Microbes Environ. Vol. 23, No. 1, 81–88, 2008
http://wwwsoc.nii.ac.jp/jsme2/ doi:10.1264/jsme2.23.81

Microbes and Environments

Distribution and Population of Free-Living Cells Related to Endosymbiont A Harbored in *Oligobrachia mashikoi* (a Siboglinid Polychaete) Inhabiting Tsukumo Bay

MASATO AIDA[1], MASAAKI KANEMORI[1], NORIHIRO KUBOTA[1], MASAHIRO MATADA[2], YUICHI SASAYAMA[2], and YOSHIHIRO FUKUMORI[1]*

[1]*Department of Life Science, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920–1192, Japan; and* [2]*Noto Marine Laboratory, Institute of Nature and Environment Technology, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920–1192, Japan*

(Received August 28, 2007—Accepted December 10, 2007)

Beard worms (Siboglinidae, Polychaeta), which lack a mouth and a digestive tract, harbor thioautotrophic or methanotrophic bacteria in special cells called bacteriocytes. These endosymbionts have been considered to be trapped at a specific larval stage from the environment. Although many species of beard worms have been discovered in various abyssal seas, *Oligobrachia mashikoi* inhabits Tsukumo Bay which is only 25 m deep. At least seven types of endosymbionts (endosymbiont A–G) have been distinguished in *O. mashikoi*. In this study, we investigated the distribution pattern of free-living cells related to the major endosymbiont (endosymbiont A) in Tsukumo Bay by quantitative PCR targeting the 16S rRNA gene. The endosymbiont A-related phylotype was detected in almost all sediment samples collected from 23 points in Tsukumo 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. Furthermore, the free-living cells 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. This is the first report on the ecological characterization of a free-living bacterium related to the endosymbiont of the siboglinid polychaete, *O. mashikoi*.

Key words: *Oligobrachia mashikoi*, Siboglinidae, beard worm, endosymbiont, free-living

Frenulates (beard worms) inhabit reducing environments such as cold seeps in abyssal seas. This animal group is related to vestimentiferans (tubeworms) within the family Siboglinidae[16,34]. More than one hundred species of beard worms have already been found. They have a very slender body that is 2–10 cm in length and 0.6–0.8 cm in width, and live in self-produced tubes which stick to the sea bottom. They lack a mouth and a digestive tract. Instead, they harbor bacterial endosymbionts in cells called bacteriocytes of the trophosome, which have been thought to supply energy sources to their host[38,39].

Chemoautotrophic endosymbioses occur ubiquitously at oxic-anoxic interfaces in the marine environment, such as in hydrothermal vents, cold seeps, whale skeletons, sunken wood, and coastal reducing sediments[42]. Bacterial endosymbionts are accommodated in the gills of some species of bivalves[9], in the gut rudiments of some marine nematodes[33] and in the subcuticle spaces of some oligochaetes[3]. In some bivalves, such as *Codakia orbicularis*, bacteria are taken into the gill epithelial cells by phagocytosis[15]. On the other hand, in some coastal bivalves, such as *Solemya reidi* and *Solemya velum*[5,24], and in some benthic bivalves, such as *Calyptogena magnifica*, *C. phaseoliformis*, and *C. pacifica*[7], bacteria are vertically transferred via eggs. In vestimentiferans and frenulates, bacterial endosymbionts are harbored in the trophosome[8–10,38,39], and not found in germ cells[6,8,13,19]. There are several reports that a mouth and a gut are transiently formed in vestimentiferan larvae[20,40] and in frenulate larvae[4]. It has been thought that the free-living bacteria are taken in through the transiently formed mouth that appears only during a specific larval stage[4,8,20,40]. Recently, Nussbaumer *et al.*[30] reported that, in some species of vestimentiferans, free-living bacteria invade through the host's skin to become the endosymbionts. 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 hosts.

One species of beard worms was identified as a new species called *Oligobrachia mashikoi* which inhabits the muddy bottom (25 m deep) of Tsukumo Bay on the Noto Peninsula, Japan[17] (Fig. 1A). When the worm is pushed out of the tube, the body is about 13 cm long and 0.6–0.8 mm wide[37]. The adult worm lacks a mouth and a digestive tract, and harbors bacterial cells in its bacteriocytes, like other beard worms[11,38]. Transmission electron micrographs show that the morphology of the endosymbiotic cells is uniform[11]. Although we 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[25]. When 65 worms were investigated, 26 individuals (40.0%) were found to have kept endosymbiont A; 10 individuals (15.4%), endosymbiont E; 9 individuals (13.8%), endosymbiont D; and 7 individuals (10.7%), endosymbiont G. The phylogenetic analysis of the 16S rRNA gene of the endosymbionts

* Corresponding author. E-mail: fukumor@kenroku.kanazawa-u.ac.jp; Tel: +81–76–264–6231; Fax: +81–76–264–6230.

