Microbiological studies on Oligobrachia mashikoi endosymbionts

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Abstract

Oligobrachia mashikoi, Siboglinidae, Annelida, is a marine invertebrate living in the reduced sediments in Tsukumo Bay, Ishikawa prefecture, Japan. The organism lacks a mouth and gut, and instead possesses specialized cells called bacteriocytes, in which endosymbiotic bacterial cells are harbored.

In the present study, I analysed 16S rRNA genes from the endosymbiotic cells, indicating that there are at least seven phylotypes of endosymbionts in *O. mashikoi*, and that one adult worm predominantly harbored one kind of the seven phylotypes of endosymbionts. Phylogenetic analysis suggested these seven identified phylotypes are closely-related gamma Proteobacteria, however, they are distinct from the endosymbionts of other marine invertebrates. The distribution patterns of the bacterial cells in adult worm bodies were very similar among at least some phylotypes of the endosymbionts.

I tried isolating the endosymbionts from sediments around the habitats of *O. mashikoi* and succeeded in long-cultivation. The nested PCR using *O. mashikoi* endosymbiont's specific primers was conducted to identify *O. mashikoi* endosymbionts in the subcultures. The sequences of PCR products exactly agreed with that of 16S rRNA genes of *O. mashikoi* endosymbionts, suggesting that *O. mashikoi* endosymbionts are freely living in the medium. Furthermore, I tried isolating them by various methods. However, I have not succeeded in isolation of *O. mashikoi* endosymbionts in the present study.

Introduction

Siboglinidae polychaetes are long, thin tube-dwelling marine invertebrates which lack a mouth and gut, and instead possess endosymbiotic bacteria. The endosymbionts form a potentially important food source for the invertebrate hosts [1]. Siboglinidae have been divided into three groups Frenulata (beard worm), Vestimentifera (tube worm) and Monilifera [2].

In 1973, one species of the Frenulate was discovered in the reduced sediments in Tsukumo Bay, Ishikawa prefecture, Japan [3], and named *Oligobrachia mashikoi.* The organism lacks a mouth, gut, and anus, suggesting that the organism seems to depend on the endosymbionts for the nutrient as well as other Siboglinidae.

However, there are few reports about molecular characterization of the endosymbionts of the Frenulata species including *O. mashikoi*, though the endosymbionts of some vestimentifera species were phylogenetically identified using 16S rRNA genes, and belonging to the gamma subdivision of the Proteobacteria [4]. Furthermore, the endosymbionts of the Siboglinidae species have not been isolated and cultivated *in vitro*.

In the present study, I analyzed phylogenetically the endosymbionts using 16S

rRNA genes and further investigated the localization of the endosymbionts in the host bodies. Secondary, I have tried isolation and cultivation of *O. mashikoi* endosymbionts *in vitro* and succeeded in their long cultivation for 12 months.

Materials and methods

• *Collection of O. mashikoi adult worms*

The adult worms of *O. mashikoi* were collected with a dredge from the muddy bottom of Tsukumo Bay (20 to 25 m depths). The bodies of *O. mashikoi* were carefully taken out of the chitin tubes and washed with autoclaved seawater repeatedly and finally with 70% ethanol.

• *DNA extraction*

O. mashikoi trophosome tissues containing endosymbiotic cells were aseptically cut off from the bodies, and homogenized in artificial seawater. Total DNA was extracted with Get pureDNA kit for blood (Dojin) according to manufacturer's instructions.

• *16S rRNA gene analyses of O. mashikoi endosymbionts*

The bacterial 16S rRNA genes were PCR-amplified using the eubacterial universal primers (27f and 1525r) [5], and the PCR products were cloned into the pT7Blue vector (Novagen), and resulting plasmids were transformed into the *Escherichia coli* XL-1 Blue strain. Inserted DNA fragments were sequenced. The seven established 16S rRNA genes were registered with the DDBJ (accession numbers: AB252051, and AB271120 - AB271125). DGGE analysis was performed according to the method of Muyzer et al [6]. Phylogenetic trees were obtained by maximum likelihood method (ML), neighbor joining method (NJ), maximum parsimony method (MP) available in the PHYLIP package. The distribution of *O. mashikoi* endosymbionts was investigated by whole mount *in situ* hybridization [7].

