In Situ Determination of Bacterial Growth in Mixing Zone of Hydrothermal Vent Field on the Hatoma Knoll, Southern Okinawa Trough

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Hiroyuki Yamamoto, Tomoko Maruyama, László G.-Tóth, Kenji Kato, Yasuo Furushima, Naoto Taira, Yoshiaki Maeda, and Kiminori Shitashima

Abstract

The doubling time of indigenous bacteria in mixing-zone of hydrothermal fluid and seawater was determined using a diffusion chamber unit deployed on the field of Hatoma Knoll (24° 51.50′N, 123° 50.50′ E), which is a submarine volcano located on southern Okinawa Trough. The diffusion chamber is a reliable tool to incubate and to directly measure the microbial growth under in situ condition of deep-sea, although an operation of submersible and a complicated preparation of seed water became the technical constraints. The doubling time at non-vent site distant from active vent site was estimated from 86 to 110 h, while at active vent sites more rapid doubling time, 21–32 h, were estimated. A potential sulfur-oxidizing bacteria belonging to *Epsilonproteobacteria* dominated the population grew in the chambers, which were incubated using the plume water obtained from the mixing zone between the vent fluid and seawater, and Bathymodiolus colony, while no detection of *Gammaproteobacteria*. The methane-oxidizing bacteria were detected only from gill and digestive tract of *Bathymodiolus platifrons*, and could not be detected from the chamber, although the chamber was placed on Bathymodiolus colony. The results of this study suggested that chemolithoautotrophic growth near by the hydrothermal vent is sustained by the rapid doubling time of *Epsilonproteobacteria* using chemical species dissolved in fluid and provides the chemo-autotrophic product to deep-sea benthopelagic community, as well as a microbial products in hydrothermal vent plume.

Keywords

Diffusion chamber • Hydrothermal vent • Microbial growth rate • Okinawa Trough

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H. Yamamoto (✉)
Environmental Impact Assessment Research Group, R&D Center for Submarine Resources, Japan Agency for Marine-Earth Science Technology (JMSTEC), 2-15 Natsushima-Cho, Yokosuka, Kanagawa 237-0061, Japan

T. Maruyama • K. Kato
Faculty of Science, Shizuoka University, Shizuoka, Japan

L. G.-Tóth
Faculty of Science, Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

Y. Furushima
Environmental Impact Assessment Research Group, R&D Center for Submarine Resources, Japan Agency for Marine-Earth Science Technology (JMSTEC), Kanagawa, Japan

N. Taira
University of the Ryukyus, Okinawa, Japan

Y. Maeda
Civil Engineering Research and Environmental Studies (CERES Inc.), Chiba, Japan

K. Shitashima
International Institute for Carbon-Neutral Energy Research, Kyushu University, Fukuoka, Japan

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33.1 Introduction

The discovery of high-density animal aggregations in the vicinity of hydrothermal vents of the Galapagos Rift (Corliss et al. 1979; Lonsdale 1977) raised many questions for the mechanism to sustain the ecosystem, especially for the source of primary production. Chemosynthesis of prokaryotes, depending on the inorganic chemical reaction between hydrothermal fluid and seawater, has been recognized major energy source of primary production in the hydrothermal ecosystems (Jannasch and Mottl 1985; Karl et al. 1980). Growth rate of microbial community in hydrothermal system is most useful data to qualify a primary production by chemosynthesis.

Potential chemical energy for the chemolithoautotrophic growth has been calculated by chemical thermodynamics based on geochemical data of hydrothermal fluids (Amend and Shock 1998; Jannasch and Mottl 1985; McCollom 2000; McCollom and Shock 1997; Schmidt et al. 2008). The primary production based on chemical energy sustains the huge biomass in megafauna and their rapid growth rate, for examples, 85 cm/year determined by tube length of *Riftia pachyptila* (Lutz et al. 1994), 10 cm/year growth and long lived of *Lamellibrachia* sp. (Bergquist et al. 2000), a biomass of the mussel bed exceeded 70 kg/m^2 of weight at Logatchev area (Gebruk et al. 2000). The biomass of endosymbiotic bacteria in such invertebrates determined by quinone biomarker suggested sustainable power of chemosynthetic primary production (Yamamoto et al. 2002), despite no data of microbial growth rate directly measured from the symbionts.

Investigation of in situ growth of free-living microbial community in ocean habitats is difficult yet due to the non-culturable or slow growing physiology in majority of marine prokaryotes. Several parameters without traditional cultivation methods have been used to estimate microbial growth rate, e.g. microscopic cell density and cell size, adenosine 5′-triphosphate (ATP), assimilation rate of isotope labeled substrates (Karl et al. 1980; Kirchman 2001; Malmstrom et al. 2005; Torrton and Dufour 1996), microbial mat formation based on time-series observation (Taylor et al. 1999), frequency of dividing cells (Newell and Christian 1981), macromolecular synthesis (Torrton and Dufour 1996), whole-genome microarray analysis (Holmes et al. 2013). These methods were applied in the hydrothermal environments (Jannasch and Mottl 1985; Karl et al. 1980) as well as general oceanic environments, and the calibration methods to convert growth rate or doubling time from these parameters have been developed (Kirchman 2001; Sherr et al. 1999; Torrton and Dufour 1996; Yokokawa et al. 2004).

