harbors bacterial cells in its bacteriocytes, like other siboglinid polychaetes. Although Kubota *et al.* classified the endosymbionts into seven types (endosymbiont A–G) on the basis of the nucleotide sequences of the 16S rRNA gene, one adult worm of *O. mashikoi* harbors just one of the seven types [5]. However, the analyses based on the 16S rRNA gene could not clarify whether the *O. mashikoi* endosymbionts are thioautotrophic or methanotrophic bacteria.

In this study, to gain further insight into the nature of the endosymbionts, I investigated the distribution and population of free-living cells related to the major endosymbiont (endosymbiont A) in Tsukumo Bay. This is the first study on the ecological characterization of a free-living bacterium related to the endosymbiont of the siboglinid polychaete. Furthermore, to elucidate energy metabolism of the *O. mashikoi* endosymbionts, I investigated the morphological features of the *O. mashikoi*

endosymbionts using TEM, and compared the sequences of the genes involved in CO_2 fixation (*cbbL*) and sulfur oxidation (*aprA* and *soxB*). This study provides the evidences indicating that the *O. mashikoi* endosymbionts are thioautotrophic bacteria.

Materials and methods

Collection of sediments and DNA extraction from sediments

The sediment samples were collected from the surface layer (0–5 cm deep) of the bottom at 44 sampling stations in Tsukumo Bay using a grab-type dredge (Fig. 1A). DNA was extracted from the sediments (0.85 gram wet-weight each) using the UltraClean Soil DNA kit (MO BIO).

Quantitative PCR of the O. mashikoi *endosymbiont A-related phylotype in sediments*

Specific primers (M450f and M860r) were designed on the basis of the 16S rRNA gene sequence of endosymbiont A. To examine the specificity of the primers, the products of PCR using the primers and the DNA extracts from sediments were cloned into pSTBlue-1 AccepTor vector (Novagen), and resulting plasmids were transformed into the *Escherichia coli* XL-1 Blue strain. Inserted DNA fragments were sequenced. The ratio of the number of clones derived from the endosymbiont A-related phylotype to the total number of clones was determined. To quantitatively detect the endosymbiont A-related phylotype, a control DNA (486bp fragment) was constructed, and used in quantitative PCR. To determine the efficiency of extraction of DNA from sediment samples, I designed the experiment using *E. coli* cells, and found that the DNA extraction efficiency was nearly 100%. To estimate the eubacterial cell number in one gram of dry-sediment from the bay, quantitative PCR using eubacterial universal primers (B341f and U533r) and a control DNA (333bp fragment) were carried out [6].

Collection of O. mashikoi, *preparation for TEM, and DNA extraction from the worm*

The adult worms of *O. mashikoi* were collected with a dredge from the muddy bottom (25 m deep) of Tsukumo Bay. The bodies of *O. mashikoi* were carefully taken out of the chitin tubes and washed with autoclaved seawater repeatedly and finally with 70% ethanol. The post-annular part containing the bacteriocytes was aseptically cut off from the body. For observations using TEM, the post-annluar part was immediately fixed, dehydrated, and embedded in Quetol-651 epoxy resin (Nisshin-EM). The ultra-thin sections (40–70 nm) were prepared using a diamond knife and an ultracut-microtome (Leica), stained with uranyl acetate and lead citrate, and viewed with JEOL 2000EX TEM (JEOL).

DNA extraction from the individual post-annular part was performed by the method as described in Kubota *et al.* [5].

Detections and comparative sequence analyses of genes coding for RubisCO, APS reductase, and soxB

Detections of three genes coding for RubisCO form I large-subunit (*cbbL*), APS reductase alpha-subunit (*aprA*) and SoxB (*soxB*) were carried out by PCR using specific primers for each gene [7] and the DNA extracts from the post-annular part of trunk. Neighbor joining analyses were carried out using Protdist program and Neighbor program in the PHYLIP package.