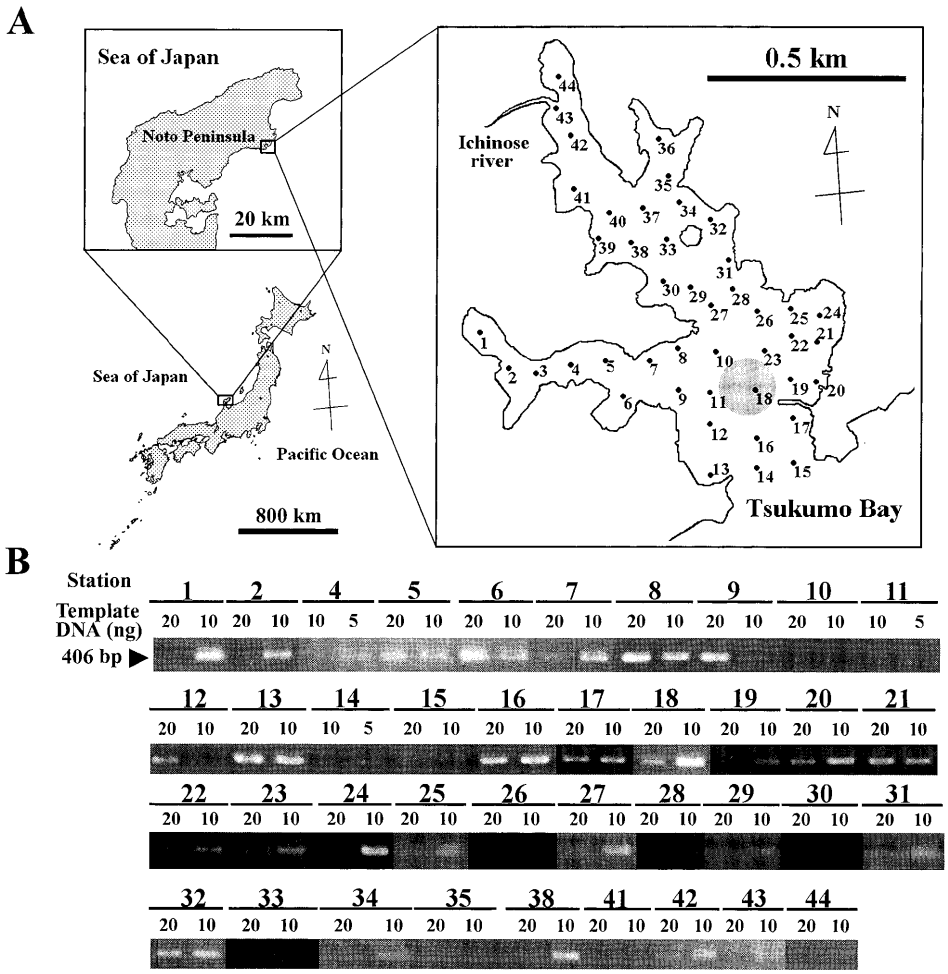

Fig. 1. (A) Sampling stations. Tsukumo Bay is located on the Noto peninsula of Japan. The bay is 0.486 square kilometers in area. The dots (#1 to 44) in the right panel show sampling stations. The gray circle is the zone in which adult worms of *O. mashikoi* cluster. (B) Qualitative detection of free-living cells of the *O. mashikoi* endosymbiont A-related phylotype. DNA was extracted from the sediment samples and 5–20 ng was used as a template in PCR. A pair of primers (M450f and M860r; Table 1) was used. The PCR products were electrophoresed on 2% agarose gels and stained with EtBr. Representative results are shown.

revealed that they are closely related to each other within the *Gammaproteobacteria* clade[22,25].

It is generally accepted that siboglinid polychaetes such as *O. mashikoi* take in the free-living cells of their endosymbionts from the environment in each generation[4,8,30]. However, it is difficult to confirm that the free-living bacterium detected by quantitative PCR targeting the 16S rRNA gene is exactly the same bacterium as endosymbiont A. So, we refer to the free-living bacterium as 'the endosymbiont A-related phylotype'. In the present study, to gain further insight into the nature of the endosymbionts, we investigated the distribution and population of free-living cells related to the major endosymbiont (endosymbiont A) in Tsukumo Bay and found that the endosymbiont A-related phylotype was detected in almost all sediment samples collected from 23 points in Tsukumo Bay.

Materials and Methods

Collection of sediment samples

Sediment samples were collected from Tsukumo Bay (37°18'N, 137°14'E), an inlet of the Noto Peninsula (Fig. 1A). Tsukumo Bay is 1,300 m long, 250 m wide, and 25 m deep in the central region. The enclosure index (EI) was calculated according to the equation $EI=(\sqrt{S}\times D1)/(W\times D2)$, where S is the bay area (in square kilometers), W is the width at the entrance of the bay (in kilometers), $D1$ is the depth at the center of the bay (in meters), and $D2$ is the depth at the entrance of the bay (in meters)[2]. EI indicates the seawater exchange capacity. The EI of Tsukumo Bay was 3.77, suggesting that the seawater exchange capacity is low.

Using a grab-type dredge (DAIKI Co, Saitama, Japan), sediment samples were collected from the surface layer of the bottom (0–5 cm depth) at 44 sampling stations, the depth of which ranged from 2.5–25.6 m (Fig. 1A). All samples were stored at 4°C.

Extraction and quantitation of DNA

DNA was extracted from the sediments (0.85 gram wet-weight each) using the UltraClean Soil DNA Kit (MO BIO Laboratories, Solana Beach, CA). Although the concentration of DNA was determined photometrically, it was also confirmed by electrophoresis, because DNA solutions containing humic acids often show higher values at A_{260} than the net values[26]. Chromosomal DNA was extracted from *E. coli* cells as previously mentioned[35].

Photographs of the pattern of electrophoresis on agarose gels stained with ethidium bromide (EtBr) were taken with the AE-6905H Image Saver HR (ATTO, Tokyo, Japan). Digitized images were analyzed using the software program Multi Gauge (Fuji Film,

Tokyo, Japan) or NIH Image.

Polymerase chain reaction (PCR)

Although seven types of endosymbionts have so far been identified in *O. mashikoi*[25], we focused on the distribution of the major endosymbiont (endosymbiont A) in this study. To detect free-living cells of the endosymbiont A-related phylotype in the sediment samples by PCR, a specific primer set (M450f and M860r; Table 1) was designed on the basis of the 16S rRNA gene sequence of endosymbiont A (accession number: AB252051). The reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, 1.25 μM of each primer, 0.5 U of Taq DNA polymerase (Takara-bio, Shiga, Japan) and 5–20 ng of template DNA. PCR amplification was done under the following conditions; the mixture was incubated for 2 min at 94°C, the subjected to a cycling reaction (usually 40 cycles) consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 2 min at 60°C. Finally, the mixture was incubated for 5 min at 72°C. The PCR products were separated on 1.5 or 2% agarose gels and stained with EtBr.