Another technical issue to determine microbial growth rate in deep-sea hydrothermal vent is an incubation method under in situ physical and chemical conditions. The so-called pressure simulated in situ incubation system equipped in laboratory was used to determine the indigenous heterotrophic marine bacteria isolated from 75 to 5,550 m depth (Carlucci and Williams 1978; Williams and Carlucci 1976). The in situ chamber approach is a convenient method to incubate the indigenous microbes under original habitat condition. It has been applied in deep-sea expeditions for hydrothermal vents, and successfully isolated and/or accumulated novel chemolithotrophic bacteria (Higashi et al. 2004; Reysenbach et al. 2000; Takai et al. 2003). Of theses several types of in situ chambers, a diffusion chamber is a very useful apparatus for in situ physiological experiments of microbes (Bollmann et al. 2007; McFeters and Stuart 1972; Vasconcelos and Swartz 1976), in situ determination of growth rate of phytoplankton (Furnas 1991) and thermophiles in alkaline geothermal pool (Kimura et al. 2010).

In this study, in situ experiments using a diffusion chamber unit were carried out on Hatoma Knoll in southern Okinawa Trough to estimate the state of microbial growth in mixing-zone of hydrothermal vent area on the knoll.

33.2 Materials and Methods

33.2.1 Sample Collection

The in situ incubation experiments were conducted during three cruises, NT01-05, NT02-07, and NT04-03 at hydrothermal and methane seep areas on the southern Ryukyu Archipelago. In these cruise, water samples were collected and prepared for the in situ experiments using diffusion chamber, and deployed the chambers in the following sites; a vent field of the Hatoma Knoll (24° 51.500′ N, 123° 50.500′ E) and a methane seep field of the Kuroshima Knoll (24° 8.000′ N, 124° 11.500′ E). Hatoma Knoll is a submarine volcano located in the southern Okinawa Trough of the southwestern Ryukyu arc. Hydrothermal activity of the Hatoma Knoll has been discovered at a rim and a center mound of horse-shoe shaped caldera on the knoll at 1,490–1,530 m in the deep-sea expedition of 1999 and 2000 (Watanabe 2001). Prominent hydrothermal activity occurs over a central mound and created two big-chimneys with discharge of high temperature fluid up to max. 300 °C (Table 33.1, Suppl. 33.1). These two big-chimneys collapsed in July of 2006. Kuroshima Knoll is a methane seep field located in the south of Ishigaki-island and isolated from island shelf. It was used as reference site for in situ growth chamber experiments in NT04-03 cruise. The gas bubbles were rising from the Bathymodiolus colony of deep-sea mussels at seafloor of 600–700 m in water-depth.

Table 33.1 Physicochemical profile and microbial cell counts in the water column on hydrothermal vent area of Hatoma Knoll determined in the dive expedition of HPD#295, NT04-03

Depth (m)	Temp. (°C)	Salinity (‰)	pH	SiO_2 (mM)	H_2S (mM)	CH_4 (nmol/kg)	delta^{13}C-CH_4 (‰)	total-Hg (ppt)	Turbidity (FTU)	TDC (cells/mL)
5	25.36	34.55	nd	nd	nd	nd	nd	nd	nd	1.9×10^5
1,365	3.94	34.54	7.65	0.112	<1	40	−45.2	14	15	1.4×10^4
1,365	3.93	34.48	7.60	0.109	<1	62	−43.5	13	19	2.4×10^4
1,400	3.91	34.54	7.60	0.089	<1	324	−49.5	24	36	9.6×10^3
1,400	3.91	34.54	7.54	0.126	<1	5,870	−49.5	56	92	2.2×10^4
1,400	3.97	34.54	7.35	0.092	2.021	4,550	−49.7	17	88	1.0×10^4
1,435	3.90	34.54	7.53	0.088	<1	3,120	nd	18	103	1.7×10^4
1,435	3.89	34.54	7.44	0.125	<1	3,140	nd	18	158	1.9×10^4
1,435	3.88	34.54	7.58	0.101	<1	135	nd	16	23	2.5×10^4
1,436	3.89	34.54	7.58	0.096	<1	354	nd	15	22	1.8×10^4
1,461	3.86	34.52	7.52	0.123	<1	1,760	−49.4	25	168	2.0×10^4
1,468	3.86	34.54	7.64	0.094	<1	742	nd	nd	143	nd
1,476	6.24	33.20	6.36	0.132	2.134	45,100	−49.9	37	156	2.8×10^4
1,475	300	nd	5.08	2.157	10.437	nd	nd	nd	nd	nd

nd no data

The water samples were collected either by a Niskin bottle sampler, ORI manifold sampler or syringe type water sampler using the human occupied vehicle (HOV) Shinkai 2000, or remotely operated vehicle (ROV) Hyper-Dolphin 3000. The sample of deep-sea mussel, *Bathymodiolus platifrons*, was collected in NT03-09 to analyze the microbial flora of gill and digestive tract. The video footages recorded whole operations on the experiment sites of NT01-05 and NT02-07 were accessible at the JAMSTEC E-library of Deep-sea Images (http://www.godac.jamstec.go.jp/jedi). The identification codes for video clips are "2K1270SHDB4028", and "2K1353SHDB6021" for Shinkai 2000 dives, respectively. The video clips of ROV dives of 2003 and 2004 are yet preparing in 2013.

