Data report: microbial activity determined by microcalorimetry and cultivation of bacteria from hydrothermally influenced subsurface marine sediments in the mid-Okinawa Trough (IODP Expedition 331)[1]

Axel Schippers,[2] Anja Breuker,[2] and Marco Blöthe[2]

Chapter contents

Abstract 1
Introduction 1
Materials and methods 2
Results and conclusions 3
Acknowledgments 4
References 4
Tables 5

[1] Schippers, A., Breuker, A., and Blöthe, M., 2016. Data report: microbial activity determined by microcalorimetry and cultivation of bacteria from hydrothermally influenced subsurface marine sediments in the mid-Okinawa Trough (IODP Expedition 331). *In* Takai, K., Mottl, M.J., Nielsen, S.H., and the Expedition 331 Scientists, *Proceedings of the Integrated Ocean Drilling Program,* 331: Tokyo (Integrated Ocean Drilling Program Management International, Inc.). doi:10.2204/iodp.proc.331.203.2016
[2] Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2, 30655 Hannover, Germany. Correspondence author: axel.schippers@bgr.de

Abstract

Using sediment samples from five Integrated Ocean Drilling Program Expedition 331 sites, bacteria were cultivated on different media, and microbial activity was measured by isothermal microcalorimetry at 25° and at 90°C to study hydrothermal influence. The data showed considerable activity of thermophilic microorganisms at 90°C, but at 25°C significant activity could only be detected for aerobic conditions. Enhanced microbial activity at 90°C was mirrored by the enrichment of thermophilic sulfate reducers in agreement with published geochemical data. Aerobic and anaerobic cultures we obtained from hydrothermally influenced, deeply buried marine sediments at 12°C were not significantly different from those of other cold and temperate deeply buried marine sediments.

Introduction

Subseafloor microbes beneath active hydrothermal vents, in the "subvent biosphere," are believed to live near the upper temperature limit for life on Earth. Hydrothermally influenced deeply buried marine sediments were sampled during Integrated Ocean Drilling Program (IODP) Expedition 331, Deep Hot Biosphere (see the **"Expedition 331 summary"** chapter [Expedition 331 Scientists, 2011a]).

Site C0013 is located ~100 m east of the main hydrothermal mound chains of the Iheya North hydrothermal field. In Hole C0013C, the core liner melted, indicating a temperature >82°C at 12.5 meters below seafloor (mbsf). Site C0014 is located ~450 m east of the high-temperature vents and mounds, and the temperature exceeded 210°C at only 50 mbsf. The temperature gradient was roughly linear from 0 to 47 mbsf, increasing from the bottom water temperature of 4.5°–145°C over that depth range, but it deviated greatly from this line at 0–9 and 47–50 mbsf, where it was clearly affected by high-temperature fluid pooling or lateral flow. Site C0015, located on a hill ~600 m northwest of the active vents, represents a potential migration path for hydrothermal fluid; however, hydrothermal alteration was absent in the cored interval. Site C0017 is located 1550 m east of the high-tempera-

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ture vents of the Iheya North hydrothermal field, in an area of low heat flow. The overall temperature profile was exponential and concave upward, which is consistent with downwelling of cold water, implying that this was an area of recharge to the hydrothermal system. A maximum temperature of 90° ± 5°C was measured at the bottom of the deepest hole (C0017D) at 151 mbsf (see the **"Expedition 331 summary"** chapter [Expedition 331 Scientists, 2011a]).

Temperature gradients decrease greatly with distance from the active vents, from >7°C/m at Site C0013 to 3°C/m at Site C0014 and 0.6°C/m at Site C0017. Site C0015 has a relatively low heat flow and a surficial temperature gradient of ~1°C/m. At Sites C0013, C0014, and C0017, the relatively high, moderate, and low heat flow areas east of the Iheya North hydrothermal field were drilled to investigate subseafloor microbial habitats and communities. Broad gradients of physical and chemical variation, both laterally and vertically, that could be affected by mixing between discharging hydrothermal solutions and recharging ambient bottom seawater (see the **"Expedition 331 summary"** chapter [Expedition 331 Scientists, 2011a]) characterizes this habitat.

