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ORIGINAL PAPER

Phylogenetic and enzymatic diversity of deep subseafloor aerobic microorganisms in organics- and methane-rich sediments off Shimokita Peninsula

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Abstract "A meta-enzyme approach" is proposed as an ecological enzymatic method to explore the potential functions of microbial communities in extreme environments such as the deep marine subsurface. We evaluated a variety of extra-cellular enzyme activities of sediment slurries and isolates from a deep subseafloor sediment core. Using the new deep-sea drilling vessel *"Chikyu"*, we obtained 365 m of core sediments that contained ~2% organic matter and considerable amounts of methane from offshore the Shimokita Peninsula in Japan at a water depth of 1,180 m. In the extra-sediment fraction of the slurry samples, phosphatase, esterase, and catalase activities were detected consistently throughout the core sediments down to the deepest slurry sample from 342.5 m below seafloor (mbsf). Detectable enzyme activities predicted the existence of a sizable population of viable aerobic microorganisms even in deep subseafloor habitats. The subsequent quantitative cultivation using solid media represented remarkably high numbers of aerobic, heterotrophic microbial populations (e.g., maximally 4.4×10^7 cells cm^{-3} at 342.5 mbsf). Analysis of 16S rRNA gene sequences revealed that the predominant cultivated microbial components were affiliated with the genera *Bacillus*, *Shewanella*, *Pseudoalteromonas*, *Halomonas*, *Pseudomonas*, *Paracoccus*, *Rhodococcus*, *Microbacterium*, and *Flexibacteracea*. Many of the predominant and scarce isolates produced a variety of extra-cellular enzymes such as proteases, amylases, lipases, chitinases, phosphatases, and deoxyribonucleases. Our results indicate that microbes in the deep subseafloor environment off Shimokita are metabolically active and that the cultivable populations may have a great potential in biotechnology.

Keywords Deep subseafloor biosphere · Subseafloor sediment · Aerobic bacteria · Meta-enzyme · Methane hydrate

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Introduction

Deep subseafloor sediments harbor an enormous microbial biomass (Parkes et al. 1994, 2000; Whitman et al. 1998). Diagenetic modeling of porewater chemical constituents showed that the metabolic activities of these subseafloor microorganisms are generally extremely low because of the low flux of nutrient supplies from the surface biosphere (D'Hondt et al. 2002, 2004), which is consistent with the results of radiotracer incubation to measure sulfate reduction and methanogenesis in deep marine sediments (e.g., Newberry et al. 2004; Parkes et al. 2005; Schippers et al. 2005; Wellsbury et al. 2000). In general, microbial activity

and turnover rates in the deep subseafloor are extremely low relative to those in other habitats on Earth. Culture-independent molecular approaches using PCR-amplified 16S rRNA genes revealed that these subseafloor microbial communities are predominantly composed of phylotypes lacking closely related cultivated strains, and consequently their physiological characteristics have remained largely unknown (Inagaki et al. 2003, 2006; Kormas et al. 2003; Parkes et al. 2005; Reed et al. 2002; Sørensen and Teske 2006).

Successful cultivations of probably indigenous microbial components from deep subseafloor sedimentary habitats more than 10 m below seafloor (mbsf) have been limited. Only three obligatory anaerobic isolates (sulfate-reducing bacterium and methanogens) were characterized as novel indigenous subseafloor microorganisms (Bale et al. 1997; Mikuchi et al. 2003; Kendall et al. 2006), while relatively diverse facultatively anaerobic heterotrophic bacteria such as *Halomonas* and *Psychrobacter* have been cultivated as sizable populations at 0.1–0.001% cultivability (Inagaki et al. 2003; D'Hondt et al. 2004; Süß et al. 2004; Biddle et al. 2005; Batzke et al. 2007). In a sediment core from the Sea of Okhotsk, over 10^5 heterotrophic cells per 1 cm^3 sediment have been obtained only from porous volcanic ash layers under the aerobic condition, while no growth was observed in pelagic clay layers (Inagaki et al. 2003). These cultivation studies on deep marine sediments demonstrated that porous layers such as sand and volcanic ash are potential habitable spaces for facultatively anaerobic heterotrophic bacteria. However, microorganisms inhabiting the clay environment remain largely unknown because of the strong resistance or extremely slow growth of predominant indigenous microbial components.

During August–October in 2006, the newly constructed deep-sea drilling vessel "*Chikyu*" Shakedown Expedition CK06-06 was conducted offshore the Shimokita Peninsula, northwestern Japan, where the presence of free hydrocarbon gases and methane hydrates had been predicted by the preliminary seismic survey (Taira 2005). Although the main purpose of the CK06-06 expedition was a system integration and operation test for deep drilling, the test core also provided an unprecedented opportunity to study the deep subseafloor microbial habitat.