• *Cultures and medium of O. mashikoi endosymbionts*

For the enrichment and isolation of *O. mashikoi* endosymbionts, I used the medium for *Thiobacillus denitrificans* [8]. Furthermore, to investigate the optimal growth conditions of *O. mashikoi* endosymbionts, the sediments around *O. mashikoi* inhabiting and the homogenates of *O. mashikoi* were inoculated into various kinds of growth media. To detect *O. mashikoi* endosymbionts in the subcultures, I designed *O. mashikoi* endosymbionts specific primers (M835f and M1257r). DNA extracts obtained from the subcultures were used as template.

• *Isolation of O. mashikoi endosymbionts*

I tried isolation of *O. mashikoi* endosymbionts by colony formation using Gellan Gum or Noble agar, and dilution to extinction technique was also used [9]. Furthermore, to isolate *O. mashikoi* endosymbionts, the filter sterilized seawater and autoclaved sediments from Tsukumo bay were added to the basal medium to simulate the natural condition. Colony hybridization was conducted for the colonies forming on these plates to detect *O. mashikoi* endosymbionts.

Results and Discussion

Identification of O. mashikoi endosymbionts

To investigate the homogeneity or heterogeneity of endosymbiotic cells in one adult body of *O. mashikoi*, the DGGE analysis was carried out. When DNA extracts from total 65 adult worms were used in the DGGE analysis, I found more than six kinds of bands on the gels (Fig. 1). On the other hand, the 16S rRNA genes (1.5 kbp) were amplified from all of the 65 DNA extracts using eubacterial universal primers (27f and 1525r) [5]. The PCR products were directly sequenced, 62 sequences were classified into seven kinds of 16S rRNA genes. Remaining three sequences could not be analyzed.

After these seven bands of 16S rRNA genes were separately cloned into the pT7Blue vector, PCR was carried out using a pair of primers (341fGC and 534r) [6] and a mixture of the seven kinds of plasmids as template. When the PCR products were analyzed by the DGGE experiment, six discrete DNA bands appeared on the gel (Fig. 2). Five of the six bands had equal fluorescence intensity, and the remaining one marked with an asterisk containing two kinds of DNA fragments (lanes 6 and 8) had a high intensity. This result indicates that if different DNA molecules carrying different 16S rRNA genes are mixed at equal concentrations and used as a template for PCR under the condition described in Materials and Methods, equal amounts of the PCR products are obtained. Therefore, it seems likely that each of 62 DNA extracts mainly contains one kind of the 16S rRNA genes, and, in turn, that one kind of endosymbiont is predominantly harbored in one adult worm. I supposed that seven 16S rRNA sequences correspond to the seven endosymbionts, and tentatively named the respective bacteria containing one of the seven rRNA genes endosymbionts A-G.

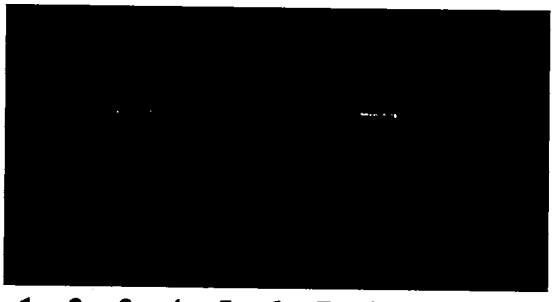

Fig. 1. The DGGE analysis of *O. mashikoi* endosymbionts. Each lane represents DGGE band patterns of DNA extracts obtained from one host worm. These bands could be divided into six groups: group 1; lane 1 and 2, group 2; lane 3 and 8, group 3; lane 4,5 and 11, group 4; lane 6 and 9, group 5; lane 7, group 6; lane 10.