Sampling of the sediments was done while applying contamination tests (Yanagawa et al., 2013). Using molecular ecology approaches, the deep biosphere of these sediments was analyzed (Yanagawa et al., 2014). Here, cultivation of bacteria with different media was done following previous Ocean Drilling Program (ODP)/IODP approaches (Batzke et al., 2007), and microbial activity was measured by isothermal microcalorimetry. The latter allows the sensitive detection of heat output generated by the sum of chemical and biological reactions in a sample. A chloroform treatment to kill the microorganisms allows for differentiation between chemical and biological activity. The higher the heat output, the higher is the activity of a sample (Schippers et al., 1995; Braissant et al., 2010; Schippers and Bosecker, 2005). To study hydrothermal influence on activity, measurements were carried out at 25°C and 90°C for comparison.

Materials and methods

Overall, 120 sediment samples from 5 sites (C0013–C0017) to a maximum depth of 110 mbsf were taken onboard the D/V *Chikyu* for microbiological research at the Federal Institute for Geosciences and Natural Resources (BGR). The samples were taken from the centermost part of the sediment cores under aseptic conditions in an anaerobic box, put in anaerobic bags, and sent to BGR at 2°–8°C as previously described in the **"Methods"** chapter (Expedition 331 Scientists, 2011b; Shipboard Scientific Party, 2003) and stored at 8°C until use.

To determine microbial activity, the heat output in microwatts per gram for each sample from Sites C0014, C0015, and C0017 was measured in an isothermal microcalorimeter (Thermal Activity Monitor Thermostat type 2277; Thermometric, Sweden) within 1 month after the expedition. A measurement lasted for 2–4 h until a stable value was obtained. After measuring the total heat output (microbial and chemical) in a ~5 g sample at 25° and 90°C, the chemical heat output at 90°C was measured after chloroform treatment (addition of 0.5 mL chloroform and 24 h incubation and subsequent removal by vacuum evaporation) to kill the microorganisms, as described elsewhere (Schippers and Bosecker, 2005). Chemical heat output was only measured at 90°C. Measurements under aerobic conditions were made in air; for anaerobic conditions, glass ampoules containing samples were closed in a nitrogen atmosphere (anaerobic box). Microbial substrates were not added for measurements. Activity values were calculated per sample dry weight (determined as weight difference after drying). Microbial activity can be calculated as the difference between total and chemical heat output (Schippers et al., 1995; Schippers and Bosecker, 2005).

For enrichment of microorganisms, liquid culture media were inoculated in a flow hood with sediment (Shipboard Scientific Party, 2003) from Sites C0013, C0014, and C0017 within 2 months after Expedition 331. The media were based on artificial seawater as described by Batzke et al. (2007) and were amended either with a polymer or monomer solution. The polymer solution contained chitin, cellulose, and peptone at 0.5 per gram each, whereas the monomer solution contained 36 different carbon sources such as amino acids, fatty acids, organic acids, alcohols, and glucose (final concentration = 0.1 mM). Media for aerobic incubations were buffered with bicarbonate/CO_2. In media for anaerobic incubations, HEPES (2.38 per gram) was added, and pH was adjusted to 7.2–7.4 by addition of NaOH before autoclaving. After autoclaving, the media were cooled under N_2 flow, and a solution of 10 vitamins (Balch et al., 1979) and sodium bicarbonate (0.2 per gram) were added from sterile stock solutions (Batzke et al., 2007). For the enrichment of sulfide-, manganese-, or iron-oxidizing bacteria, 1 mL of a 100 mM sterilized filter and anoxic (0.2 µm) Na_2S, $MnSO_4$, or $FeCl_2$

solution was added in gradient tubes. The enrichment cultures were incubated at 12°, 60°, 70°, or 80°C.

Growth was continuously checked by visual inspection (turbidity) and/or phase contrast microscopy, and in case of growth, colonies were picked and transferred to fresh media. Several aerobic and anaerobic enrichments were obtained. To identify isolates, 16S rRNA gene sequencing and analysis was done. The amplified polymerase chain reaction products of bacterial gene fragments were purified and sequenced at Microsynth sequencing company, Switzerland. Primers GM3F (5′AGAGTTTGATCMTGGC3′) and GM4R (5′TACCTTGTTACGACTT3′) were used for sequencing. The sequences obtained were edited with Geneious 6 software and compared with the NCBI database through BLAST searches.