33.2.2 Preparation and In Situ Experiment Using a Diffusion Chamber

The diffusion-chamber based approach was applied for determination of microbial growth under in situ condition of deep-sea hydrothermal vent field. The chamber was designed to make a cultivation space (ca. 35 mL) sealed with polycarbonate membrane filter (0.2 μm-pore-size), which permit to exchange chemicals and organic matters between inside and outside of the chamber (Fig. 33.1). The chamber was filled with 0.2 μm filter-sterilized seawater, which was collected from the experimental site. No artificial supplement was added in the chamber. Both of the prepared chamber and the seeds-water were preserved in 4 °C until the time to next dive for chamber experiment. Just before a dive the chambers were inoculated with the seed-water (Fig. 33.1), the chambers set in the canister were settled in the payload container filled with cold seawater, transported by the deep-sea submersibles, and placed on the experiment sites. For this experiment, three kinds of seeds-water specimens were collected respectively from the mixing zone of 1 m heights on vent, within the Bathymodiolus colony, and the surface seawater as negative reference. In the growth experiment, a seed-water filtered by 1 μm pore size membrane filter was prepared to examine an effect of protista predation on microbial growth rate. In the cruise of NT04-03, compact thermometer (MDS-MkV/T, JFE Advantech Co., Ltd.) was attached on the canister for determination of temperature fluctuation during in situ incubation experiments (Suppl. 33.2).

33.2.3 Microbial Population and Growth

The cells fixed with 1 % paraformaldehyde were collected on polycarbonate membrane (0.2 μm-pore-size), and stained with acridine orange or DAPI (Hobbie et al. 1977). The microbial cells were observed using epifluorescence microscopy (BX50, Olympus Corp.) and counted 100 microscopic fields per filter sample.

Doubling time and growth rate constant are index for microbial growth rate, and generally calculated from the data during the exponential growth phase having a constant interval of cell dividing (Powell 1956). Our experiment did not provide the time course population growth and we could not determine the exponential growth phase precisely. In this study, we used the initial and final microbial cell densities by regarding that they were in exponential phase. The incubation time was counted from the time of the chamber deployed on seafloor. The generation time, doubling time,

