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Estimation of Abundance and Diversity of Bacteria Associating with Burrow Lining of *Capitella* sp. I in Seawater Soft-Agar Microcosm

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Abstract—An annelid polychaetes, *Capitella* sp. I is one of the most common components of macro faunal communities in organically enriched, sulfide-rich marine sediments. The burrow and protruding tubes made by the polychaetes have been known to function as oxidation/reduction boundaries on which numbers of metabolically active bacteria colonize, and therefore play central roles in material cycles and energy flows in the soft bottom environments. In order to obtain insights into the abundance and structure of bacterial community on burrow lining of the worm, we employed experimental microcosms composed of soft-agar containing ca. 5 mM sodium sulfide to mimic sulfidic sediment inhabited by *Capitella* sp. I. Direct counting of the bacterial cells retrieved from burrow in the agar microcosms revealed that up to nearly 10^7 bacteria cells per mm^2 were found on the burrow lining. DNA extraction and subsequent amplification of 16Sr RNA gene from microbial cells on the burrow enabled clone analyses of bacterial community present on the burrow lining of *Capitella* sp. I. Preliminary results demonstrated that gamma proteobacteria was the most abundant phylogenetic component of the bacterial community. Alpha- and epsilon proteobacteria, and CFB group bacteria were nearly equally contributing to the remaining bacterial flora. These results imply that burrow lining of *Capitella* sp. I harbors highly abundant and phylogenetically diverse bacterial cells, which is consistent with the previous reports on mutual relationship between bacteria and the worm.

Keywords: *Capitella* sp. I, burrow, bacterial abundance, sulfur oxidizing bacteria, *Arcobacter*

1. INTRODUCTION

In marine sediments, diverse types of micro- and macro benthic organisms (benthos) reside and interact with one another. Burrowing and irrigating activities of the macro benthos, such as polychates and crustaceans, often create oxic/anoxic interfaces in otherwise totally anoxic subsurface sediment, whereby enhanced metabolic processes of microorganisms, mainly bacteria, can be achieved over the burrow wall and nearby sediments (Aller, 1982). In some cases, the bacterial biomass itself or organic matter derived from bacteria in return can be exploited by the benthos as nutrition (Hylleberg, 1975; Tsutsumi et al., 2001). Therefore, interactions between the two organisms should exert substantial impacts on material cycles and energy transfer in the sediment ecosystems.

In order to clarify their ecological importance, we have studied interactions between bacteria and a small annelid polychaete, *Capitella* sp. I. *Capitella* sp. I often dominates the macro faunal communities in sulfide-rich environment of the organically enriched sediments (Pearson and Rosenberg 1978; Diaz and Rosenberg 1991) and actively create numerous burrows in the subsurface and extruding tubes over the surface, forming a burrow network through which oxygenated water overlying the sediment is irrigated into otherwise anoxic subsurface. The irrigation activity of *Capitella* thus facilitates aerobic bacterial activity (Wu et al., 2003) and growth (Alongi, 1985) on the burrow system, which seem to benefit the worms in detoxifying high levels of ammonium or sulfide that had been built up in anoxic subsurface, and/or other hazardous chemicals such as polychlolinated aromatic hydrocarbon (Holmer et al., 1997). Alternatively, bacterial population colonizing over the burrow structures may be of nutritional value for *Capitella* sp. I, as suggested by the long-held concept of "microbial gardening by deposit feeding polychate" (Hylleberg, 1975).

Thus, studies on the microbial activities and community structure over and near the burrow system of *Capitella* sp. I should yield insights into the biogeochemical processes in the inhabited sediment. Due mostly to the small size of the burrow system, however, it is often difficult to subsample regions of interest without destroying the intact structure of the burrow, and therefore least destructive approaches to assess the microbial activities are needed. Wu et al. (2003) reported a sediment microcosm approach in which they visualized microbial activity around the *Capitella* burrow by staining with a tetrazolium dye, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT). It was demonstrated that INT was intensively reduced to form red crystals of INT-formazan by bacterial cells on the burrow lining, extruding tubes, feces, and mucus excreted from the worms. These observations clearly demonstrated versatility of the combination of sediment microcosm and histochemical approach for studying aerobic respiratory metabolism of the bacteria on the *Capitella*'s burrow system.

Wada et al. (2006a) modified this approach by using 0.7% soft-agar as a substitute for sediment in order to improve spatial resolution of visual assessment of, and to gain better access to the area of high microbial activity on the burrow system of *Capitella* sp. I. As the soft-agar microcosm is simple in its constituents, impacts of a given chemical on microbes and host organisms can be easily assessed.