Results and conclusions

The results for the microcalorimetric activity measurements of Expedition 331 samples are shown in Tables T1 and T2. Microbial activity can be calculated as the difference between total and chemical heat output. The data show considerable activity at 90°C, which is partly attributed to microbial activity (50% for aerobic conditions). At 25°C, significant activity could only be detected for aerobic conditions. Overall, microcalorimetric measurements reveal activity of thermophilic microorganisms in the Expedition 331 samples.

Table T3 gives an overview of enrichment cultures and isolates; details are described in the following text. Overall, enhanced microbial activity at 90°C was mirrored by the enrichment of thermophilic sulfate reducers in agreement with geochemical data (Aoyama et al., 2014). The cultures we obtained from hydrothermally influenced, deeply buried marine sediments at 12°C were not significantly different from those of cold and temperate deeply buried marine sediments. We were not able to cultivate *Archaea*, for example, despite the fact that they are broadly found with 16S rRNA gene approaches (Yanagawa et al., 2014).

Thermophiles

The first attempts to cultivate thermophiles at 70° and 80°C in liquid media under aerobic and anaerobic conditions with monomer or polymer solutions as a carbon source were unsuccessful; no growth was observed after 3 months of incubation. In a second enrichment attempt, incubation temperature was decreased to 60°C under anoxic conditions. Over an incubation period of 10 months, nearly all inoculated tubes showed activity by formation of black sulfide precipitation. We interpreted the formation of this precipitation as a sign of an ongoing sulfate reduction, because in control tubes with autoclaved or no inoculums, no formation of sulfide precipitation was observed. However, subculturing remained unsuccessful.

Aerobic litho(hetero)trophs

No (stable) sulfide- or iron-oxidizing enrichment culture at 12°C was obtained from the investigated depth intervals in the three cores. For microaerophilic Fe(II) oxidizers, only culture tubes inoculated with samples from Sites C0013 (0.2 and 0.3 mbsf), C0014 (2.1 mbsf), and C0017 (6.4 and 10.9 mbsf) showed a sharp band of Fe(III) precipitation, which indicates a microbially enhanced Fe(II) oxidation, in contrast to the fuzzy band in the chemical control tubes. Similar observations were made for the enriched aerobic sulfide oxidizers. None of these enrichment cultures could be successfully maintained. On the other hand, stable Mn(II)-oxidizing enrichment cultures were obtained from all core depth intervals. In all of these enrichment cultures, a sharp brown band 1–2 cm beneath the top of the agar in the gradient tubes developed over 3–4 weeks. In the chemical controls, only a weak and fuzzy band was observed. These putative Mn(II)-oxidizing bacteria could successfully be transferred to agar plates with the polymer mix as the carbon and energy source. The 16S rRNA gene of five isolates was partially sequenced (320–430 bp), and the isolates were affiliated to *Bacillus oceani* (91%), whereas two isolates were affiliated to *Bacillus aquimaris* (92%). The obtained Mn(II)-oxidizing cultures could grow on agar plates in the presence of 5 mM Mn(II) but without an additional carbon source.

Aerobe and anaerobe heterotrophic mesophiles

Aerobe heterotrophic mesophiles were successfully cultivated from all samples at 12°C with monomer and polymer as the carbon source on agar plates. From Site C0013, two isolates were obtained affiliated to *Halobacillus litoralis* (92%) and one to *Bacillus niacini* (95%); both are species originally isolated from deep-sea samples. Two additional isolates from Site C0013 are affiliated to *Marinobacter salsuginis* (90%) and *Marinobacter hydrocarbonoclasticus* (93%). From Hole C0017, we obtained two isolates affiliated to *Shewanella benthica* (89%), three isolates affiliated to *Cytophaga fermentans* (92%), and one isolate affili-

ated to *Idiomarina* spp. (92%) and *Psychrobacter* spp. (95%). None of the obtained isolates could grow at 60°C.