To examine the specificity of the PCR primer set (M450f and M860r) for quantifying free-living cells of the endosymbiont A-related phylotype in the sediment samples, the PCR products from six sampling stations (#1, 6, 13, 18, 24, and 44) were cloned and sequenced. Since sufficient PCR products to be used for cloning could not be obtained due to the presence of PCR inhibitors, such as humic acid, in the first PCR, a second PCR was done using the same primers and the products of the first PCR as templates and in this case, the PCR cycling number was reduced to 30 cycles to decrease PCR bias. The second PCR products were cloned into the pSTBlue-1 AccepTor vector (Novagen, Madison, USA), and the resulting plasmids were introduced into an *Escherichia coli*, strain XL-1 Blue. Twenty transformants from each sediment sample were removed and plasmids were prepared. The DNA inserts were sequenced with the ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster city, CA, USA). The results showed that the PCR products contained not only a DNA fragment derived from the endosymbiont A-related phylotype, but also other fragments from other unidentified bacteria. Since the experiment was repeated two times, 40 clones were analyzed at each sampling station. The ratio of the number of clones derived from the endosymbiont A-related phylotype to the total number of clones was 32.5% for station #1 (the percentage is a mean value calculated from two experiments), 65.0% for #6, 80.0% for #13, 82.5% for #18, 97.5% for #24, and 32.5% for #44. Percentages were used to correct the values obtained in this study (Table 3).

To quantitatively detect the endosymbiont A-related phylotype, quantitative PCR was conducted under the above conditions except that the reaction mixture contained 0.002% (wt/vol) bovine serum albumin which is known to alleviate the inhibition of PCR by humic acids[44]. A DNA fragment (486bp in length) with a part of the *mam22* gene[32] flanked by DNA sequences of the primers (M450f and M860r) was used as a control which was amplified by PCR using the *mam22* gene of *Magnetospirillum magnetotacticum* MS-1 cloned on a plasmid as a template and a pair of primers (compet-Mf and compet-Mr; Table 1). Since the compet-Mf and compet-Mr primers have regions complementary to the *mam22* gene in the 3' region and the same sequence as the M450f (in the case of compet-Mf) and M860r (in the case of compet-Mr) primers in the 5' region, the resulting PCR product (486bp) was flanked by the DNA sequences of the M450f and M860r primers. To evaluate the validity of the 486bp fragment as a control, we prepared another DNA fragment 406bp in length which was also amplified by PCR using the 16S rRNA gene of endosymbiont A cloned on a plasmid as a template and the primers (M450f and M860r).

To estimate the copy numbers of the 16S rRNA gene of all eubacteria in the sediment samples, the quantitative PCR was also carried out by using a set of primers (Bacteria-specific primer: B341f and universal primer: U533r; Table 1)[2]. A part of the 16S rRNA gene of almost all eubacteria can be amplified with the primer set. A control DNA fragment (333bp in length) which included a part of the unrelated λ-DNA flanked by the sequences of the primers was prepared using the Competitive DNA Construction Kit (Takara-bio).

To determine the amplification and extraction efficiencies, real-time PCR was carried out according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The reaction mixture (20 μl) contained 3 mM $MgCl_2$, 0.5 μM of each primer, and 2.0 μl of the FastStart DNA Master SYBR Green I (Roche Diagnostics). One microliter of each DNA solution (5–11 ng DNA) was added as a template. PCR amplification was done under the following conditions. First, the DNA templates were denatured for 10 min at 95°C. The subsequent cycling reaction (usually 32 cycles) consisted of denaturation for 10 sec at 95°C, annealing for 5 sec at 61°C, and elongation for 20 sec at 72°C.

Amplification efficiency

Since the short linear DNA fragments (486bp and 333bp) were used as a control for quantitative PCR, the amplification efficiency was compared between a short linear DNA fragment (473bp) and chromosomal DNA. Also, since *O. mashikoi* endosymbiont A has not been isolated, *Escherichia coli* strain KY2039 (Δ*hslVU*::*tet*) was used. KY2039 lacks HslVU, an ATP-dependent protease, in which the *hslVU* operon encoding HslVU is partially replaced with the tetracycline resistance gene[21]. The amplification efficiency was determined by real-time PCR using a pair of primers (hslU3031f and tet3424r; Table 1) to amplify the artificial chromosomal region (473bp) including the tetracycline resistance gene. The DNA fragment (473bp) was PCR-amplified in advance, and used as the short linear template DNA in real-time PCR. The amplification efficiency (E) was calculated according to the following formula: $E=10^{-1/S}$, where S is the slope of a line graph which was drawn by plotting the threshold cycle numbers (Ct) obtained from reactions with serial dilutions of a template against common logarithmic values of known copy numbers of a target gene. An efficiency of 2.0 means the doubling of products in each cycle. The two DNA templates gave similar results when each was added to the reaction mixture at 10^4–10^6 copies; E was 1.876 for the short linear template and 1.856

Table 1. PCR primers used

Primer	Application	Sequence (5'–3')
M450f	Specific detection of *O. mashikoi* endosymbiont A-related phylotype	CAAGATTAATACTCTTGGTC
M860r	Specific detection of *O. mashikoi* endosymbiont A-related phylotype	TAGCTGCGTCACTAAGCTCCG
compet-Mf	Preparation of the control DNA for quantitative PCR	CAAGATTAATACTCTTGGTCTGT-CTAGCAAGC-CGTCG
compet-Mr	Preparation of the control DNA for quantitative PCR	TAGCTGCGTCACTAAGCTCCGT-TGAAATTGATGG-GATTGG
hslU3031f	Specific detection of *E. coli* strain KY2039	TCGATAGCCTGGACGCGTACC
tet3424r	Specific detection of *E. coli* strain KY2039	GGGACAGCTTCAAGGATCGC
B341f	Detection of almost all bacterial phylotypes	CCTACGGGIGGCIGCA
U533r	Detection of almost all bacterial phylotypes	TTACCGCGGCKGCTGRCAC

for the chromosomal DNA template. When the *M. magnetotacticum* chromosomal DNA was added to the reaction mixtures at up to 10 ng to test whether extraneous chromosomal DNA affects the amplification efficiency, similar results were obtained; 1.883 for the short linear DNA template and 1.873 for the chromosomal DNA template. These results indicated that a short linear DNA fragment functions well as a control in the quantitative PCR.