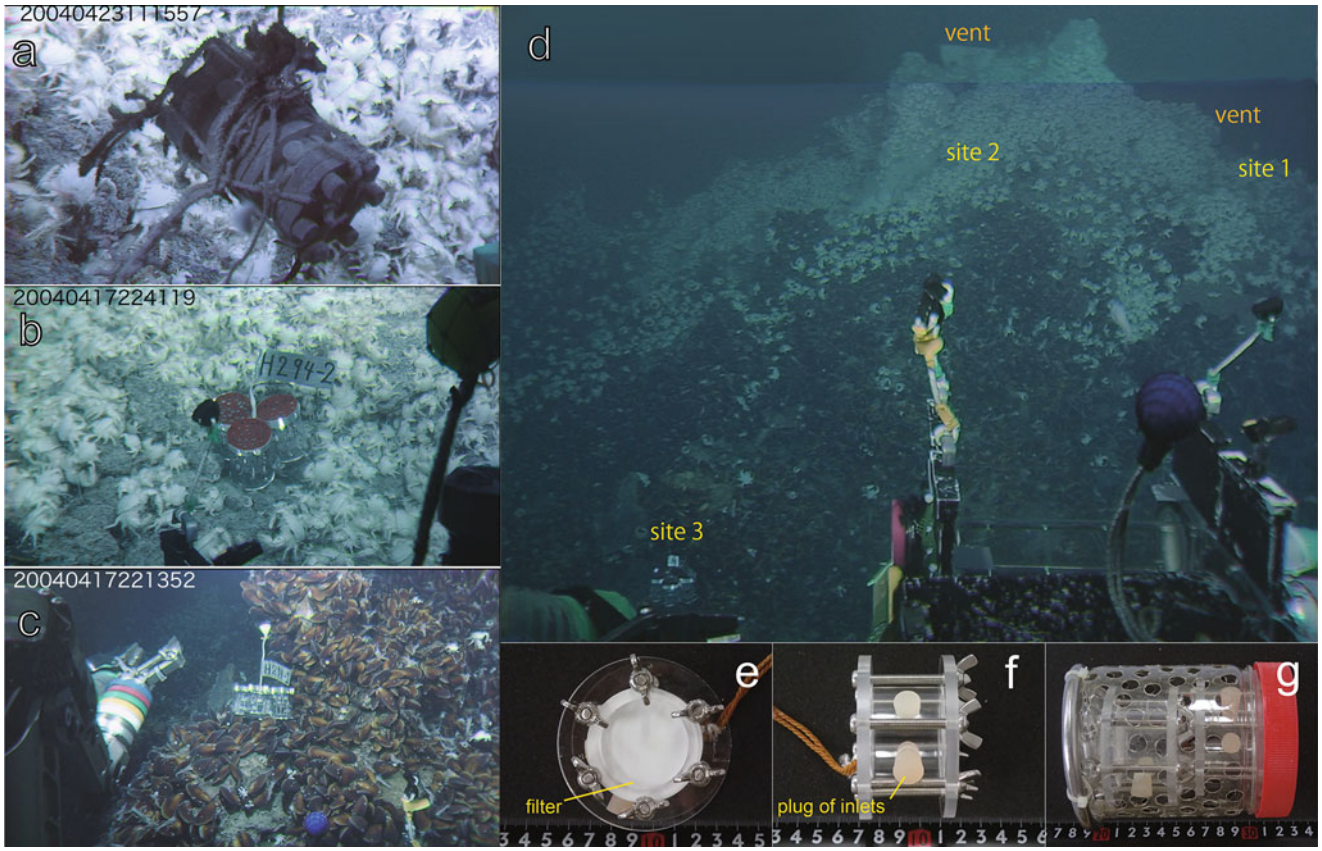

Fig 33.1 In situ experiment conducted in NT04-03 cruise at hydothermal vent of Hatoma Knoll. (**a**) Chamber on hydrothermal vent (site 1), (**b**) canisters contained chambers in aggregation of *Shinkaia crosnieri* (site 2), (**c**) canisters contained chambers settled in Bathymodiolus colony (site 3), (**d**) location of hydrothermal vents and experiments sites on the moud, (**e**, **f**) profile of chamber with membrane filter, and (**g**) punched canisters contained chambers

and growth rate constant are calculated from the following equation:

$$generations = \frac{log_{10}N_t - log_{10}N_0}{log_{10}2}$$

$$doubling\ time = \frac{incubation\ time}{generations}$$

$$growth\ rate\ constant\ (\mu) = \frac{\ln 2}{doubling\ time}$$

N_0: initial cell density, N_t: cell density at the end of indubation

33.2.4 16S rRNA Gene Based Analysis by DGGE and DNA Sequencing

The chamber-incubated-microbes were precipitated by centrifugation. Crude DNA of the microbial cell was extracted by phenol-chloroform-isoamylalcohol solution and purified with a GFX genomic blood DNA purification kit (Amersham Pharmacia biotech, USA). Digestive tract contents and gill tissue specimens were collected from dissected sample of *Bathymodiolus platifrons,* and washed in TE-buffer (10 mM Tris-hydrochloride, 1 mM EDTA (pH 8.0)) and DNA was extracted according to a previously described method (Yamamoto et al. 2002). Denaturing gradient gel electrophoresis (DGGE) was performed on a D-code apparatus (Bio-Rad, Hercules, Calif.). Extracted and purified DNA was used as templates for amplification by the universal DGGE primers targeted to V3 region of 16S rRNA gene of domain Bacteria (341F-GC: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGGGCCTACGGGAGGCAGCAG and 53 4R: 5′-ATTACCGCGGCTGCTGG). The annealing temperature was set at 10 °C above the expected annealing temperature and lowered with 1 °C every second cycle until a touchdown at 55 °C (30 cycle) (Muyzer et al. 1993). PCR products were loaded onto 10 % (wt/vol) polyacrylamide gels (37.5:1, acrylamide-bisacrylamide) in 1× Tris-acetate-EDTA (TAE), (containing 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) with a denaturing gradient ranging from 30 to 55 % denaturant (100 % denaturant contains 7 M urea and 40 % (vol/vol) formamide in 1× TAE). The PCR amplicons were electrophoresed at 60 °C and 200 V for 3.5 h. The gel was

stained with SYBR Green I (Molecular Probes) for 30 min. DGGE bands were excised from the gel and re-amplified using the aforementioned primer set. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Germany). The sequences of the DGGE bands were determined for both strands using an ABI model 377 (Applied Biosystems, USA) or a CEQ2000XL DNA analysis system (Beckman Coulter, USA). The sequence data were checked for chimeric artifacts and were analyzed by a modular system for evolutionary analysis, MESQUITE v2.75 (http://mesquiteproject.org) (Maddison 2004).

33.2.5 Physicochemical Conditions

The conductivity, temperature, and pressure were determined by SBE 911 plus (Sea-Bird Electronics, Inc.) or Micro-CTD (Falmouth Scientific, Inc.). The turbidity was measured by turbidity meter (Seapoint, Inc.). We also used a pH sensor which uses an ion-sensitive field- effect transistor (ISFET) as the pH electrode, and a chloride ion selective electrode (Cl-ISE) as the reference electrode (Shitashima et al. 2008). The concentration of H_2S was measured with colorimetry using the methylene blue method (Guenther et al. 2001). Determination of CH_4 was conducted using an automated CH_4 analysis system (DKK corporation, GAS-1061), which consists of a purge unit, a trap unit, and a gas chromatograph with FID (flame ionization detector) after separation by a packed column (Porapak Q 60/80 mesh, 3 mm i.d. × 4 m) (Ishibashi et al. 1997). Sensitivity of the FID was calibrated every day using a working standard gas from which CH_4 concentration had determined. Dissolved total mercury was determined by cold-vapor atomic absorption spectrometry (CVAAS). Dissolved mercury is reduced to zerovalent Hg (Hg^0) by $SnCl_2$ under the acid condition. The zerovalent mercury ion has low solubility and rapidly vaporized by air bubbling. Vaporized mercury is introduced to detection cell and measured the absorbance in 253.7 nm. We used a CVAAS system (RA-3220, Nippon Instruments Co.) and vaporized procedure was modified with Bloom and Crecelius (1983) (Bloom and Crecelius 1983). 5 mL of seawater sample were mixed with 0.5 mL of 50 % H_2SO_4 (analytical grade, Kanto Chemical) and 0.5 mL of 10 % $SnCl_2$ (analytical grade, Kanto Chemical) in closed glass vial. We started the bubbling with mercury free air and logging of Hg^0 absorbance (253.7 nm) just after the reagents addition. 1,000 ppm $HgCl_2$ standard solution for atomic absorption spectrometry (Kanto Chemical) diluted for calibration. To prevent mercury oxidation and/or reduction during storage of standard solution, L-cysteine was added into each standard solution.