Therefore, the soft-agar microcosms are considered to be better suited for estimating the burrow-associated microbial biomass and activity. Taking advantage of this, Wada et al. (2006b) found that the burrowing activity of *Capitella* and thus the respiratory activity of bacteria in the subsurface were enhanced in the presence of sodium sulfide up to 5 mM. Under the similar condition, they found "tiny white granules (Wada et al., 2006b)" among the INT-formazan positive bacterial cells (therefore respiratory active) on the mucus layer and the burrow lining, which suggested sulfide oxidation to elemental sulfur actively took place around these micro-environments.

In the current study, we applied this microcosm to quantify bacterial abundance on the burrow lining by a direct counting method and used molecular techniques to unveil the bacterial community structure that associated with *Capitella* sp. I in the soft-agar microcosms containing sodium sulfide. Based on the results, we discussed ecological implications of the bacterial community associating with *Capitella* sp. I.

2. MATERIAL AND METHODS

Construction of seawater soft-agar microcosms

We made 0.7% soft-agar microcosms containing 5 mM sodium sulfide according to Wada et al. (2006b) with slight modifications. Briefly, 0.24 g $Na_2S{\cdot}9H_2O$ was dissolved in 100 mL seawater (30 psu), filtered through 0.22 μm pore size filter, and mixed with equal volume of 1.4% seawater agar molten that had been autoclaved and cooled down to ca. 50°C. The final concentration of sulfide in the soft-agar was 5 mM. We dispensed the sulfide-containing agar molten (20 mL) into glass tubes (35 mm in diameter) with a silicon stopper at the bottom, allowed the agar solidified over night and poured filter-sterilized seawater (30 mL) onto the top of the solidified agar.

Laboratory cultivation and handling of Capitella *sp. I*

Capitella sp. I used for the experiments in the present study was originally collected from the sediment of Kusuura Bay, Amakusa, Kumamoto, Japan. The worms have been reared in seawater (30 psu) and sediment under the laboratory condition at 20°C, being fed periodically with moist fish food pellets. Prior to the following experiments, we sieved the sediment of the containers to collect the worms and left them in sterile seawater for overnight at 20°C under the dark condition. The *Capitella* worms that seemed in good condition were then gently picked up with sterile forceps and carefully rinsed several times in sterile seawater just before introducing into the agar microcosms described above.

Microcosm Experiment I

We initiated an experiment to estimate abundance of bacterial cells on burrow lining of *Capitella* sp. I developed in the soft-agar microcosms containing sodium sulfide. A total of 15 individuals of *Capitella* sp. I was placed onto the top of soft-agar and incubated for 3 days at 20°C with aeration of 0.22 μm-filtered air in dark

conditions. In order to minimize evaporation due to the aeration in the overlying water, head space of each microcosm was sealed with a plastic film. The volume and the salinity of the water were manually adjusted if necessary throughout the incubation period. This experiment was conducted in duplicate microcosms.

At the end of the incubation period, overlying water was discarded and a whole soft-agar was aseptically extruded, transected as slices with 0.5 cm thickness with a stainless steel blade. The agar slices were preserved in sterile saline solution (0.9% NaCl) containing 10% formaldehyde (v/v) and kept at 4°C for a week. Each of the preserved agar slices was then stained with 10 mL of filter sterilized 0.1% crystal violet solution for 10 min or more (up to 1 hour). The stained agar slices were rinsed with an extensive volume of filter sterilized DW (more than 100 mL per agar slice). We carefully trimmed agar area surrounding the burrow lining into small cubes (ca. 20 μg in weight, approximately 2–3 mm on each side), held each agar specimen in position so as to make sure the burrow align on the focal plane as much as possible under a dissecting microscope (SZX-12, Olympus) and photographed the burrow. A total of 11 agar cubes was produced and kept in micro tubes (1.5 mL/tube). The images of the agar cubes were analyzed by Image J 1.33 package (http://rsb.info.nih.gov/ij/index.html) to estimate the burrow areas and express them in mm^2. Each of the agar cubes was then suspended in 0.5 mL of 0.9% NaCl solution, melted at 98°C for 3 min and cooled down to a room temperature (ca. 23°C). After 0.01 mL of filter sterilized TritonX-100 solution (0.5% v/v) was added, the melted agar suspension was dispersed by a sonicator (26W with a microtip of 2 mm in diameter, UD-200, TOMY, Japan) for 15 sec on ice. Each of the dispersed suspensions was then stained with 10 μL of 0.01% DAPI solution for 30 min. The resultant suspensions were finally filtered through 0.22 μm black polycarbonate filters (Nuclepore). The bacterial cells on the filters were counted with an epifluorescent microscope (Olympus BX50) to give more than 400 cells for at least 10 randomly chosen microscopic fields. The numbers of bacterial cells on the filters were further divided by the estimated area of the burrow lining to give bacterial population density as cells/mm^2. Bacterial abundance in overlying water of the microcosms was also estimated by the direct cell count method of Porter and Feig (1980). Five mL seawater overlying the microcosms at beginning and the end of the incubation period was sub-sampled and used for the counting.