Results and Discussion

Distribution and population of free-living cells related to O. mashikoi *endosymbiont A*

To investigate the existence of endosymbiont A-related phylotype in sediments of Tsukumo Bay, PCR using DNA extracts from sediments as templates was carried out. The target nucleotide sequences were amplified from almost all DNA extracts, suggesting that the endosymbiont A-related phylotype are distributed in sediment over

the entire this bay. The target nucleotide sequences were also detected in total bacterial cells retrieved from the sediment sample (station #24), indicating that the bacterium related to endosymbiont A inhabits the sediments of the bay in a free-living form. To investigate the population of the endosymbiont A-related phylotype in the sediments, the quantitative PCR using a control DNA (486bp fragment) was carried out. The endosymbiont A-related phylotype was detected in almost all sediment samples collected from 23 points in the bay, ranging in copy number of the 16S rRNA gene from 2.22×10^4 to 1.42×10^6 copies per gram of dry-sediment (Fig. 1B). When the average copy number is computed on the basis of information from the rRNA Operon Copy Number Database, it is 5.7 in 108 species from *Gammaproteobacteria*. Kubota *et al.* reported that the endosymbionts A–G belongs to *Gammaproteobacteria* [5]. If the endosymbiont A-related phylotype has 5 copies of the 16S rRNA gene, the cell number in one gram of dry-sediment is between 4.44×10^3 and 2.84×10^5. Furthermore, the copy number of eubacterial 16S rRNA gene in one gram dry-sediment was estimated by the quantitative PCR using eubacterial universal primers (B341f and U533r) and a control DNA (333bp fragment) (Fig. 1B). Assuming that a bacterial cell has from 1 to 7 copies of the 16S rRNA gene, one gram of dry-sediment from the bay contains from 3.13×10^6 to 2.06×10^8 bacterial cells. Therefore, the free-living cells of endosymbiont A-related phylotype made up 0.002–9.073% of the total eubacterial population, suggesting that the *O. mashikoi* larvae precisely select candidates for their endosymbiont from bacterial flora in the environment.