33.2.6 Nucleotide Sequence Accession Numbers

The nucleotide sequences of 16S rRNA genes amplified from the DGGE bands (150–190 bp) have been deposited in the DDBJ database under accession numbers AB207847 to AB207866.

33.3 Results

33.3.1 Environmental Conditions of Hydrothermal Vent Site

Physicochemical factors were measured in NT04-03 cruise. Ambient temperature during in situ incubation on vent fauna habitat fluctuated between 3.9 °C and 4.9 °C with an interval corresponding to tidal cycle (Suppl. 33.2). High concentration of methane (45 μmol kg^{-1}) and hydrogen sulfide (2 mM) dissolved in the vent fluid were detected in the mixing zone of vent side and in the plume of Hatoma Knoll (Table 33.1). The vent plume at 1-m height from vent showed physical and chemical anomaly in cell count, turbidity, total Hg and methane. The high concentration of hydrogen sulfide was detected in the vent fluid and two samples of the hydrothermal plum from 1,476 and 1,400 m. The 324 nmol kg^{-1} of methane concentration was detected, and $\delta^{13}C$ of the methane was range within 49 ‰. A 56 ppt of total Hg was detected even at 1,400 m in water depth. The signatures of hydrothermal fluid clearly declined at water depth of 1,365 m.

33.3.2 Estimation of the Growth Rate Using Diffusion Chamber System

The in situ incubation experiments using the diffusion chambers were carried out in three cruises under different settings (Table 33.2). The onboard preservation time of a prepared chamber and a seeds-water was varied from 16 to 18 h for next submersible dive to deploy the chambers onto seafloor. In the result of NT01-05 cruise, the filtered seed-water induced an effect to shorten the doubling time, and similar effect was detected in the following experiments in NT04-03. The results of NT02-07 showed longer doubling time, 31–32 h, in two times longer incubation days than the case of NT01-05. The results of NT04-03 showed that the most rapid rate, 21 h, was recorded from the chambers placed at vent side, while the growth rate slowed down significantly to 110 h at Bathymodiolus colony in 3–5 m distance from hydrothermal vent (Fig. 33.1). The microbial growth in the chambers inoculated with seed-water from the surface (5 m depth) failed completely under hydrothermal

Table 33.2 In situ experiments of diffusion chamber for determination of microbial growth

Origin of seed-water	Incubation sites	Depth (m)	Temp. (°C)	TDC (cells/mL)		Incubation (day)	DT (h)	GR (day^{-1})
				At initial	At end			
Hatoma Knoll								
NT01-05 (2K1270)								
Colony	Vent side	1,523	150 [*1]	1.9×10^4	3.4×10^6	7	22.5	0.74
Colony	Vent side	1,523	150 [*1]	1.9×10^4	3.7×10^6	7	22.2	0.75
f-colony	Vent side	1,523	150 [*1]	1.6×10^4	3.4×10^6	7	21.9	0.76
f-colony	Vent side	1,523	150 [*1]	1.6×10^4	3.6×10^6	7	21.6	0.77
NT02-07 (2K1354)								
Plume	Vent side	1,473	80 [*1]	4.3×10^3	4.5×10^6	13	31.2	0.53
Plume	vent side	1,473	80 [*1]	4.3×10^3	3.2×10^6	13	32.8	0.51
NT04-03 (HPD294)								
Plume	Vent side	1,477	97.27[*1]	9.2×10^3	9.7×10^5	6	21.4	0.78
Plume	Shinkia site	1,477	4.17	9.2×10^3	2.9×10^4	6	86.6	0.19
Plume	Bathymodiolus site	1,479	3.93	9.2×10^3	2.3×10^4	6	109.8	0.15
Colony	Shinkia site	1,477	4.20	5.5×10^3	1.4×10^4	6	110.2	0.15
f-colony	Shinkia site	1,477	4.20	5.5×10^3	1.7×10^4	6	89.6	0.19
Colony	Bathymodiolus site	1,479	3.90	5.5×10^3	1.5×10^4	6	100.4	0.17
f-colony	Bathymodiolus site	1,479	3.90	5.5×10^3	1.2×10^3	6	−67.6	−0.25
Surface	Bathymodiolus site	1,479	3.90	1.5×10^4	4.7×10^3	6	−86.1	−0.19
f-surface	Bathymodiolus site	1,479	3.90	1.5×10^4	4.4×10^3	6	−81.0	−0.21
Kuroshima Knoll								
NT04-03 (HPD291)								
Seep	Bathymodiolus site	641	7.56	3.3×10^4	5.7×10^6	10	32.4	0.51
Colony	Bathymodiolus site	641	7.56	7.9×10^3	4.5×10^6	10	26.2	0.63
Surface	Bathymodiolus site	641	7.56	3.0×10^4	2.1×10^6	10	39.0	0.43
f-seep	Bathymodiolus site	641	7.56	1.3×10^4	6.8×10^5	10	42.4	0.39
f-colony	Bathymodiolus site	641	7.56	7.8×10^3	5.6×10^6	10	25.3	0.66
f-surface	Bathymodiolus site	641	7.56	4.7×10^4	5.1×10^5	10	69.8	0.24