Efficiency of extraction of DNA from sediment samples

Since *O. mashikoi* endosymbiont A has not been isolated, we can not determine the precise DNA extraction efficiency. Therefore, we assumed that the cell destruction efficiency of endosymbiont A was equal to that of *E. coli*, and estimated the DNA extraction efficiency of the method by using *E. coli* cells as follows. KY2039 cells were grown to the stationary phase in L broth[27] at 37°C, harvested by centrifugation (13,000×*g* for 1 min at 4°C), washed five times with 0.9% (wt/vol) NaCl, and resuspended in the 0.9% NaCl solution. After the cell number had been determined by microscopically counting the cells, cells were added to 0.85 grams (wet weight) of the sediment samples from four representative sampling stations (#1, 6, 18, and 24) (Fig. 1A). These samples could be divided into two classes by their external appearance; muddy sediment (#1 and 18) and sandy sediment (#6 and 24). After the addition of KY2039 cells, the sediment samples were thoroughly mixed. DNA was extracted using the UltraClean Soil DNA Kit. Copy numbers of the KY2039 chromosomal DNA in the DNA extracts were determined by real-time PCR using a set of primers (hslU3031f and tet3424r; Table 1). The efficiency with which DNA was extracted from the four samples was calculated by dividing the number of copies of the target gene in the DNA extracts by the number of cells added. The efficiency was very high, over 100%, and was similar among the four samples (Table 2). This result was consistent with a previous report that the extraction of DNA using the UltraClean Soil DNA Kit was highly efficient[28]. Since many cells probably contain two copies of the chromosomal DNA, the efficiency might be not less than 100%. On the basis of these results, we judged that the DNA extraction efficiency was nearly 100%, and a value of 100% was taken into account in this study.

In the control experiments, when the KY2039 chromosomal DNA was prepared and mixed with the DNA extracts from the sediment samples, the amplification efficiency for the DNA fragment (473bp) was 1.864 for station #1, 1.839 for #6, 1.861 for #18, and 1.856 for #24. There were only slight differences in the amplification efficiency among the four samples, and the values were similar to that for KY2039 chromosomal DNA in the absence of any DNA extract from the sediment samples (1.883), indicating that when calculating the extraction efficiency, the amplification efficiency in each reaction can be ignored.

Results and Discussion

Qualitative detection of free-living cells of the O. mashikoi *endosymbiont A-related phylotype in the sediment samples collected from Tsukumo Bay*

When the existence of the endosymbiont A-related phylotype was investigated by PCR using DNA extracts from sediment samples as templates and a pair of primers (M450f and M860r; Table 1), the target nucleotide sequences were amplified from 35 points except for #3, 10, 11, 30, 33, 36, 37, 39, and 40 (Fig. 1B), although the band intensity of the PCR products on agarose gels differed among the sampling stations. Therefore, this result suggests that the free-living cells which ultimately compose endosymbiont A are distributed in sediment over the entire Tsukumo Bay. On the other hand, however, we could not detect the endosymbiont A-related phylotype in seawater samples (one liter each) collected above the sediment-sampling points (about 10–20 m in depth), even from areas where the endosymbiont A-related phylotype was detected (data not shown).

When sediment samples from the area in which no adult worms were found (i.e. station #24) were gently mixed with the same volume of artificial seawater and left at room temperature for 10 min, we found more than 10^5 eukaryotic microorganisms per milliliter of supernatant. After removing these eukaryotic microorganisms from the supernatant by low-speed centrifugation (100×*g* for 10 min at 10°C), the supernatant was centrifuged at 500×*g* for 10 min at 10°C to remove the suspended sediment particles. The supernatant obtained was further centrifuged at 10,000×*g* for 10 min at 4°C, and the pellet containing bacterial cells was washed two times with phosphate-buffered saline (PBS; pH 7.4) to avoid contamination from free-DNA. Total DNA was extracted from the resulting pellet with Lyso-N-Go PCR Reagent (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions. We could also detect the endosymbiont A-related phylotype in the extracted DNA by PCR (data not shown). This result suggests that a bacterium related to endosymbiont A inhabits the area in which no adult worms were found in a free-living form. Since we tried to determine the proportion of viable cells but failed to discriminate between the viable cells and dead cells, dead cells could be included in the following results.

Evaluation of the validity of a control DNA fragment used in quantitative PCR

When DNA extracts from sediment samples are used as templates, PCR is often inhibited by humic acids[18,43,44]. To avoid the effects of PCR inhibitors and precisely measure the numbers of free-living cells of the endosymbiont A-related phylotype in each sample, we performed quantitative PCR with the 486bp DNA fragment as a control which included a part of the *mam22* gene of *Magnetospirillum magnetotacticum* MS-1 flanked by sequences of primers (M450f and M860r). To evaluate the validity of the 486bp fragment as a control, the 406bp PCR-amplified fragment was prepared as mentioned in the Materials and Methods. To directly com-

Table 2. Efficiency of DNA extraction from sediment samples

Sampling station	Sediment type	Numbers of *E. coli* cells added	Numbers of estimated *E. coli* cells	Extraction efficiencies (%)	Replicate
1	Muddy	1.905×10^7	$(3.117\pm0.821)\times10^7$	164±43	n=3
6	Sandy	1.905×10^7	$(2.139\pm0.317)\times10^7$	112±17	n=3
18	Muddy	2.151×10^6	$(3.353\pm1.735)\times10^6$	156±81	n=3
		2.453×10^6	$(3.716\pm0.542)\times10^6$	151±22	n=3
24	Sandy	1.707×10^7	$(2.435\pm0.629)\times10^7$	143±37	n=3