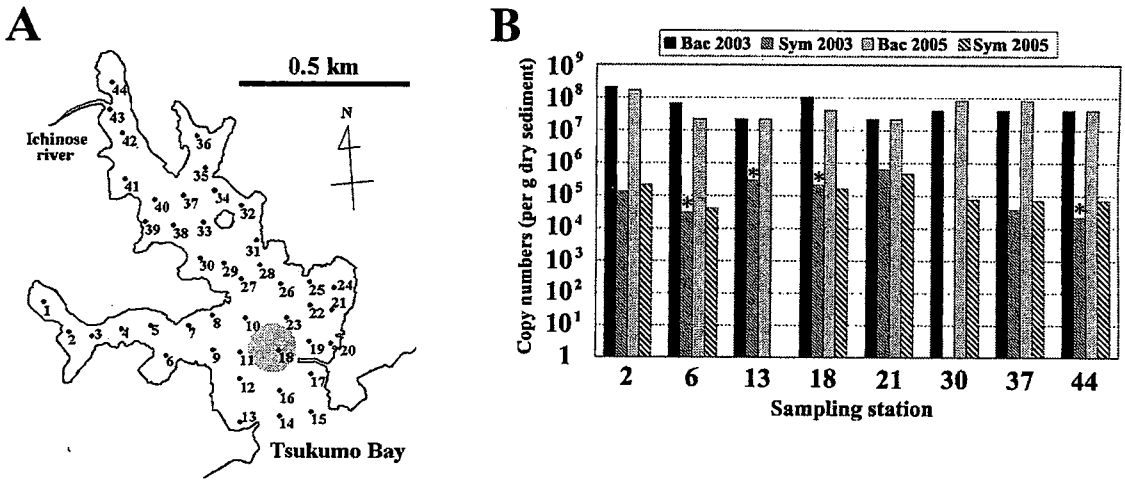

Fig. 1. (A) Sampling stations. The dots (#1 to 44) show sampling stations of sediments. The gray circle is the zone in which adult worms of *O. mashikoi* cluster. (B) Representative results of quantitative PCR. The copy numbers of the 16S rRNA gene of all eubacteria estimated by quantitative PCR were plotted together with the copy numbers of the *O. mashikoi* endosymbiont A 16S rRNA gene in the same sample. Numbers with asterisks were corrected by multiplying the quantitative PCR-estimated copy numbers by the ratios of the PCR products derived from the endosymbiont A-related phylotype to the total PCR products. The copy numbers of the 16S rRNA gene of all eubacteria in samples collected in 2003 and 2005 are shown as 'Bac 2003' and 'Bac 2005', respectively. The copy numbers of the *O. mashikoi* endosymbiont A 16S rRNA gene are shown as 'Sym 2003' and 'Sym 2005'.

Observations of endosymbionts in the bacteriocytes of O. mashikoi *using TEM*

TEM observations of the bacteriocytes in four individuals (three individuals for endosymbiont A, and one individual for endosymbiont E) revealed that the endosymbiotic bacteria were morphologically single in the bacteriocytes and the morphology was slender rod-shaped, about 1 2 μm long and about 0.5 μm wide (Fig. 2A and B). The intracytoplasmic-stacked membranes specifying all of methanotrophic bacteria (i.e. type I, II, and X) were not observed in the *O. mashikoi* endosymbionts (Fig. 2B and C). Interestingly, the electron-translucent vesicles were often found in the *O. mashikoi* endosymbionts (Fig. 2A and B). The vesicles of the *O. mashikoi*

endosymbionts were similar to those of endosymbiotic thioautotrophic bacteria of marine invertebrates such as a vestimentiferan tubeworm *Riftia pachyptila* (Fig. 2D).

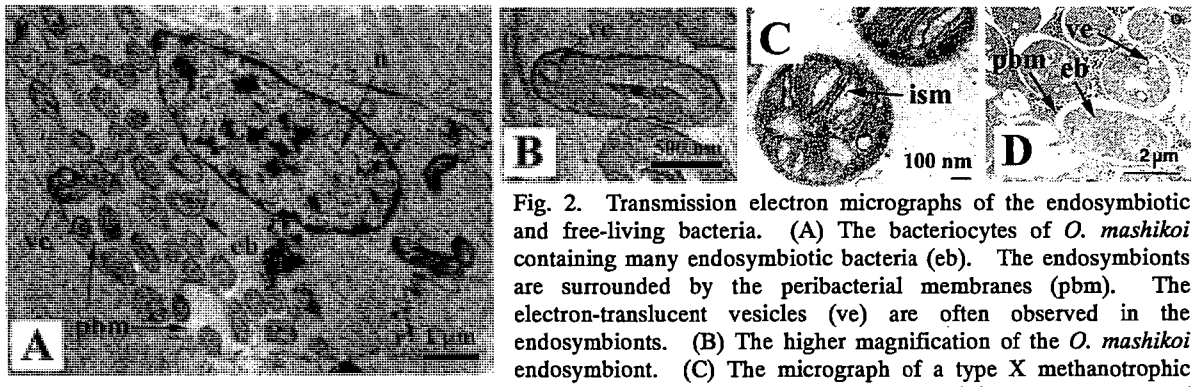

Fig. 2. Transmission electron micrographs of the endosymbiotic and free-living bacteria. (A) The bacteriocytes of *O. mashikoi* containing many endosymbiotic bacteria (eb). The endosymbionts are surrounded by the peribacterial membranes (pbm). The electron-translucent vesicles (ve) are often observed in the endosymbionts. (B) The higher magnification of the *O. mashikoi* endosymbiont. (C) The micrograph of a type X methanotrophic bacterium *Methylococcus capsulatus* [8]. (D) The micrograph of endosymbiotic bacteria of a vestimentiferan tubeworm *Riftia pachyptila* [9].