f-: filtrated by 1 μm pore-sized-filter
TDC total direct count, *DT* doubling time, *GR* growth rate constant
NT: cruise ID of R/V Natsushima, 2K: dive ID of Shinkai 2000, HPD: dive ID of Hyper-Dolphin 3000
[*1]The temperature records on vent side were measured in vent orifice, not in the chamber

vent conditions in 1,449 m depth on Hatoma Knoll, while they grew under methane seep field in 641 m depth on Kuroshima Knoll.

33.3.3 Phylogenetic Analysis of Indigenous Bacteria

The seed-waters contained many bacterial taxon groups and the physicochemical condition in mixing zone accelerated the growth of several group in the chambers (Fig. 33.2). The bacterial phylogeny of 16S rRNA gene isolated in the DGGE bands from the samples of incubated chambers and the specimens of *B. platifrons* shown that three taxa, *Mollicutes*, *Gammaproteobacteria*, and *Epsilonproteobacteria* were dominant groups (Fig. 33.3). The 16S rRNA gene sequences belonging to *Mollicutes* were found only in the specimens from digestive tract of *B. platifrons*. The 16S rRNA gene sequences belonging to methanotroph of *Gammaproteobacteria* were only isolated from *B. platifrons*, and no thioautotroph type of *Gammaproteobacteria* appeared in the chambers incubated under the condition of mixing zone on the vent area. The 16S rRNA gene sequences belonging to *Epsilonproteobacteria* were discovered from specimens of the digestive tract and the chambers incubated with seed water from plume and Bathymodiolus colony.

33.4 Discussion

The data of microbial growth rate of deep-sea zone were previously determined by a simulated in situ incubation system equipped in laboratory. As shown in Table 33.3, the microbial growth rate in ocean gradually declined with water depth (Carlucci and Williams 1978; Lochte and Turley 1988). The oligotrophic growth of indigenous bacteria in unsupplemented seawater of North Pacific Ocean was quite longer doubling time, 145 h, under 1,500 m depth pressured

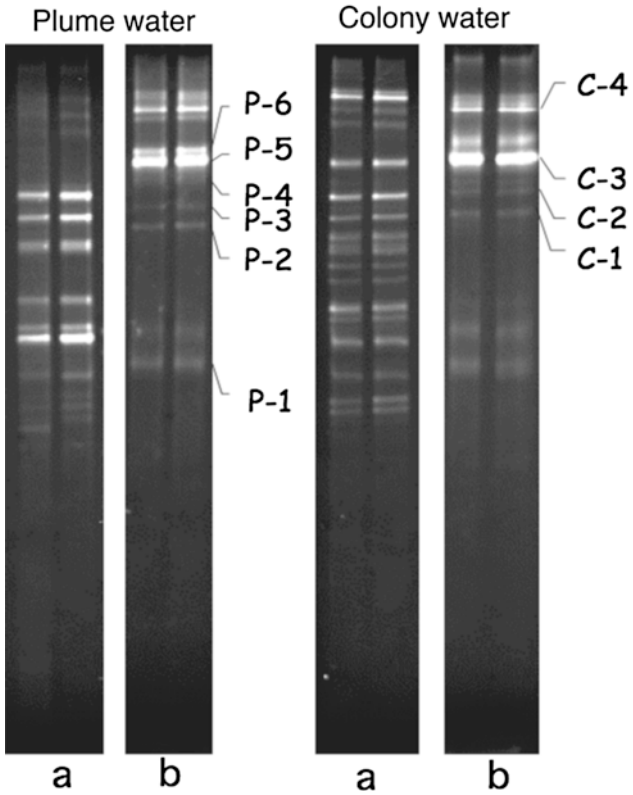

Fig 33.2 DGGE band patterns of the water sample before (**a**) and after (**b**) the incubation with the seeds water collected from plume and Bathymodiolus colony. P is meaning "plume water", C is meaning "colony water". The DGGE bands with ID numbers, P- and C-, in the lane (**b**) were applied to DNA sequencing

condition (Carlucci and Williams 1978). In the same depth zone of hydrothermal vent area in Hatoma Knoll, the doubling time at non-vent site distant from the active vent were estimated from 86 to 110 h, while at active vent sites 21–32 h doubling time were estimated. The phytodetritus and marine snow provides eutrophic microhabitat for microbial community. The microbial growth determined in such microhabitats under simulated deep-sea condition showed quite rapid doubling times from 10 to 11 h (Lochte and Turley 1988). The photosynthetic product is an effective substrate of bacterial community in pelagic ocean, but its efficiency to sustain the population size declined with water depth (Steinberg et al. 2008).