We propose that a "meta-enzyme" is an assemblage of extra- and intra-cellular enzymes of a microbial community in an extreme habitat such as deep marine subsurface and deep subseafloor sediment cores, and we report here the meta-enzyme activities of supernatants of the 365 m of organic- and methane-rich sediment core slurries from offshore the Shimokita Peninsula. Additionally, we evaluated the phylogenetic and enzymatic diversity of the remarkably high number of cultivable aerobic bacteria from the deep marine sediments.

Materials and methods

Drilling site description and sample collection

The drilling site C9001C is located in the Hidaka Trough, a forearc basin between the northeastern part of Honshu Island of Japan and the Japan Trench (41°10.6380′N, 142°12.081E). The site is approximately 80 km from the eastern coast of the Shimokita Peninsula. The water depth at site C9001C is 1,180, and 365 m of sediment cores were recovered at site C9001C. At most of the depths, the core was obtained by a hydraulic piston coring system (HPCS), producing the least external chemical and microbiological contamination during the drilling operation. The innermost core section (50 cm^3) of the whole round cores (WRC) was collected by a sterile tip-cut plastic syringe and was suspended in 50 ml of filter-sterilized and autoclaved synthetic seawater (NaCl 25 g, $MgCl_2{\cdot}6H_2O$ 5 g, KCl 3 g per liter of distilled and deionized water) in the presence or absence of a reducing reagent (neutralized $Na_2S{\cdot}9H_2O$ solution, final concentration of 0.025% w/v) in a 250 ml of glass bottle with a butyl rubber stopper. All procedures were performed under anaerobic conditions onboard the *"Chikyu"*, and the slurry was preserved under a gas phase of 100% N_2 (120 kPa) at 4°C until experiments. Totally, 11 core sediment slurries from different depths were used in this study; the preliminary characterizations of the sediments are shown in Table 1.

Extra-sediment enzyme assays

A portion (4 ml) of each sediment suspended without the reducing reagent was centrifuged at 14,000*g* for 10 min at 4°C. The supernatant was passed through a 0.22-μm pore size filter, and the filtrate was then diluted with an equal volume of sterilized artificial seawater (Nissui, Japan). The resultant solution was incubated at 30°C for 48 h with a kit for detection of enzyme activities (API ZYM, Bio Merieux, France).

For further detection of enzyme activity, acid phosphatase activity was measured at 30°C in 100-mM acetate buffer (pH 5.0) containing 4-mM *p*-nitrophenyl phosphate (Sigma) and 0.15 ml of the supernatant of each sediment suspension in a total volume of 0.25 ml. After incubation for 6 h, 1 ml of 2-M Na_2CO_3 was added to stop the reaction, and then the absorbance of the solution was measured at 410 nm. C4-esterase activity was measured at 30°C in 100-mM MOPS buffer (pH 7.0) containing 4-mM *p*-nitrophenyl butyrate (Sigma) and 0.1 ml of the supernatant in a total volume of 0.25 ml. After incubation for 3 h, 1 ml of 10-mM MOPS buffer (pH 7.0) was added to dilute the reaction mixture, and then the absorbance of the solution was measured at 410 nm. One mU of acid

Table 1 Characterizations of deep subseafloor sediments at Site C9001

Core-section	Depth (mbsf)	Total organic carbon (wt%)[a]	CH4 concentration (μM)[a]	Remarks
1-1	0.5	1.62	11.2	Sulfate reduction zone, silty clay
1-4	4.8	1.32	1,000.4	Silty clay
2-1	8.0	1.30	3,010.7	Silty clay
3-2	13.4	1.24	2,095.3	Silty clay
6-3	48.2	1.79	1,375.9	Silty clay
12-4	106.7	1.91	2,071.5	Silty clay
21-4	190.4	nd[b]	nd[b]	Hydrate-bearing ash layer
24-4	212.5	1.13	1,658.9	Silty clay
24-4′	216.9	1.13	nd[b]	Hydrate-bearing silt and sand
27-6	247.1	0.99	1,744.6	Hydrate-bearing silt and sand
38-7	342.5	1.02	2,866.0	Hydrate-bearing sand

[a] Concentrations of total organic carbon and methane were inferred from the closest core section used for geochemical analyses

[b] Not determined

phosphatase and C4-esterase activities was defined as the amount of the protein that released 1 nmol *p*-nitrophenol per min under the assay condition. Catalase activity was measured in 100-mM Tris–HCl buffer (pH 7.5) containing 0.1% (v/v) H_2O_2 and 0.16 ml of the supernatant in a total volume of 0.2 ml, and the absorbance of the solution at 240 nm was measured. After incubation at 30°C for 1 h, changes in the absorbance of the solution were measured, and the decreasing rate of absorbency per min was calculated. One mU of catalase activity was defined as the amount of protein that degraded 1 nmol H_2O_2 per min under the assay condition. Supernatants of all sediment suspensions were incubated at 100°C for 15 min and were used for blank reactions. Protein concentrations were measured in all extra-sediments by a protein assay kit (Bio-Rad) using bovine serum albumin as the standard.