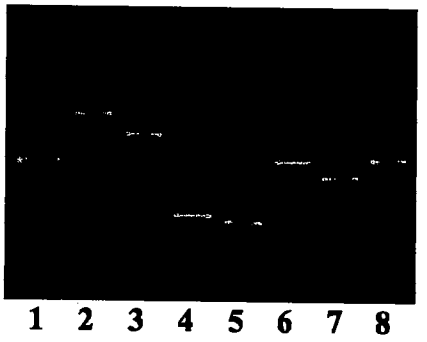

Fig. 2. DGGE analysis of 16S rRNA genes cloned on plasmids. A part of the 16S rRNA genes (about 200 bp length) was PCR-amplified by using a set of primers (341fGC and 534r) and plasmids carrying 16S rRNA genes from the *O. mashikoi* endosymbionts as a template. PCR products were analyzed by DGGE. Template DNA contained 16S rRNA genes from following endosymbionts, lane 1; all seven endosymbionts, lane 2; endosymbiont A, lane 3; endosymbiont B, lane 4; endosymbiont C, lane 5; endosymbiont D, lane 6; endosymbiont E, lane 7; endosymbiont F, lane 8; endosymbiont G. The band marked with * contained two kinds of PCR products.

To determine the sequences of the 16S rRNA genes from the *O. mashikoi* endosymbionts, the 16S rRNA genes (1.5 kbp) which were amplified from 17 DNA extracts of 65 samples by PCR were cloned and sequenced. One kind of 16S rRNA gene was found in each DNA extract. This result supports the proposal that these 16S rRNA gene phylotypes are from seven distinct endosymbionts, not from multiple copies in a single endosymbiont. And one kind of endosymbiont is predominantly maintained in one adult worm.

Phylogenetic tree was constructed by the maximum likelihood method (Fig. 3). The results showed that *O. mashikoi* endosymbionts are within the gamma subdivision of Proteobacteria, in which many thioautotrophic and methanotrophic symbionts of marine invertebrates are included. However, I could not infer the nutrient source of the endosymbionts by phylogenetic analysis. Furthermore, phylogenetic analysis indicated that *O. mashikoi* endosymbionts are not closely related to the endosymbionts of many marine invertebrates such as *Bathymodiolus japonicus* and *Riftia pachyptila* (Fig. 3). To compare the distribution patterns among the endosymbionts in host worms, after identification of an endosymbiont type by PCR amplification and DNA sequencing

experiments using a DNA extract from a small part of a host worm, the distribution of the endosymbiotic cells in the same *O. mashikoi* adult worm was determined. However, the distribution patterns of the bacterial cells in adult worm bodies were very similar among at least some phylotypes of the endosymbionts.