Microcosm Experiment II

We conducted another set of experiment using the soft-agar microcosm to reveal the bacterial community structure on the burrow lining by molecular cloning techniques. We used duplicate microcosms for each analysis (4 microcosms in total). Microcosms were prepared and incubated in the same way as in the experiment I, except that the microcosms contained 4.2 mM sulfide and were incubated at 18°C for 7 days. At the 7th day, each of the soft-agar was aseptically transected as slices with 0.5 cm thickness with a stainless steel blade. The agar slices were preserved in 70% ethanol. All the preserved agar slices were kept at 4°C until use.

DNA extraction, clone library construction and sequencing

Genomic DNA of microbial cells present on burrow lining in the sliced agar (total volume ≈ 50 μL) was extracted by using InstaGene matrix (Bio-rad). Sliced agar samples were soaked in 200 μL of well mixed InstaGene matrix and incubated for 30 min at 56°C. Agar samples were then heated for 8 min at 100°C, centrifuged for 5 min at 12,000 rpm and supernatants were recovered into 1.5 mL tubes for storage at –20°C. For comparison, DNA was also extracted from microbial cells on fecal pellets and a single specimen of *Capitella* sp. I in the same way described above.

About 560 bp 16S rRNA gene sequences covering V3-V6 hyper variable region were then PCR amplified from the extracted DNA as a temperate by using the domain bacteria-specific oligonulceotide primer 341F (5′ CCT ACG GGA GGC AGC AG 3′) and 907R (5′CCG TCA ATT CMT TTG AGT TT 3′) (Muyzer, 1998). The PCR system consisted of the following: 1 μL template DNA, 2.5 U of Z-Taq DNA polymerase (Takara), 1× Z-Taq buffer, each deoxynucleotide triphosphate at a concentration of 0.2 mM, and 0.2 μM of each primer in 50 μL mixture. Thermal cycling was carried out with the following protocol: 30 cycles of 94°C for 5 second, 55°C for 10 second, 72°C for 20 second in PTC-100 thermal cycler (Bio-rad). In order to avoid PCR bias, each sample was prepared in triplicate. The triplicate PCR amplimers of each sample, 150 μL in total, were pooled together, ligated into the pTAC-1 vector, and transformed into *E. coli* strain DH 5α jet competent cell according to the manufacturer's instruction (TA PCR Cloning Kit, BioDynamics). Transformants were screened using blue-white selection on LB agar plate containing X-gal and 100 μg mL^{-1} ampicillin. M13 primers provided within this cloning kit were used to check the correct insertion. PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (USB), and then used for bidirectional sequencing with flanking vector primer M13BDFw (5′CAG GGT TTT CCC AGT CAC GAC 3′) and M13BDRev (5′CGG ATA ACA ATT TCA CAC AGG 3′). DNA sequencing was performed on an ABI 3130 genetic analyzer (Applied Biosystems) with BigDye terminator version 3.1 cycle sequencing kit. Sequences were screened for similarity with other published bacterial 16S rRNA gene sequences at the NCBI database (http://www.ncbi.nlm.nih.gov) to determine their taxonomical affiliation (Altschul et al., 1997).

3. RESULTS AND DISCUSSION

Bacterial abundance on the burrow lining of Capitella *sp. I*

During the incubation periods of experiment I, *Capitella* worms were frequently found as an aggregate in the top layer (<5 mm depth) of the agar, while they sometimes move along burrow path by stretching and contracting their body down to the subsurface of 3 cm depth. After the agar microcosms were sliced, burrow paths of *Capitella* sp. I were made visible by crystal violet staining. Due to elastic nature of agar matrix, most of the burrow structures preserved in agar were flattened in shape from one end to the other. Estimated numbers of bacterial cells on the burrow linings were summarized in Table 1. Eight out of eleven agar cubes examined produced

Table 1. Bacterial abundance on burrow lining of *Capitella* sp. I in agar microcosm amended with 5 mM Na_2S.