Detections and comparative sequence analyses of the genes coding for RubisCO, APS reductase, and SoxB

I searched for the genes coding a protein involved in CO_2 fixation (RubisCO), and two proteins involved in sulfur oxidation (APS reductase and SoxB) on genomes of *O. mashikoi* endosymbionts A–G by PCR using specific primers for each gene. To distinguish the types of the endosymbionts, DNA extracts were individually prepared from the post-annular region of the trunk of one adult worm, and the types of the endosymbionts were confirmed by DGGE analyses and/or sequencing of the 16S rRNA gene as described in Kubota *et al.* (2007) [5]. The analyses indicated that the DNA extracts contained only one sequence of 16S rRNA gene.

The three genes (*cbbL*, *aprA*, and *soxB*) were amplified from each of the DNA extracts containing chromosomes of the endosymbionts A–G. The three genes were not detected in DNA extracted from the pre-annular parts containing no bacteriocytes. Although the small extent of the nucleotide sequence variation of the three genes among the endosymbionts A–G was found, the nucleotide sequences of the three genes were identical among each types of the endosymbiont. Phylogenetic trees based on the amino acid sequences deduced from the three genes were constructed by the neighbor joining method. The trees based on the amino acid sequences deduced from *cbbL* and *aprA* are shown in Fig. 3. Three phylogenetic trees strongly suggest that the *O. mashikoi* endosymbionts are thioautotrophic bacteria.

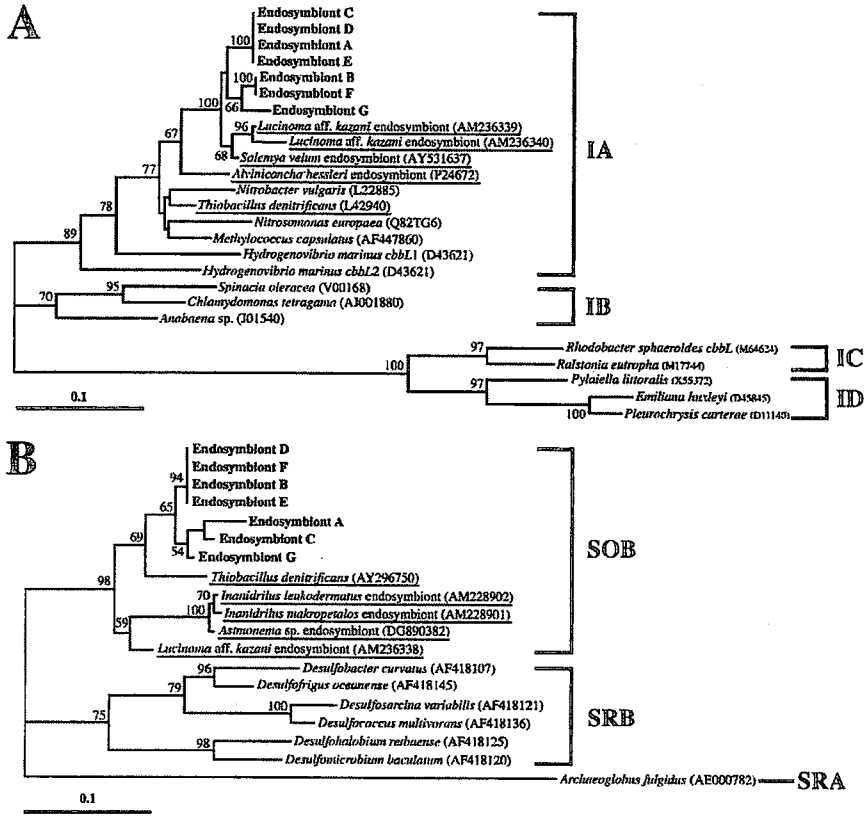

Fig. 3. Phylogenetic placements of RubisCO large-subunit (A), and APS reductase alpha-subunit (B) sequences from *O. mashikoi* endosymbionts A–G based on the neighbor joining analyses. The scale bars represents 10% estimated sequence divergence. Percentage of 100 bootstrap resampling is shown at each branch (value higher than 50% are shown). The single underline indicates the sequences from thioautotrophic bacteria. IA; RubisCO form IA, IB; form IB, IC; form IC, ID; form ID. SOB; thioautotrophic (sulfur-oxidizing) bacteria, SRB; sulfate-reducing bacteria, SRA; sulfate-reducing archaeon.