The bacterivorous protozoa are common member of microbial community even in deep-sea zone (Turley and Carstens 1991; Turley et al. 1988). In this study, although any protozoa by microscopic observation could not be observed, probably due to small population size in the sample, the evidence of bacterivorous activity in hydrothermal system was detected as rapid growth rate in the result of chamber incubation inoculated with filtered seedwater, which was eliminated protozoan sized cells. The microbes of surface seawater did not grow in the chambers placed on the hydrothermal vent field, while grow on the methane seep area on Kuroshima Knoll. The toxicity of hydrogen sulfide and mercury (Pracejus and Halbach 1996) in hydrothermal fluid may be an effective agent to regulate the microbial growth, rather than the water depth and temperature.

The growth rate of thermophiles inhabiting an 85 °C geothermal pool has been measured by the similar in situ diffusion chamber tool, and recorded a range from 20 to 39 h doubling time under 0.2 mM sulfide in geothermal fluid (Kimura et al. 2010). This range was a comparable result determined in 2 mM sulfide in the hydrothermal vent of Hatoma Knoll. While the phylogenetic composition and the physical conditions are quite different between geothermal and hydrothermal systems, they showed very similar range of the doubling times.

The onboard preservation time of the prepared chamber and the seeds-water was unavoidable technical issue of deep-sea submersible operation. This factor may induce unlike physiological condition and be affected the results of growth rate estimation, although the inoculation of seeds-water was performed just before each dive operation. To determine accurate growth rate, the time course in situ experiment with frequent sampling will be needed.

The the 16S rRNA gene sequences of potential sulfur-oxidation bacteria belonging to *Epsilonproteobacteria* were detected in the chambers incubated with plume water and colony water (Fig. 33.3), while the sequences of *Gammaproteobacteria* appeared in the chamber incubated with the colony water. The *Epsilonproteobacteria* have been known as common member discovered from benthic mixing zone of hydrothermal system (Campbell et al. 2006), and a majority of epibiotic microbial community on *Sinkaia crosnieri* (Tsuchida et al. 2010; Watsuji et al. 2010). The free-living *Gammaproteobacteria*, SUP05 potential sulfur oxidizing phylotype, dwelled in hydrothermal system have been discovered as common member in chemoautotrophic group of hydrothermal plume (Dick et al. 2013; Sunamura et al. 2004). The different ecophysiological functions between *Epsilonproteobacteria* and *Gammaproteobacteria* probably induced the habitat segregation within hydrothermal system (Nakagawa and Takai 2008; Yamamoto and Takai 2011).

The clade of *Epsilonproteobacteria* consists of many ecotypes include symbiont with marine invertebrates (Dubilier et al. 2008; Tokuda et al. 2008; Tsuchida et al. 2010), pathogen or normal flora of animals, and free-livings (Campbell et al. 2006). In this study, the 16S rRNA gene sequences of *Epsilonproteobacteria* from a digestive tract of the Bathymodiolus have either possibility of a feed from mixing-zone bacterial population or a member of normal flora (Egas et al. 2012; Van Horn et al. 2011).