Depth range	Bacterial abundance on burrow lining (cells/mm^2)			
(cm)	Average (stdev)	Max	Min	*n*
1 – 1.5	1.09×10^6			1
1.5 – 2	1.08×10^6 (4.2×10^5)	1.53×10^6	7.0×10^5	3
2 – 2.5	2.44×10^6 (3.6×10^6)	7.75×10^6	2.07×10^4	4

countable bacterial cells under an epifluorescence microscope. Maximum number (7.8×10^6 cells/mm^2) was found in the burrow lining that had been in contact with a specimen of *Capitella* sp. I from the time of fixation, while mimimum number (2.1×10^4 cells/mm^2) was found on another burrow lining in the same agar slice. Except for these values, bacterial abundance on burrow lining was rather constant (on average 1.05×10^6 cells/mm^2) within 1–2.5 cm depth.

The present results demonstrates that bacterial abundance on burrow lining of *Capitella* worms was comparable to that found on other substrata, such as tissue of Mediterranean coral, *Oculina patagonica* (8×10^6 cells/mm^2, Koren and Rosenberg, 2006) or artificial ceramic plates in stream (1.3×10^5 to 1.1×10^6 cells/mm^2, Besemer et al., 2007). On the other hand, as bacterial abundance on burrow wall sediment of other benthic animals has been expressed as cells per unit weight or volume of sediment mass near burrow lining, direct comparison of the data can not be made. However, if we assume a burrow lining of the present study has 1 mm thickness, bacterial abundance were to range from 2×10^7 to 7.8×10^9 cells/cm^3, which is comparable to that reported for the burrow of Tylorrhynchus polychaete (Satoh et al., 2007).

Bacterial community structure on burrow lining of Capitella sp. I

A total of 70 clones were analyzed for the bacterial community on burrow lining, fecal pellets and body of *Capitella* sp. I (Table 2). Among the 23 clones analyzed for the bacterial community on burrow lining, clones belonging to gamma-proteobacteria were most abundant (39%). Alpha and epsilon proteobacteria, and CFB group clones were equally contributing to the remaining clones. Similar patterns of bacterial diversity were seen for the clones from the bacterial community of *Capitella* body. On the other hand, more than half (57%) of the clones from fecal pellets were gamma, and the remaining fecal clones did not include epsilon proteobacteria, but were nearly equally occupied by alpha, beta, delta and CFB.

Clone analysis of the bacterial community on burrow, fecal pellets and the body of *Capitella* sp. I in the present study suggests at least two types of mutualistic interactions exist between bacteria and the worm. Firstly, the fact that more than half of the gamma clones examined (57%) were closely related to species of Vibrionacea was consistent with a well-known phenomenun that Vibrios comprise one of the

Table 2. Number of clones and their phylogenetic affiliation of the bacterial community on burrow, fecal pellets and body of *Capitella* sp. I.

Phylogenetic group	Burrow	Fecal pellets	*Capitella* body
Proteobacteria			
alpha	5	3	5
beta	0	2	0
gamma*	9	13	8
delta	0	2	2
epsilon**	4	0	4
CFB	5	3	5
Total	23	23	24

*Nineteen out of the 30 clones were closely related to strains of Vibrionacea.
**All of the 8 clones were closely related to a sulfur oxidizing bacterium, *Arcobacter sulfidicus*.

common components of the bacterial flora of marine animals. Due to their versatility in hydrolyzing various organic compounds, they may help their host (*Capitella*) digest complex organic polymers, thus contributing efficient loss of organics in the presence of the worms in the organically enriched sediments. Secondly, presence of eight clones belonging to epsilon proteobacteria that were closely related to an unique sulfur oxidizing bacterium, *Arcobacter sulfidicus* (Wirsen et al., 2002) suggests that part of the bacterial community on burrow lining and body of *Capitella* sp. I are capable of oxidizing inorganic sulfur as chemo-lithotrophic microorganisms. This is in support of the previous reports that described possible nutritional exploitation of sulfur oxidizing bacteria by *Capitella* sp. I in the presence of high levels of sulfide (Tsutsumi et al., 2001). It is likely that sulfur oxidizing bacterial community of epsilon proteobacteria developed on burrow lining can serve as food for the worms.