Isolation of cultivable microorganisms

A series of dilution of the core sediment suspended in sterilized artificial seawater were spread onto a marine agar 2216 (MA; Difco), a marine agar diluted to one-tenth with 2% NaCl (1/10 MA), and a nutrient agar (Difco) diluted to one-tenth with distilled water (1/10 NA), respectively. The diluted media were supplemented with 1.5% agar. The pH of each medium was adjusted to pH 4.5, 7, and 9 with diluted HCl or Na_2CO_3 before autoclaving. The plates were incubated at 4, 15, 30, and 50°C for 1–4 weeks, respectively. The colonies were counted, and several colonies grown on each medium were isolated. The isolates were purified by transferring them to each corresponding medium and growth condition several times.

Plate assay for enzyme-producing microorganisms

Protease, α-amylase, endoglucanase (CMCase), lipase, chitinase, ligninase, and deoxyribonuclease (DNase)-producing microorganisms were detected by the dissolved zones around cells grown on marine agar containing 1% (w/v) skim milk (Difco), 0.5% starch azure (Sigma), 1% CMC (Sigma) plus 0.005% trypan blue (Sigma), 1% tributyline (Wako Pure Chemical), 1% colloidal chitin (Sigma), 0.5% lignin (Sigma) plus 0.5% CMC for dispersion, or 1% DNA from salmon (Sigma), respectively. For the detection of DNase activity, a solution of 1N HCl was poured onto cells grown on a marine agar 2216. To detect the production of xylanase, pectinase, alginase, chondroitinase, and carrageenase, a soft agar (0.8%) containing 50-mM Tris–HCl buffer (pH 7.5) and 0.2% of each substrate [xylan (Sigma), pectin (Sigma), alginate (Wako Pure Chemical), chondroitin C (Wako Pure Chemical), or κ-carrageenan (Wako Pure Chemical)] was poured onto cells grown on marine agar containing 0.2–1% of the corresponding substrate. In the case of phosphatase production, 10-mM *p*-nitrophenyl phosphate (Sigma) was added to a soft agar after cells were grown on a marine agar 2216. Once the soft agar layer had solidified, they were incubated at 30°C overnight. The following solutions were poured onto the soft agar layer to detect the enzyme produced: 0.1% Congo red (for xylanase), 0.1% ruthenium red (for pectinase), 0.5% cetylpyridinium chloride (for alginase and carrageenase), 0.1% bovine serum albumin in 2-N acetic acid (for chondroitinase), and 1-M sodium carbonate (for phosphatase). Catalase-producing microorganisms were detected using 3% H_2O_2 in 50-mM Tris–HCl buffer (pH 7.0). Cytochrome oxidase-producing microorganisms were examined using a test paper (Nissui, Japan).

Sequencing and phylogenetic analysis of 16S rRNA genes

The nearly complete 16S rRNA genes of the purified isolates were amplified using the bacterial primers 27f and 1492r (Lane 1991), and frozen-and-melted cells or genomic

DNA prepared by Ampdirect plus (Shimadzu, Japan) as the templates with an LA Taq DNA polymerase (Takara Bio, Japan) in a DNA thermal Cycler (Gene Amp PCR System 9700, Applied Biosystems). Nucleotide sequencing was performed using an ABI Prism Big Dye Terminator Cycle Sequencing kit and an ABI 3100 sequencer (Applied Biosystems). The similarity among the sequences (approximately 500 bp) was analyzed using the BLAST program, and the isolates with ≥97% sequence similarity were assigned to the same phylogenetic group. The representative 16S rRNA gene sequence (approx. 1,400 bp) in each group was determined for both strands. Phylogenetic analysis of each sequence was determined using the ARB program (Ludwig et al. 2004). The nucleotide substitution rate (k*nuc*) was calculated and a distance matrix tree was constructed by the neighbor-joining method (Saitou and Nei 1987) with the Clustal X program (Thompson et al. 1997). The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1,000 replications.

Nucleotide sequence accession numbers

All 16S rRNA gene sequences of the representative clones determined in this study have been submitted to DDBJ/EMBL/GenBank data bases under accession numbers AB362247 to AB362308.

Results and discussion

Characteristics of the samples of the core C9001C

The cored sediments were mainly composed of diatom-rich hemi-pelagic clay and silt, intercalated commonly with volcanic ash and sand layers (Table 1). Preliminary biostratigraphic age models indicate very high sedimentation rates, ranging from 54 to 95 cm ky^{-1} (kilo-years) and an approximate core-bottom age of 640 ka (kilo-ages). The sediments contain abundant organics (an average total organic carbon content through the depth of 1.35 wt%) and high concentrations of methane gas (1,000–3,000 μM in porewater). Using the Thermo-View camera onboard, the existence of methane hydrates was observed in several porous layers (ca. 190, 217, 247, 275, and 343 mbsf). Detailed sedimentological and biogeochemical information will be reported elsewhere soon.