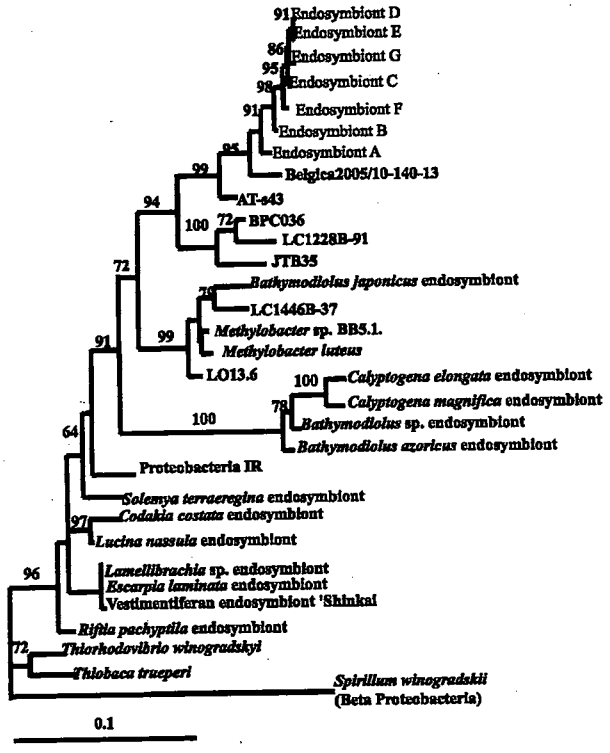

Fig. 3. Phylogenetic tree of 16S rRNA genes. The sequences of 16S rRNA genes from the endosymbionts of *O. mashikoi* and other free-living or symbiotic bacteria within the gamma subdivision of Proteobacteria were analyzed with the maximum likelihood method. *Spirillum winogradskii* was used as outgroups. Scale bar is equal to 0.1 nucleotide substitutions per sequence position.

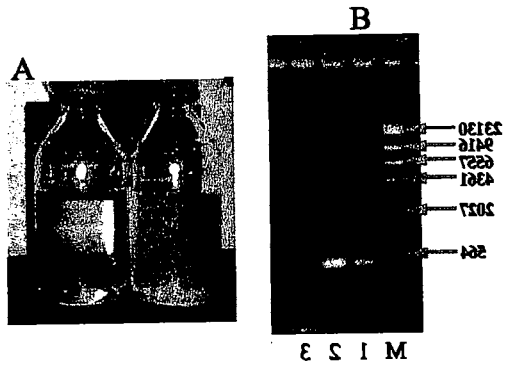

Fig. 4. The subcultured medium and detection of the *O. mashikoi* endosymbionts by PCR using oligonucleotide primers (M835f and M1257r) complimentary to *O. mashikoi* endosymbionts-specific regions of the 16S rRNA gene sequences. A. left bottle; subcultured medium incubated for 3 weeks at 25℃. Right bottle; the medium not inoculated the sediments. B. PCR products were analyzed by agarose gel electrophoresis and stained with ehidium bromide. Lane M; Marker, Lane 1; Subcultured bacteria, Lane 2; *O. mashikoi* homogenate as template DNA, Lane 3; *E. coli* as template DNA.

Enrichment cultures of O. mashikoi endosymbionts

In the present study, I used the liquid medium for *Thiobacillus denitrificans* [8] as the culture medium for enrichments and isolation of *O. mashikoi* endosymbionts. The sediments obtained from Tsukumo Bay were added to the medium and incubated at 25℃, pH 7.0. To confirm whether *O. mashikoi* endosymbionts exist in the subcultures, I designed *O. mashikoi* endosymbiont's specific primers (M835f and M1257r) and performed nested PCR using DNA fragments amplified with universal primers for eubacterial 16S rRNA genes (27f and 1525r). The PCR products were sequenced directly and these sequences exactly agreed with 16S rRNA gene sequences of *O. mashikoi* endosymbiont A. This result suggests that *O. mashikoi* endosymbiont A are freely and chemoautotrophically living in the medium with thiosulfate under anaerobic condition. The medium in which the growth of bacteria was confirmed by nested PCR using *O. mashikoi* endosymbiont's specific primers was further enriched by about 10 passages through 1:100 dilutions for 12 months.

I tested various culture methods. I used sulfide and various kinds of organic compounds such as yeast extract, glucose, thiotaurine etc. as alternative respiratory substrate. Although I repeatedly conducted PCR using *O. mashikoi* endosymbionts specific primers and DNA extracts from the cells in the medium as template, PCR products were not detected (data not shown). Therefore, it seems likely that the endosymbionts might be obligatory chemolithoautotrophic bacteria using thiosulfate. Although the homogenates of *O. mashikoi* trophosome tissues were also inoculated into all media

described above, I could not observe the growth of the endosymbionts.