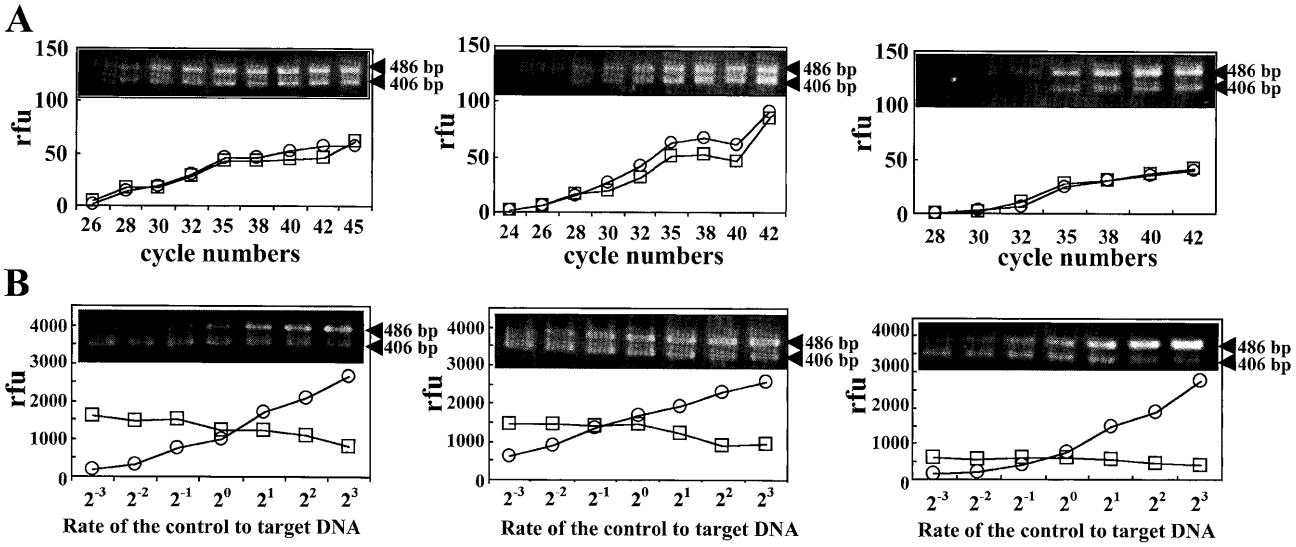

Fig. 2. Validity of the short linear DNA fragment (486bp) as a control DNA for quantitative PCR. (A) Both 406bp and 486bp fragments were added to the reaction mixtures at the same copy number: 1.0×10^4 copies (left panel), 1.0×10^3 copies (middle panel), and 1.0×10^2 copies (right panel). PCR-amplification was performed as mentioned in the Materials and Methods. Aliquots were taken at indicated cycle numbers and electrophoresed on 2% agarose gels. The intensity of the EtBr-stained PCR products was quantified with NIH Image. Relative fluorescence units (rfu) are shown. Symbols: squares, 406bp products; circles, 486bp products. (B) Both 406bp and 486bp fragments were PCR-amplified (40 cycles) and the PCR products were analyzed as mentioned in (A). The copy number of the 406bp fragment added was 5.4×10^5 copies (left panel), 5.4×10^4 copies (middle panel), or 5.4×10^3 copies (right panel). The 486bp fragment was serially diluted and added to the reaction mixtures. The numbers below each graph show the relative amount of the 486bp fragment to the 406bp fragment. 2^0 means the same copy number as the 406bp fragment. Symbols: squares, 406bp products; circles, 486bp products.

pare the PCR-amplification efficiency between the 406bp fragment and the 486bp fragment, they were mixed at the same copy numbers and subjected to PCR with the primer set (M450f and M860r). Since both fragments were amplified to a similar extent in 28 to 42 cycles (Fig. 2A), the sequence derived from the *mam22* gene was shown not to affect the amplification efficiency. We performed quantitative PCR for 40 cycles in the subsequent experiments to increase the sensitivity of detection.

Furthermore, the validity of the control DNA was tested by serially diluting it and PCR-amplifying it with various concentrations of the 406bp fragment. The intensity of the PCR products was similar at around the point of the same copy number of both templates (Fig. 2B). These results showed that the control DNA fragment can be used to quantify the free-living cells of the endosymbiont A-related phylotype in Tsukumo Bay.

Quantitative detection of the O. mashikoi *endosymbiont A-related phylotype in sediment samples*

We chose 23 points from the initial 44 points, extracted DNA from the sediment samples, and carried out quantitative PCR (Fig. 3). The results are summarized in Table 3. Since the endosymbiont A-related phylotype has not yet been isolated, the number of copies of the 16S rRNA gene in its genome is not known. Therefore, we give estimated copy numbers of the endosymbiont A 16S rRNA gene in the sediment samples. The *O. mashikoi* endosymbiont A 16S rRNA gene was detected in samples from 21 points. The copy number of the 16S rRNA gene ranged from 2.22×10^4 to 1.42×10^6 copies per gram of dry-sediment. Although the bacterium was not detected at sampling stations #30 and 33, we could not exclude its existence there, since it was found in preliminary experiments that less than 2×10^4 copies of the target gene could not be detected under the PCR conditions. In fact, we repeated the same experiment using other sediment sam-

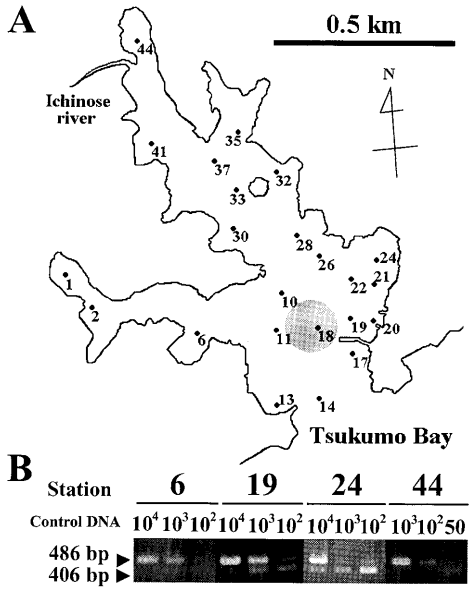

Fig. 3. Estimation of the copy numbers of the *O. mashikoi* endosymbiont A 16S rRNA gene in the sediment samples.
(A) 23 sampling stations are shown. (B) DNA extracts from the 23 sediment samples were used as templates for quantitative PCR. Representative results of quantitative PCR are shown. The 486bp PCR products were amplified from the control DNA (the short linear 486bp fragment), whereas the 406bp PCR products were amplified from the chromosomal DNA of the endosymbiont A-related phylotype in DNA extracts from the sediment samples. The values above photographs indicate copy numbers of the control DNA fragment added to the reaction mixtures.