In conclusion, it is demonstrated that soft-agar microcosm approach allowed us to quantify bacterial abundance on the burrow lining and to reveal the bacterial community structure associating with *Capitella* sp. I under the conditions similar to sulfidic sediment in natural environments. It is of course necessary to interpret the results obtained from the microcosm experiments with cautions, as we do not know how agar might ever alter the behavior of the animals and the resultant effects on microbial community to be examined. Nevertheless, the present data seem to be consistent with the previous data obtained in natural sediment environments. Therefore, *Capitella* sp. I in a soft-agar microcosm should be regarded as a experimental model system that provide fundamental insights into the interaction between *Capitella* and the associated bacterial population.

REFERENCES

Aller, R. C. 1982. The effects of macrobenthos on chemical properties of marine sediment and overlying water. Animal-sediment relations, the biogenic alteration of sediments. *Topics in Geobiology* **2**: 53–102.

Alongi, D. M. 1985. Microbes, meiofauna, and bacterial productivity on tubes constructed by the polychaete *Capitella capitata*. *Marine Ecology Progress Series* **23**: 207–208.

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipmam. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Besemer, K., G. Singer, R. Limberger, A.-K. Chlup, G. Hochedlinger, I. Hödl, C. Baranyi and T. J. Battin. 2007. Biophysical controls on community succession in stream biofilms. *Applied and Environmental Microbiology* **73**: 4966–4974.

Diaz, R. J. and R. Rosenberg. 1991. Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna. *Oceanography and Marine Biology: An Annual Review* **33**: 245–303.

Holmer, M., V.E. Forbes and T.L. Forbes. 1997. Impact of the polychaete *Capitella* sp. I on microbial activity in an organic-rich marine sediment contaminated with the polycyclic aromatic hydrocarbon fluoranthene. *Marine Biology* **128**: 679–688.

Hylleberg, J. 1975. Selective feeding by *Abarenicola pacifica* with notes on *Abarenicola vagabunda* and a concept of gardening in lugworms. *Ophelia* **14**: 113–137.

Koren, O. and E. Rosenberg. 2006. Bacteria associated with mucus and tissues of the coral *Oculina patagonica* in summer and winter. *Applied and Environmental Microbiology* **72**: 5254–5259.

Muyzer, G. 1998. Structure, function and dynamics of microbial communities: the molecular biological approach. In: *Advances in Molecular Ecology*, G.R. Carvalho (Ed.), NATO Science Series, pp. 87–117.

Pearson, T. H. and R. Rosenberg. 1978. Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. *Oceanography and Marine Biology: An Annual Review* **16**: 229–311.

Porter, K. G. and Y. S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* **25**: 943–948.

Satoh, H., Y. Nakamura and S. Okabe. 2007. Influences of Infaunal Burrows on the Community Structure and Activity of Ammonia-Oxidizing Bacteria in Intertidal Sediments. *Applied and Environmental Microbiology* **73**: 1341–1348.

Tsustumi, H., S. Wainright, S. Montani, M. Saga, S. Ichihara and K. Kogure. 2001. Exploitation of a chemosynthetic food resource by a polychaete, *Capitella* sp. I. *Marine Ecology Progress Series* **216**: 119–127.

Wada, M., S. S. Wu, H. Tsutsumi, K. Kita-tsukamoto, H. K. Do, H. Nomura, K. Ohwada and K. Kogure. 2006a. Visual assessment of the respiring microorganisms associated with burrow structures of *Capitella* sp. I in seawater soft-agar microcosms. *Plankton Benthos Research* **1**: 54–58.

Wada, M., S. S. Wu, H. Tsutsumi, K. Kita-tsukamoto, H. K. Do, H. Nomura, K. Ohwada and K. Kogure. 2006b. Effects of sodium sulfide on burrowing activity of *Capitella* sp. I and bacterial respiratory activity in seawater soft-agar microcosms. *Plankton Benthos Research* **1**: 117–122.

Wirsen, C. O., S. M. Sievert, C. M. Cavanaugh, S. J. Molyneaux, A. Ahmad, L. T. Taylor, E. F. DeLong and C. D. Taylor. 2002. Characterization of an Autotrophic Sulfide-Oxidizing Marine *Arcobacter* sp. That Produces Filamentous Sulfur" *Applied and Environmental Microbiology* **68**: 316–325.

Wu, S. S., H. Tsutsumi, K. Kita-Tsukamoto, K. Kogure, K. Ohwada and M. Wada. 2003. Visualization of the respiring bacteria in sediments inhabited by *Capitella* sp. I. *Fisheries Science* **69**: 170–175.

M. Wada (e-mail: miwada@nagasaki-u.ac.jp), Z. Dan, H.-K. Do, H. Nomura, T. Kunihiro, M. Nishimura, K. Kogure, K. Ohwada and H. Tsutsumi