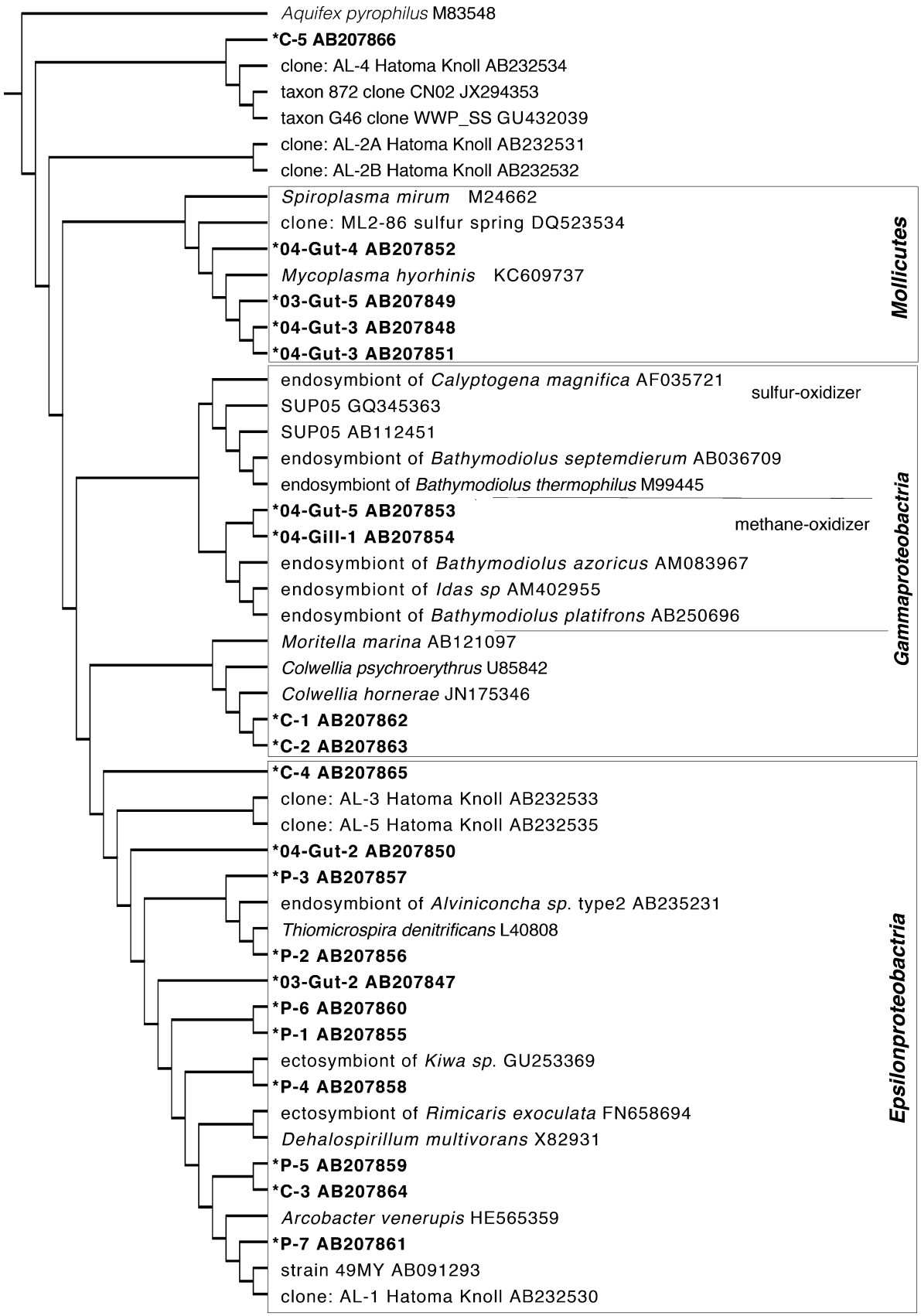

Fig 33.3 Cladogram of phylogenetic clusters for 16S rRNA sequences. The sequences with *asterisk* are DNA from DGGE gel in this study. Abbreviation of the sequences: "Gill" is the gill tissue, "Gut" is the content of digestive tract, "P" is the chamber samples incubated with seed-water of plume, "C" is the chamber samples incubated with seed-water from Bathymodiolus colony

Table 33.3 Comparison between bacterial growth rates and aquatic environments

Site	Incubation methods	Depth (m)	Temp. (°C)	Incubation (day)	DT (h)	GR (day^{-1})	References
Vent sides	In situ incubation	1,477–1,523	80–150	6–13	21–33	0.77–0.50	This study
Colony	In situ incubation	1,477–1,479	3.9–4.8	6	86–110	0.15–0.92	This study
Methane seep	In situ incubation	641	6.5–9.5	10	26–39	0.63–0.42	This study
Geothermal pool	In situ incubation	Terrestrial	84–88	3	20–38	0.43–0.82	Kimura et al. (2010)
Pacific Ocean	Simulated incubation	Surface	22.6	nd	14	1.18	Carlucci and Williams (1978)
Pacific Ocean	Simulated incubation	500	7.5	nd	67	0.24	Carlucci and Williams (1978)
Pacific Ocean	Simulated incubation	1,500	2.5	nd	145	0.11	Carlucci and Williams (1978)
Phytodetritus	Simulated incubation	4,500	2	1	11.5	1.45	Lochte and Turley (1988)
Phytodetritus	Simulated incubation	Surface	15	1	10.1	1.50	Lochte and Turley (1988)
Oregon coast	Simulated incubation	Surface	10	2–3	19.1	0.86	Sherr et al. (1999)
Oregon coast	Simulated incubation	Surface	15	2–3	38.3	0.43	Sherr et al. (1999)
Oregon coast	Simulated incubation	Surface	18	2–3	43.3	0.38	Sherr et al. (1999)
Delaware estuary	Simulated incubation	Surface	11.9–27.6	3	nd	1.7–3.0	Yokokawa et al. (2004)
Delaware estuary	Simulated incubation	Surface	12.8–28.5	3	nd	0.4–3.5	Yokokawa et al. (2004)

Another digestive tract associated the 16S rRNA gene sequences belonging to *Mollicute* were probably parasitic or potential pathogenic group for mollusks (King et al. 2012; Van Horn et al. 2011; Waltzek et al. 2012).

Methane is proposed as a significant energy source of microbial growth in hydrothermal system (McCollom 2000). The 16S rRNA gene sequence of methane-oxidizing bacteria were detected only from gill and digestive tract, and could not detect from the chamber, despite the chamber was placed on the Bathymodiolus colony. The reasons why the chamber incubation did not allow the growth of methane-oxidizing bacteria was unknown whether caused by methane-permeability of membrane filter or environment constrain for proliferation, or some others.

In conclusion, the results of this study suggested that chemoautotrophic growth in the hydrothermal vent area has an equivalent primary production in the surface seawater. Although an extent of vigorous chemosynthetic growth is constrained to the size of hydrothermal system, the primary production from the growth continuously delivers by hydrothermal plume and bottom current into deep-sea benthopelagic environments. Thus, the microbial growth sustained by chemosynthesis is an important element to estimate the productivity of marine ecosystem.

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