Table 3. Copy numbers of the *O. mashikoi* endosymbiont A 16S rRNA gene in the sediment samples

Sampling station	Sediment type[a]	Water depth (m)[b]	Copy numbers[c]		Ratios of endosymbiont A-related phylotype (%)[d]	Corrected copy numbers[e]
			Sep 2003	May 2005		Sep 2003
1	O	12.4	1.31×10^5		32.5	4.26×10^4
2	O	11.6	1.31×10^5	2.14×10^5		
6	×	11.9	4.72×10^4	4.16×10^4	65.0	3.07×10^4
10	O	23.6	9.65×10^4			
11	O	25.3	9.65×10^4			
13	×	8.7	3.67×10^5	ND	80.0	2.94×10^5
14	O	22.4	5.96×10^5			
17	O	17.1	4.57×10^5			
18	O	24.2	2.55×10^5	1.65×10^5	82.5	2.10×10^5
19	O	15.1	2.20×10^5			
20	×	4.4	1.16×10^6			
21	×	2.5	6.33×10^5	4.71×10^5		
22	O	16.2	4.22×10^5			
24	×	10.3	1.46×10^6		97.5	1.42×10^6
26	O	25.6	4.04×10^5			
28	O	16.2	5.49×10^4			
30	O	19.8	ND	7.63×10^4		
32	O	5.6	5.71×10^4			
33	O	22.6	ND			
35	O	16.2	1.01×10^5			
37	O	20.3	3.84×10^4	7.40×10^4		
41	O	15.9	7.24×10^4			
44	O	8.0	6.82×10^4	7.20×10^4	32.5	2.22×10^4

[a] O: muddy sediment, ×: sandy sediment.
[b] The depth of sampling stations was measured with a fishfinder.
[c] Copy numbers of the PCR products (copies per gram of dry-sediment) were estimated by quantitative PCR. ND: not detected.
[d] Ratios of the PCR products derived from the endosymbiont A-related phylotype to the total PCR products were estimated by cloning and sequencing the PCR products as mentioned in the Materials and Methods.
[e] Corrected copy numbers of the endosymbiont A 16S rRNA gene (copies per gram of dry-sediment) were obtained by multiplying the 'copy numbers' by the 'ratios of endosymbiont A-related phylotype'.

ples, which were collected in May 2005, and detected the endosymbiont A-related phylotype in a sediment sample from #30 (Table 3).

Bacterial genomes usually have up to 15 copies of the 16S rRNA gene and generally up to 7 copies[1]. When the average copy number is computed on the basis of information from the rRNA Operon Copy Number Database (rrndb) (http://rrndb.cme.msu.edu)[23], it is 5.7 in 108 species from *Gammaproteobacteria*. Previous phylogenetic analyses of the 16S rRNA gene indicated that the *O. mashikoi* endosymbiont belongs to *Gammaproteobacteria*[22,25]. If the *O. mashikoi* 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.

Ratio of the O. mashikoi *endosymbiont to all eubacteria in sediment samples*

To estimate the eubacterial cell number in one gram of dry-sediment from Tsukumo Bay, we performed quantitative PCR using the same DNA extracts as those used in the above experiments. A pair of primers (B341f and U533r; Table 1) has been used to amplify the 16S rRNA gene of almost all eubacteria[2]. From 2.19×10^7 to 2.06×10^8 copies of the gene per gram of dry-sediment were detected at 8 points (Fig. 4). Assuming that a bacterial cell has from 1 to 7 copies of the 16S rRNA gene, one gram of sediment from Tsukumo Bay contains from 3.13×10^6 to 2.06×10^8 bacterial cells. This result is consistent with the report that 1 cm^3 of sediment contains 10^8–10^9 bacterial cells[14].

Assuming that the endosymbiont A-related phylotype has 5 copies of the 16S rRNA gene, the phylotype accounts for 0.002–9.073% of all the eubacteria in Tsukumo Bay, suggesting that *O. mashikoi* has a strict mechanism to select the endosymbiont from among many bacteria. *Vibrio fischeri* is attracted by some components of the light organ mucus secreted from the host squid *Euprymna scolopes*[12,31]. *O. mashikoi* endosymbionts may also approach a host larva in response to a certain chemical and invade through the host's transiently formed mouth or skin into bacteriocytes in the trophosome. To elucidate the mechanisms of infection, we should isolate and cultivate the *O. mashikoi* endosymbionts.

In some species of vestimentiferans, the larvae are thought to be able to swim for some weeks without feeding[41], until they can settle in a suitable place where they would take in the free-living cells of endosymbionts. Assuming that *O. mashikoi* larvae swim like the vestimentiferan larvae, it is expected from our results that the transmission of free-living cells of the endosymbionts to *O. mashikoi* larvae occurs