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Table T1. Total (biological + chemical) and chemical activity in samples measured as heat output by microcalorimetry, Sites C0014, C0015, and C0017.

Hole	Depth (mbsf)	Anaerobic			Aerobic		
		Total activity at 25°C (µW/g)	Total activity at 90°C (µW/g)	Chemical activity at 90°C (µW/g)	Total activity at 25°C (µW/g)	Total activity at 90°C (µW/g)	Chemical activity at 90°C (µW/g)
C0014B	0.4	0	24	0	46	221	98
	1.3	0	66	2	65	361	213
	2.6	0	0	0	ND	ND	ND
	4.0	0	32	12	ND	ND	ND
	5.4	0	0	0	ND	ND	ND
	6.5	0	0	0	ND	ND	ND
	8.8	0	0	0	ND	ND	ND
	15.3	0	0	0	ND	ND	ND
	17.5	0	0	0	ND	ND	ND
	20.0	0	87	0	ND	ND	ND
	23.0	0	42	0	ND	ND	ND
	24.9	0	0	0	ND	ND	ND
	26.3	0	0	0	ND	ND	ND
C0014D	0.3	0	0	0	67	158	0
	2.1	0	0	64	59	362	482
	3.2	0	0	0	ND	ND	ND
	4.3	0	0	28	ND	ND	ND
	6.7	0	18	0	ND	ND	ND
	8.7	0	0	4	ND	ND	ND
	10.2	0	0	92	ND	ND	ND
	11.5	0	0	53	ND	ND	ND
	12.9	0	0	123	ND	ND	ND
C0015B	0.4	0	0	148	0	0	0
	3.5	0	0	47	48	288	222
C0015C	7.0	0	0	222	1	4	0
	9.0	0	32	59	4	0	0
C0017A	0.7	0	96	28	25	80	20
	6.4	0	0	15	19	63	18
C0017B	10.9	0	42	9	12	42	0
	14.9	0	0	39	12	59	19
C0017C	20.2	0	4	0	0	71	3
	24.7	0	1	0	0	156	11
	26.7	0	24	0	0	47	0
	28.4	0	350	20	0	2	0
	30.1	0	198	0	0	0	0
C0017D	63.8	0	85	0	0	0	0
	66.6	0	0	51	0	0	0
	68.3	0	12	0	5	8	0
	68.4	0	55	0	7	29	0
	75.0	0	31	0	35	244	0

ND = not determined.

Table T2. Mean total (biological + chemical) and chemical activity in samples measured as heat output by microcalorimetry, Sites C0014, C0015, and C0017.

	Total activity at 25°C (µW/g)	Total activity at 90°C (µW/g)	Chemical activity at 90°C (µW/g)	Number of samples measured
Anaerobic conditions	<0.1	30	25	40
Aerobic conditions	18	100	50	22

Table T3. Overview of enrichment cultures and isolates at 12°C, Sites C0013, C0014, and C0017.

Substrate in medium		Enrichments							Number of isolates		
		Anaerobic		Aerobic		Microaerophilic			Aerobic		
Hole, core, section	Depth (mbsf)	Monomer	Polymer	Monomer	Polymer	Na_2S	Mn(II)	Fe(II)	Mn(II)	Monomer	Polymer
331-											
C0013E-1H-2	0.2			x	x	x	x	x	1	3	
C0013F-1H-2	0.3	x	x	x	x	x	x	x	10		6
C0013D-1H-4	7.0	x	x	x	x						
C0014B-2H-3	8.8	x	x	x	x	x	x		11	1	6
C0014D-2H-3	10.2	x	x	x	x						
C0014D-2H-4	11.5	x	x	x	x		x			3	2
C0014D-2H-6	12.9	x	x	x	x						
C0014B-2H-10	15.3			x	x						
C0017A-1H-5	6.4	x	x	x	x	x	x	x	2	6	6
C0017B-1H-2	10.9			x	x	x	x	x	2	3	4
C0017B-1H-5	14.9	x	x	x	x		x		1	4	7
C0017C-2H-2	30.1	x	x	x	x		x		1	6	12
C0017D-2H-5	75.0			x	x		x			2	3
C0017D-7H-4	108.5	x	x	x	x						

x = successful enrichment.