Enzyme activities in extra-sediments

The supernatants prepared from the sediment slurries were subjected to a kit for detection of enzyme activities (API ZYM). Almost all supernatants showed naphthol-AS-BI-phosphate (7-bromo-3-hydroxy-2-naphthoic-*o*-anisidide phosphate) hydrolysis, indicating the presence of acid phosphatase activities in situ. Furthermore, 2-naphthyl phosphate hydrolyzing activity was detected in many horizons but not in 1-4, 2-1, and 38-7. Only one supernatant of core 6-3 showed esterase (2-naphthyl butyrate as the substrate) activity. Catalase activity was detected in all sections, and cytochrome oxidase activity was detected in cores 21-4 and 38-7. To detect the enzyme activities more precisely, we performed assays for acid phosphatase, C4-esterase, and catalase using centrifugal supernatants (14,000*g* for 10 min at 4°C) of sediment slurries.

Acid phosphatase, C-4 esterase, and catalase activities were detected in almost all samples, as shown in Table 2. A variety of levels of phosphatase activity (0.08–390 nmol PO_4^{3-} cm^{-3} h^{-1}) have been reported from various marine environments (such as the Baltic Sea and Indian Ocean) (Hoppe 2003). The acid phosphatase activities in the core

Table 2 Enzymatic activities in the supernatant of core sediments

Core section (mbsf)	Protein (mg cm^{-3})	Acid phosphatase (mU cm^{-3})	SA[a] (mU mg^{-1})	C4-esterase (mU cm^{-3})	SA (mU mg^{-1})	Catalase (mU cm^{-3})	SA (mU mg^{-1})
1-1 (0.5)	0.112	0.05	0.446	<0.01	–	<10	–
1-4 (4.8)	0.632	0.072	0.114	0.178	0.282	52	82.3
2-1 (8.0)	0.788	<0.01	–	0.644	0.817	46	58.4
3-2 (13.4)	0.57	0.12	0.211	0.128	0.225	126	221.1
6-3 (48.2)	0.652	0.042	0.064	0.758	1.16	<10	–
12-4 (106.7)	0.62	0.068	0.110	0.87	1.40	480	774.2
21-4 (190.4)	0.15	0.042	0.280	0.064	0.427	<10	–
24-4 (212.5)	0.212	<0.01	–	0.66	3.11	60	283.0
24-4′ (216.9)	0.464	0.038	0.082	0.322	0.694	776	1672.4
38-7 (342.5)	0.22	0.072	0.327	1.02	4.64	58	263.6

[a] SA: specific activity

sediments at different depths were within this range. In water column environments, extra-cellular phosphatases are recognized to be derived from alga and marine bacteria, and their activities play an important role in the recycling of organic phosphorus to avoid phosphorus limitations in marine ecosystem (Hoppe 2003). Therefore, the detectable activities of extra-cellular acid phosphatase would also play a role in taking up, resolving, or recycling biological phosphates and/or phosphorous in deep subseafloor microbial ecosystems. Esterase activity could be a good index for estimation of microbial biomass and/or organic matter content in sediments of sea, lakes, and reservoirs (Poremba and Hoppe 1995; Boschker and Cappenberg 1998; Wobus et al. 2003). Catalase is generally involved in removal of hydrogen peroxide by aerobic microorganisms. One of the unexpected findings was the observation of significantly high catalase activities even in deep-pelagic clay samples such as 12-4, as well as in hydrate-bearing porous layers. The detection of high catalase activity throughout the core suggests the presence of microbes that can alternatively grow on oxygen as an electron donor. Indeed, we observed sizable aerobic microbial populations by cultivation experiments, as described below. The culture-independent measurement of catalase activities showed good agreement with cultivation results.

Cultivated microbial populations and phylogenetic diversity of isolates

The cultivable populations and the predominant cultivated members from the subseafloor sediments are summarized in Fig. 1 (supplementary Tables I–IV). A comparison of the maximum yields of cultivable populations among the slurry samples revealed that the deeper zones of porous sediments harbored a greater number of aerobic heterotrophs than the shallower sediments (Fig. 1). The most cultivable populations were all obtained from the core sections of 24-4 or 24-4′ (210–220 mbsf) at any of the incubation temperatures (maximally 2.4×10^8 cells cm^{-3}). In the relationship between cultivable cell numbers and the agar plates, pHs, and incubation temperatures, the cultivable cell numbers were lower at pH 4.5 in any agar plates than at pH 7 and 9 when incubated at 4, 15, and 30°C (supplementary Tables I–III). The cultivable cell numbers were quite low in any conditions incubated at 50°C excepting core section 24-4′ (maximally 2.6×10^5 cells cm^{-3}) (supplementary Table IV).