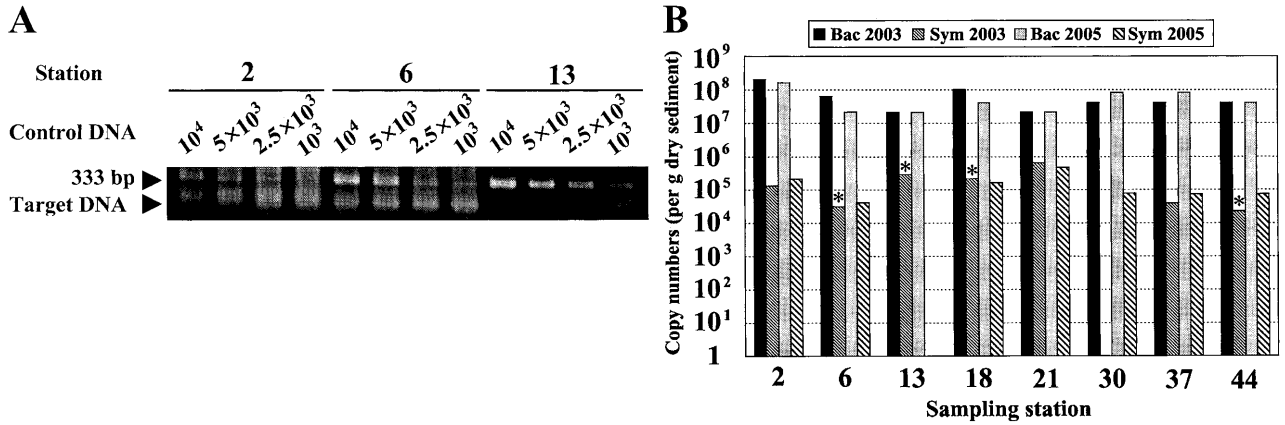

Fig. 4. Estimation of the copy numbers of the 16S rRNA gene of all eubacteria in the sediment samples. The same DNA extracts used in Fig. 3 and Table 3 were used as templates in quantitative PCR. (A) Representative results of quantitative PCR. The upper PCR products (333bp) were amplified from the control DNA fragment, whereas the lower PCR products were amplified from the 16S rRNA gene on chromosomal DNA extracted from all eubacteria in the sediment samples. The values above photographs indicate copy numbers of the control DNA fragment added. (B) 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. 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'. The endosymbiont A 16S rRNA gene was not detected in two samples, #13 in 2005 and #30 in 2003. 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.

ubiquitously at the bottom of Tsukumo Bay. However, *O. mashikoi* adult worms inhabit the mud, not sand, in Tsukumo Bay. Although we thoroughly examined whether *O. mashikoi* adult worms live in sandy areas by intensively collecting sediment samples from 20 points around station #24 in which many free-living cells of the endosymbiont A-related phylotype were found, no adult worms of *O. mashikoi* were found, suggesting that *O. mashikoi* can not inhabit areas covered with sand. Therefore, in addition to free-living cells of the endosymbionts, other factors may be required for the growth of *O. mashikoi*. Judging from the morphology of the endosymbiotic cells[11] and the existence of the extracellular giant hemoglobin, which is thought to convey sulfides to the endosymbionts[29], the *O. mashikoi* endosymbionts seem to be sulfur-oxidizing bacteria. Total sulfide levels in sediment collected from a region in which *O. mashikoi* adults live were moderate[36]. Physical and/or chemical conditions, such as temperature, sulfide concentration, and pH of the sediment, may be important for determining the distribution of *O. mashikoi*, not the distribution of endosymbionts.

References

1) Acinas, S.G., L.A. Marcelino, V. Klepac-Ceraj, and M.F. Polz. 2004. Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. J. Bacteriol. **186**:2629–2635.
2) Asami, H., M. Aida, and K. Watanabe. 2005. Accelerated sulfur cycle in coastal marine sediment beneath areas of intensive shellfish aquaculture. Appl. Environ. Microbiol. **71**:2925–2933.
3) Bright, M., and O. Giere. 2005. Microbial symbiosis in Annelida. Symbiosis **38**:1–45.
4) Callsen-Cencic, P., and H.J. Flügel. 1995. Larval development and the formation of the gut of *Siboglinum poseidoni* Flügel & Langhof (Pogonophora, Perviata). Evidence of protostomian affinity. Sarsia **80**:73–89.
5) Cary, S.C. 1994. Vertical transmission of a chemoautotrophic symbiont in the protobranch bivalve, *Solemya reidi*. Mol. Mar. Biol. Biotechnol. **3**:121–130.
6) Cary, S.C., H. Felbeck, and N.D. Holland. 1989. Observations on the reproductive biology of the hydrothermal vent tube worm *Riftia pachyptila*. Mar. Ecol. Prog. Ser. **52**:89–94.
7) Cary, S.C., and S.J. Giovannoni. 1993. Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. Proc. Natl. Acad. Sci. USA **90**:5695–5699.
8) Cary, S.C., W. Warren, E. Anderson, and S.J. Giovannoni. 1993. Identification and localization of bacterial endosymbionts in hydrothermal vent taxa with symbiont-specific polymerase chain reaction amplification and *in situ* hybridization techniques. Mol. Mar. Biol. Biotechnol. **2**:51–62.
9) Cavanaugh, C.M. 1983. Symbiotic chemoautotrophic bacteria in marine invertebrates from sulphide-rich habitats. Nature **302**:58–61.
10) Cavanaugh, C.M., S.L. Gardiner, M.L. Jones, H.W. Jannasch, and J.B. Waterbury. 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: Possible chemoautotrophic symbionts. Science **213**:340–342.
11) Deguchi, M., N. Kubota, A. Matsuno, M. Kanemori, Y. Fukumori, and Y. Sasayama. 2007. Actual distribution of bacteriocytes in the trophosome of a beard worm (*Oligobrachia mashikoi*, Siboglinidae, Annelida): Clarification using whole-mount *in situ* hybridization. Acta. Zool. **88**:129–135.
12) DeLoney-Marino, C.R., A.J. Wolfe, and K.L. Visick. 2003. Chemoattraction of *Vibrio fischeri* to serine, nucleosides, and *N*-acetylneuraminic acid, a component of squid light-organ mucus. Appl. Environ. Microbiol. **69**:7527–7530.
13) Flügel, H.J., and I. Langhof. 1983. A new hermaphroditic pogonophore from the Skagerrak. Sarsia **68**:131–138.
14) Grimes, D.J., Mills, A.L., and Nealson, K.H. 2000. The importance of viable but nonculturable bacteria in biogeochemistry, p. 209–227. *In* R.R. Colwell and D.J. Grimes (ed.), Nonculturable microorganisms in the environment. American Society Microbiology, Washington, D.C.
15) Gros. O., A. Darrasse, P. Durand, L. Frenkiel, and M. Mouëza. 1996. Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. Appl. Environ. Microbiol. **62**:2324–2330.
16) Halanych, K.M. 2005. Molecular phylogeny of siboglinids annelids (a.k.a. pogonophorans): a review. Hydrobiologia. **535/536**:297–307.
17) Imajima, M. 1973. A new species of the genus *Oligobrachia* (Pogonophora) collected from Tsukumo Bay, Noto Peninsula. Ann. Rep. Noto. Mar. Lab. **13**:7–12. (In Japanese with English summary)
18) Jacobsen, C.S., and O.F. Rasmussen. 1992. Development and application of a new method to extract bacterial DNA from soil based on separation of bacteria from soil with cation-exchange resin. Appl. Environ. Microbiol. **58**:2458–2462.
19) Jones, M.L., and S.L. Gardiner. 1985. Light and scanning electron