Try isolation of O. mashikoi endosymbionts

I have tried isolation of *O. mashikoi* endosymbionts from the subcultures by colony formation using the Gellan Gum plates and dilution to extinction technique. Partial 16S rRNA genes were amplified by PCR and sequenced directly using the DNA extracted from the colonies forming on the Gellan Gum plates, and the DNA extracted from the cultures in the tube showing growth at the highest dilution in the dilution to extinction experiment. However, these DNA sequences mainly showed the highest homology to the 16S rRNA gene sequences of *T. denitrificans* (accession number: AJ243144). These results suggest that although *O. mashikoi* endosymbionts are alive in the subcultures, the contents of the bacteria may be too low to isolate them.

There are some reports that previously uncultivated bacteria could be isolated by simulating the natural environment [10]. Therefore, I tried isolation of *O. mashikoi* endosymbionts by adding filter sterilized natural seawater and autoclaved sediments to the basal medium. Furthermore, charcoal and dithiothreitol (DTT) were added to the medium to reduce both H_2O_2 and O_2^-. To detect *O. mashikoi* endosymbionts, colony hybridization was conducted. After the existence of *O. mashikoi* endosymbionts was confirmed in the subcultures by PCR, the subcultures were spread over the surface of the plates and incubated at 15℃ for more than 12 weeks (Fig. 5A). And then, colony hybridization was conducted, but the positive signals were not confirmed on the plates (Fig. 5B).

In the present study, I succeeded in the long cultivation of *O. mashikoi* endosymbionts, but could not isolate them. It may be due to small populations of *O. mashikoi* endosymbionts in the subcultures. Further improvement of the culture conditions will be required to increase the populations of *O. mashikoi* endosymbionts in the subcultures and isolate them.

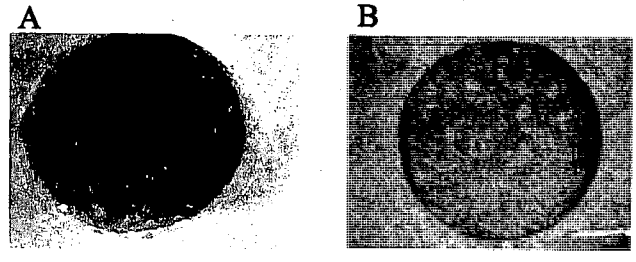

Fig. 5. Colony hybridization of the plate inoculated the subcultured medium at 15℃. The plates were incubated more than 12 weeks, and colonies were transferred to the nylon membrane. Thereafter, DNA had to be released in lysozyme containing buffer, washed with 2 x SSC, and fixed to the membrane by UV cross-linking. Hybridization was conducted using the anti mashi probe [7] at 55℃ overnight. A; the plate incubated more than 12weeks at 15℃. B; the result of colony hybridization. There are many colonies transferred to the membrane. However, positive signals were not detected.

References

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学位論文審査結果の要旨

本論文は、有鬚動物 *Oligobrachia mashikoi*（マシコヒゲムシ）の栄養体に共生する細菌の 16S rDNA の塩基配列を決定し、その系統解析と細胞内分布から、宿主１個体には、１種類の細菌しか共生しないが、その種類は、少なくとも７種類存在することを見いだし、有鬚動物と細菌の新しい共生関係を明らかにした論文である。

マシコヒゲムシは能登半島先端付近に位置する九十九湾の水深約 20- 30m の硫化物に富む泥中に生息している。これらの生物は口や肛門、消化器官を全く持たない代わりに、体内に化学合成独立栄養細菌を共生させており、その共生細菌が炭酸固定し合成した有機物を用いて生育に必要なエネルギを獲得していると考えられている。本研究では、まず共生細菌の 16S rRNA 遺伝子の配列を決定し、その分子系統を考察した。その結果、少なくとも７種類の共生細菌がおり、これらはきわめて近縁でありガンマプロテオバクテリアに属していることを明らかにした。また、宿主は各一個体が７種類の共生細菌のうちの１種類を優先的に保持していること、さらに、宿主個体内での共生細菌のタイプによる分布の違いを調べたところ、すべて同様に細胞内分布していることを明らかにした。

以上、本研究により、これまで知られていなかった有鬚動物と細菌との新しい共生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。