In general, cultivability (cultivable population vs. total cell population) of the deep subseafloor microbial community ranges from 0.1% to 0.001% (Inagaki et al. 2003; D'Hondt et al. 2004; Süß et al. 2004), and the cultivable

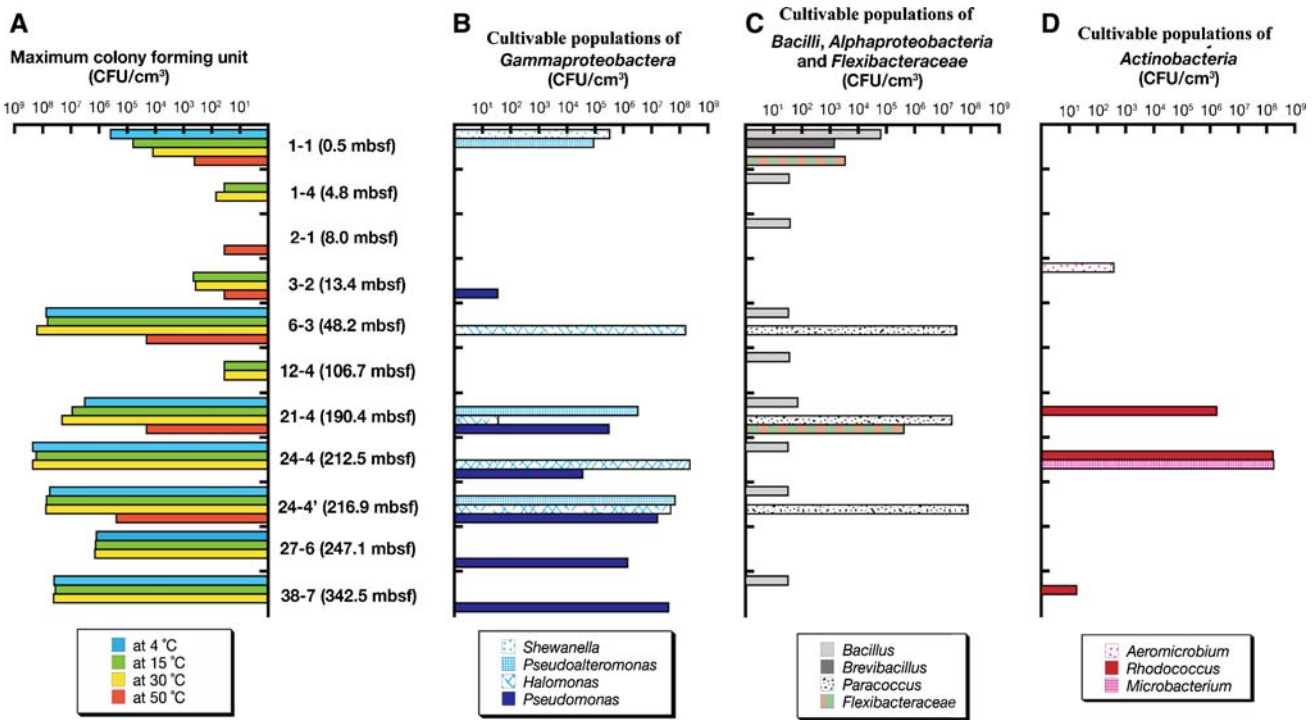


Fig. 1 Cultivable populations of aerobic heterotrophic bacteria and emerging patterns of predominant members isolated from core sediments. **a** Maximum colony forming units of aerobic heterotrophic bacteria at different temperatures in the core sections. **b** Maximum cultivable populations of predominant members of *Gammaproteobacteria* at different temperatures in the core sections. **c** Maximum cultivable populations of predominant members of *Bacilli*, *Alphaproteobacteria*, and *Flexibacteraceae* at different temperatures in the core sections. **d** Maximum cultivable populations of predominant members of *Actinobacteria* at different temperatures in the core sections