Conclusion

I found that the free-living cells of the *O. mashikoi* endosymbiont A-related phylotype inhabited almost all sediment samples collected from 23 points in Tsukumo Bay and made up less than 9% of the total eubacterial population, suggesting that the *O. mashikoi* larvae precisely select candidates for their endosymbiont from bacterial flora in the environment. Secondly, I found that the endosymbionts contain the electron-translucent vesicles typically observed in thioautotrophic bacteria, but not contain the intracytoplasmic-stacked membranes specifying methanotrophic bacteria. Furthermore, I found the genes involved in CO_2 fixation (*cbbL*) and sulfur oxidation (*aprA* and *soxB*) on genomes of the *O. mashikoi* endosymbionts. These results strongly suggest that the *O. mashikoi* endosymbionts are thioautotrophic bacteria.

References

[1] Southward, 1981. Nature 293:616–620.
[2] Rouse, 2001. Zool. J. Linn. Soc. 132:55–80.
[3] Nussbaumer *et al.*, 2006. Nature 441:345–348.
[4] Imajima, 1973. Ann. Rep. Noto Mar. Lab. 13:7–12.
[5] Kubota *et al.*, 2007. Microbes Environ. 22:136–144.
[6] Asami *et al.*, 2005. Appl. Environ. Microbiol. 71:2925–2933.
[7] Blazejak *et al.*, 2006. Appl. Environ. Microbiol. 72:5527–5536.
[8] Fjellbirkeland *et al.*, 1997. Arch. Microbiol. 168:128–135.
[9] Bright *et al.*, 2003. Invert. Biol. 122:347–368.

学位論文審査結果の要旨

本論文は、能登半島九十九湾に棲息する環形動物マシコヒゲムシとその細胞内共生細菌との共生関係成立の要因を考察するため、九十九湾における共生細菌の分布とマシコヒゲムシの分布との相関性、さらに共生細菌が硫黄酸化細菌と近縁であることを明らかにした論文である。

深海に生息するハオリムシ類と同じ環形動物ヒゲムシ類は口や消化管を持たず、バクテリオサイトと呼ばれる細胞の内部に細菌を保持している。これまでの研究によりヒゲムシの共生細菌は幼生期に周辺環境から獲得される事が示唆されている。そこで本研究では、共生細菌の分布が共生関係成立の要因になり得るかを考察するため、マシコヒゲムシの共生細菌の九十九湾における分布と個体数を検討した。その結果、共生細菌の 16S rRNA 遺伝子は、海底土壌 1 g あたり 2.22×10^{4} - 1.42×10^{6} コピーの範囲で、湾内のほぼ全域から検出されることを明らかにした。また、共生細菌は海底土壌中の真正細菌の 9%以下であることを見いだし、マシコヒゲムシの幼生が環境中の細菌群から共生細菌を厳密に認識し、獲得していることを明らかにした。さらに、マシコヒゲムシ共生細菌のゲノムには、硫黄酸化や炭酸固定に関わる遺伝子が存在することを見いだし、マシコヒゲムシ共生細菌が硫黄酸化細菌と近縁であることを明らかにした。

以上、本研究により、マシコヒゲムシ共生細菌の生息分布や硫黄酸化細菌としての系統関係が明らかとなり、それらの知見は当該分野の研究発展に大いに寄与するものと考えられる。従って、審査委員会は、本研究が博士（理学）に値すると判断した。