microscopic studies of spermatogenesis in the vestimentiferan tube worm *Riftia pachyptila* (Pogonophora: Obturata). Trans. Am. Microsc. Soc. **104**:1–18.
20) Jones, M.L., and S.L. Gardiner. 1988. Evidence for a transient digestive tract in vestimentifera. Proc. Biol. Soc. Wash. **101**:423–433.
21) Kanemori, M., K. Nishihara, H. Yanagi, and T. Yura. 1997. Synergistic roles of HslVU and other ATP-dependent proteases in controlling in vivo turnover of σ^{32} and abnormal proteins in *Escherichia coli*. J. Bacteriol. **179**:7219–7225.
22) Kimura, H., M. Sato, Y. Sasayama, and T. Naganuma. 2003. Molecular characterization and *in situ* localization of endosymbiotic 16S ribosomal RNA and RuBisCO genes in the pogonophoran tissue. Mar. Biotechnol. **5**:261–269.
23) Klappenbach, J.A., P.R. Saxman, J.R. Cole, and T.M. Schmidt. 2001. rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. **29**:181–184.
24) Krueger, D.M., R.G. Gustafson, and C.M. Cavanaugh. 1996. Vertical transmission of chemoautotrophic symbionts in the bivalve *Solemya velum* (Bivalvia: Protobranchia). Biol. Bull. **190**:195–202.
25) Kubota, N., M. Kanemori, Y. Sasayama, M. Aida, and Y. Fukumori. 2007. Identification of endosymbionts in *Oligobrachia mashikoi* (Siboglinidae, Annelida). Microbes Environ. **22**:136–144.
26) Kuske, C.R., K.L. Banton, D.L. Adorada, P.C. Stark, K.K. Hill, and P.J. Jackson. 1998. Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol. **64**:2463–2472.
27) Lennox, E.S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology. **1**:190–206.
28) Mummey, D.L., and P.D. Stahl. 2003. Candidate division BD: Phylogeny, distribution and abundance in soil ecosystems. System. Appl. Microbiol. **26**:228–235.
29) Numoto, N., T. Nakagawa, A. Kita, Y. Sasayama, Y. Fukumori, and K. Miki. 2005. Structure of an extracellular giant hemoglobin of the gutless beard worm *Oligobrachia mashikoi*. Proc. Natl. Acad. Sci. USA **102**:14521–14526.
30) Nussbaumer, A.D., C.R. Fisher, and M. Bright. 2006. Horizontal endosymbiont transmission in hydrothermal vent tubeworms. Nature **441**:345–348.
31) Nyholm, S.V., E.V. Stabb, E.G. Ruby, and M.J. McFall-Ngai. 2000. Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. Proc. Natl. Acad. Sci. USA **97**:10231–10235.
32) Okuda, Y., K. Denda, and Y. Fukumori. 1996. Cloning and sequencing of a gene encoding a new member of the tetratricopeptide protein family from magnetosomes of *Magnetospirillum magnetotacticum*. Gene **171**:99–102.
33) Ott, J., M. Bright, and S. Bulgheresi. 2004. Symbioses between marine nematodes and sulfur-oxidizing chemoautotrophic bacteria. Symbiosis **36**:103–126.
34) Rouse, G.W. 2001. A cladistic analysis of Siboglinidae Caullery, 1914 (Polychaeta, Annelida): formerly the phyla Pogonophora and Vestimentifera. Zool. J. Linn. Soc. **132**:55–80.
35) Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
36) Sasayama, Y., Y. Higashide, M. Sakai, M. Matada, and Y. Fukumori. 2007. Relationship between the lifestyle of a siboglinid (pogonophoran) polychaete, *Oligobrachia mashikoi*, and the total sulfide and nitrogen levels in its habitat. Zool. Sci. **24**:131–136.
37) Sasayama, Y., M. Matada, Y. Fukumori, M. Umebayashi, A. Matsuno, T. Nakagawa, and M. Imajima. 2003. External morphology of the posterior end, the 'Opisthosoma', of the beard worm *Oligobrachia mashikoi* (Pogonophora). Zool. Sci. **20**:1411–1416.
38) Southward, A.J., E.C. Southward, P.R. Dando, G.H. Rau, H. Felbeck, and H. Flügel. 1981. Bacterial symbionts and low $^{13}C/^{12}C$ ratios in tissues of Pogonophora indicate unusual nutrition and metabolism. Nature **293**:616–620.
39) Southward, E.C. 1982. Bacterial symbionts in Pogonophora. J. Mar. Biol. Ass. U.K. **62**:889–906.
40) Southward, E.C. 1988. Development of the gut and segmentation of newly settled stages of *Ridgeia* (Vestimentifera): Implications for relationship between Vestimentifera and Pogonophora. J. Mar. Biol. Ass. U.K. **68**:465–487.
41) Southward, E.C., A. Schulze, and S.L. Gardiner. 2005. Pogonophora (Annelida): form and function. Hydrobiologia **535/536**:227–251.
42) Stewart, F.J., I.L.G. Newton, and C.M. Cavanaugh. 2005. Chemosynthetic endosymbioses: adaptations to oxic-anoxic interfaces. Trends Microbiol. **13**:439–448.
43) Tsai, Y., and B.H. Olson. 1992. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. Appl. Environ. Microbiol. **58**:2292–2295.
44) Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63**:3741–3751.