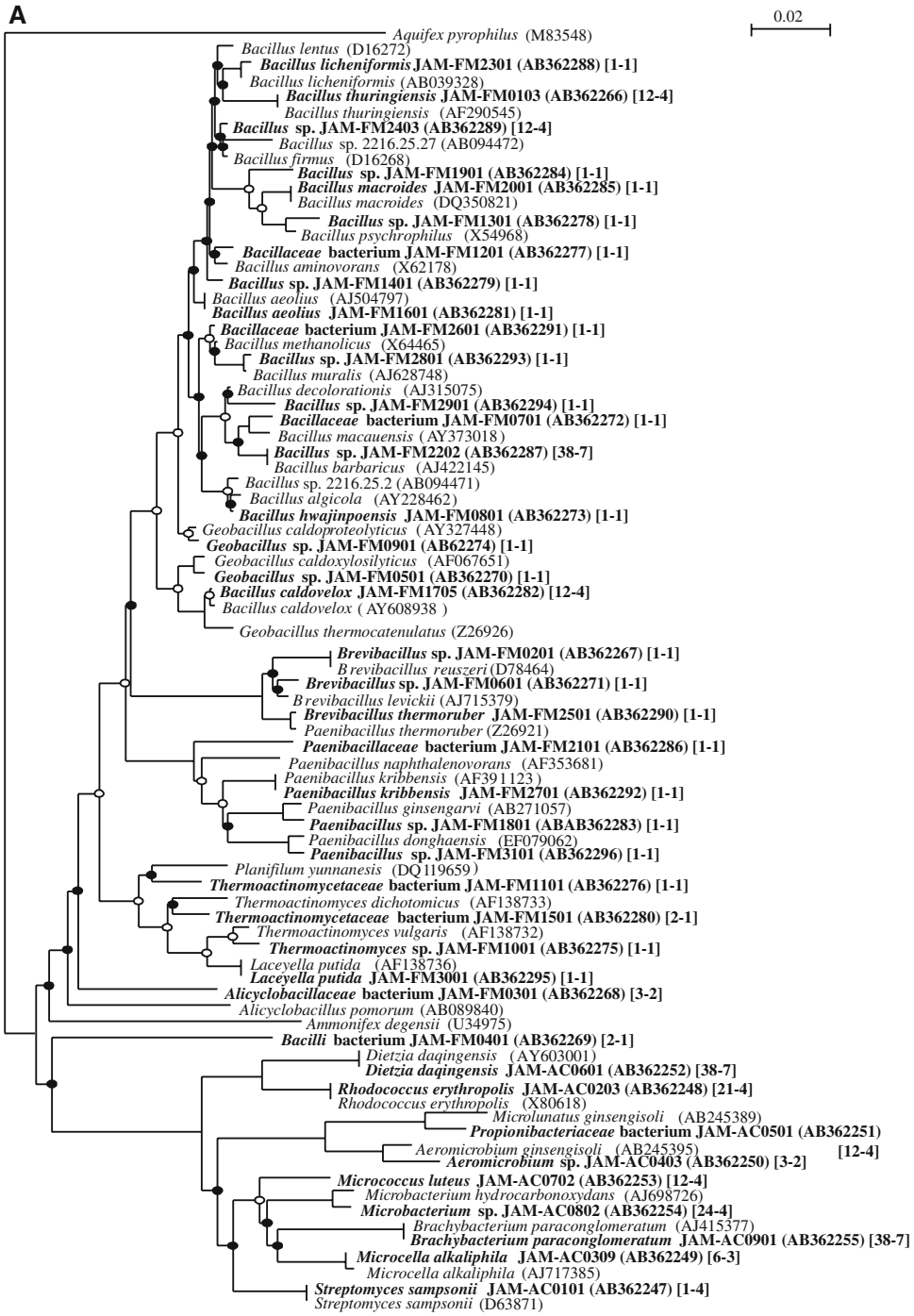
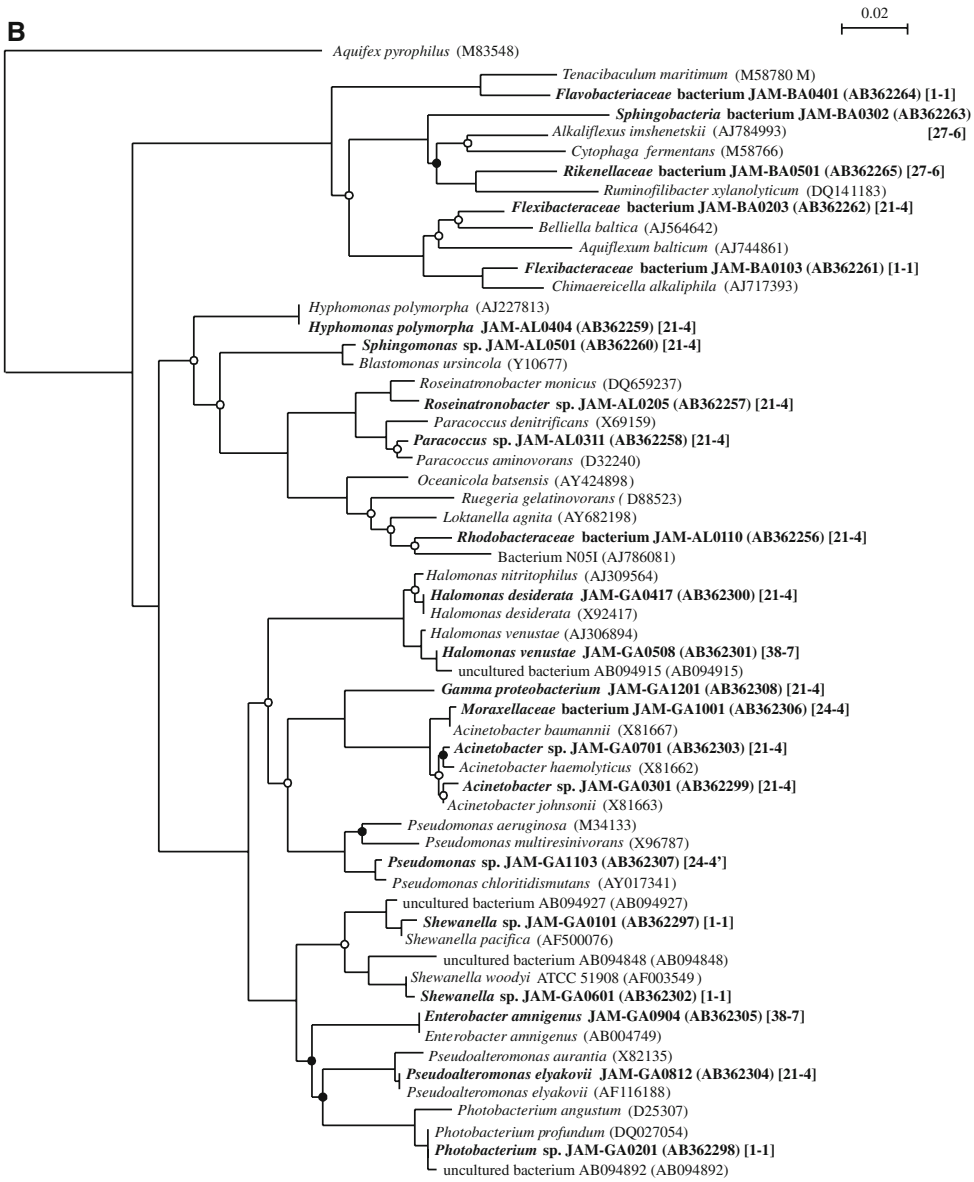


Fig. 2 Phylogenetic tree of representative isolates from core sediments off Shimokita based on the partial 16S rRNA gene sequences. Trees for the phyla *Firmicutes* and *Actinobacteria* **a**, the phyla *Bacteroides/Chlorobi* and the phylum *Proteobacteria* **b** are indicated. The trees were inferred by the neighbor-joining method. The representative isolates determined in this study are shown in bold-faced type. The reproduction of each branch in 1,000 bootstrap analyses is indicated by no mark (>800 bootstrap values), open circles (between 500 and 800 bootstrap values) and closed circles (<500 bootstrap values) at branch points. The numbers in parentheses are accession numbers of the sequences. The core numbers in brackets show sampling core section of the isolates. Scale bar represents *knuc* units

populations from deep marine sediments (>10 mbsf) number between 10^4 and 10^6 cells cm^{-3}. In this study, the cultivable populations from surface sediments were equivalent to the average range of previous demonstrations, while those below 190 mbsf (below the core section of 21-4) were unusually high in comparison to any other subseafloor sediment habitat. In addition, Inagaki et al. (2003) revealed that cultivable populations are generally higher in numbers in fluid-transportable porous layers than those in pelagic clay environments, which is consistent with the observation in this study that the most cultivable populations were obtained from hydrate-bearing porous layers.

Based on the sequence analysis of nearly complete 16S rRNA genes of 552 isolates, the isolates were classified into 9, 5, 31, 5, and 12 phylotypes within the *Actinobacteria, Bacteroidetes/Chlorobi, Firmicutes* (low G + C Gram-positive group), *Alphaproteobacteria*, and

Fig. 2 continued



Gammaproteobacteria, respectively (Fig. 2). With regard to the cultivated population of each strain, the emerging patterns varied with sediment depth or lithostratigraphic characteristics (Fig. 1). For instance, in near-surface sediments, the members of *Shewanella*, *Pseudoalteromonas*, *Bacillus*, *Brevibacillus*, and *Flexibacteraceae* dominated the cultivable population. Among these components, only the *Pseudoalteromonas* and *Flexibacteraceae* members more abundantly populated the deeper sediments below 190 m (Fig. 1); thus, *Shewanella*, *Bacillus*, and *Brevibacillus* members may preferentially inhabit the shallower subsurface sediments. In sharp contrast, *Pseudomonas* species were predominant only in the core sediments below 247 m (Fig. 1). In the methane hydrate-bearing zone at 190–220 mbsf, the phylogenetically most diverse aerobic, heterotrophic microbial communities were identified by cultivation. The communities were composed of *Gammaproteobacteria* (*Halomonas*, *Pseudoalteromonas*, and *Pseudomonas*), *Alphaproteobacteria* (*Paracoccus*), and *Actinobacteria* (*Rhodococcus* and *Microbacterium*). Most of the predominant aerobic, heterotrophic microorganisms were likely members of non-spore-forming bacterial groups, except for *Bacilli* members. Thus, the culture sources of these bacteria could be presently active or dormant cells rather than buried spores.

Most of the isolates were phylogenetically closely related to the previously cultivated species (Fig. 2). Although not many bacterial strains have been isolated from the deep subseafloor sediments (>50 mbsf) (Inagaki et al. 2003; D'Hondt et al. 2004; Süß et al., 2004; Bale et al. 1997; Mikuchi et al. 2003; Kendall et al. 2006), members of the *Rhodococcus*, *Aeromicrobium*,

Table 3 Number of extra-cellular enzymes-producing bacteria

Core	Number of isolates	Number of enzyme-producing isolates								
		Protease	α-Amylase	CMCase	Xylanase	Alginase	Phophatase	Chitinase	Lipase	DNase
1-1		35	26	5	2	4	56	20	41	44
1-4	4	3	3	1	0	0	3	1	3	1
2-1	9	0	4	0	0	0	1	0	4	0
3-2	16	1	2	0	1	0	15	0	11	1
6-3	54	2	13	3	5	0	27	3	18	15
12-4	5	5	5	1	0	0	5	2	2	4
21-4	94	13	34	3	0	0	58	7	54	8
24-4	89	2	30	1	1	0	54	0	41	5
24-4′	68	13	32	0	0	0	47	18	40	18
27-6	15	0	13	3	3	0	12	0	10	1
38-7	76	0	50	9	0	0	48	1	47	1
Total	499	74	212	26	12	4	326	52	271	98

Flexibacteraceae, *Sphingobacteria*, *Hyphomonas*, *Sphingomonas*, *Roseinatronobacter*, *Pseudomonas*, *Pseudoalteromonas*, and *Acinetobacter* were newly identified as deep subseafloor microorganisms from the C9001 core sediments off Shimokita. In addition, several isolates exhibited a distant relationship with any of the previously identified species. For instance, the 16S rRNA gene sequences of the *Rikenellaceae* bacterium JAM-BA0501, *Bacilli* strain JAM-FM0401, and *Sphingobacteria* strain JAM-BA0302 were distantly related with any sequences of the previously described genera (<92%). These bacteria might represent a potentially novel genera of microorganisms in the subseafloor environments.

Extra-cellular enzyme activities of subseafloor isolates

The potentials of the isolates for extra-cellular enzyme production were also characterized. The activities of protease, α-amylase, lipase, CMCase, xylanase, alginase, pectinase, phosphatase, agarase, carrageenase, chondroitinase, ligninase, chitinase, and DNase were tested. Most of the aerobic, heterotrophic strains from different core sections appeared to have a variety of extra-cellular enzyme activities (Table 3). Protease activity was identified in the *Pseudoalteromonas* and *Bacillus* strains (74 strains/499 isolates). α-Amylase-producing strains (212/499) were also predominantly found in the genus *Pseudomonas*, and lipase-producing strains (271/499) were members of the genera *Pseudoalteromonas*, *Pseudomonas*, and *Aeromicrobium*. More than 60% of the isolates produced phosphatase. DNase activity was also detected in many isolates, dominantly from the genera *Pseudoalteromonas* and *Microcella*. In contrast, around 10% or less of the isolates produced CMCase, xylanase, and chitinase, and these enzyme activities were detected mainly in members of the genera *Pseudomonas*, *Microcella*, and *Pseudoalteromonas*. Interestingly, no isolate showed agarose- and carrageenan-degrading activities although these polysaccharides were expected to be buried in the sediments, probably derived from sea algae. In addition, only *Shewanella* sp. strain JAM-GA0101, which was isolated from a near seafloor section (1-1), produced an alginase (alginate lyase). No chondroitinase, ligninase, or pectinase activity was found among the isolates.

Conclusion and future prospects

Our meta-enzyme assay of sediment slurry samples and isolates from the deep subseafloor off Shimokita clearly suggests that the subseafloor environment is an active microbial habitat and that their extra-cellular enzymes play important roles in degrading or recycling the consumable substrates in a nutrient-limited, low energy-flux microbial ecosystem. This study shows preliminary pictures of bulk core sediments as well as aerobic, heterotrophic bacterial isolates, and the meta-enzyme approach provides new insights into the functioning of the least-explored subseafloor microbial communities. Additionally, the genetic, molecular, and functional resources of these subseafloor isolates may have great potential for future industrial application. A detailed survey of these isolates and further efforts in screening the industrially applicable microbial sources are our ongoing foci.

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