Deep Terrestrial Subsurface Bacterial Unique Distribution Constrained by Geological Setting

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Running Title: Bacterial unique distribution in deep subsurface

Summary

We obtained deep-water samples at depths of up to 482 m from three boreholes drilled in sedimentary rock in two formations in Hokkaido, Japan. Prokaryotes in the samples amounted to 4.61×10^4 to 5.06×10^6 cells/mL by total direct count (TDC), which is similar to that encountered at the marine subsurface. However, the vertical distribution of prokaryotes did not simply reveal a decrease in abundance with increased depth from the surface. Considerable activity of representatives of the domain *Bacteria* was revealed using fluorescence in situ hybridization (FISH) at the transition zone of the two sedimentary rock formations. Cloning-sequencing analysis showed the difference in dominant sub-phylum of

Proteobacteria between depths of 281 to 312 m and 364 to 409 m. Horizontal heterogeneity of microbial distribution in subsurface environment was also shown by a relatively high abundance observed in members of the *Archaea* from another borehole drilled only 1.5 km northeast in the same formation.

Introduction

The rapid progress in environmental microbiology during the last decade, supported by culture-independent molecular analyses, has presented researchers with a new and unexpected view of the distribution of prokaryotes and function (Hinrichs *et al.*, 1999; Jeon *et al.*, 2003). Identification of prokaryotes at the level of individual cells by fluorescence in situ hybridization (FISH) (DeLong *et al.*, 1989) has provided us with an image of prokaryotes and their environmental interactions as well as characteristics of the distribution of prokaryotes *in situ* at very fine scale (Boetius *et al.*, 2000; Raghoebarsing *et al.*, 2006).

While our knowledge of the deep marine subsurface biosphere has developed rapidly (Parkes *et al.*, 1994; Inagaki *et al.*, 2006; Lipp *et al.*, 2008; Roussel *et al.*, 2008), our understanding of the microbiology of the deep terrestrial subsurface environment is still limited to geochemical considerations on the distribution of functional prokaryotes within a given geological setting (Colwell, 2001; Fredrickson and Onstott, 2001). Some studies on the distribution of prokaryotes conducted using molecular techniques have been undertaken, but these were restricted to igneous hard rock environments (Lehman *et al.*, 2001; Pedersen, 2002a). The impetus for studying terrestrial subsurface environments arises in response to social interest which has increased from considerations of aquifer pollution to include site selection for depositing nuclear waste

(Ghiorse, 1997; Pedersen, 2002b)**.** Knowledge of the distribution of prokaryotes and its regulating environment at the scale of several hundred or thousand meters in the deep subsurface environments that are ideally suited to nuclear waste disposal is essential given the negative legacy of the nuclear power that provided a significant portion of the electricity generated by developed countries in the 20th and 21st centuries. Such considerations are particularly relevant in Japan, which has yet to decide on the suitability of a site in which to deposit nuclear waste. An understanding of deep terrestrial subsurface microbiology in both igneous and sedimentary rock environments is thus important (Amend and Teske, 2005).

This study was conducted to increase our understanding of the distribution of prokaryotes and possible activity in different geological settings of deep, subsurface terrestrial sites in sedimentary rock.

Results and Discussion

Figure 1 shows the distributions of prokaryotes in groundwater in three boreholes drilled into two geological formations in Hokkaido, Japan (Supporting Information Figure 1). Total prokaryotic abundance in the upper layer (35.5 to 37.5 m) of the Koetoi Formation (HDB-17) amounted to 2.57×10^6 cells/ml, which was higher than the levels of 2.20×10^5 cells/ml observed in the lower layer (281 to 312 m) of the Wakkanai Formation (HDB-6). Abundance decreased to 4.61×10^4 cells/ml at depths of 364 to 409 m in the Wakkanai Formation. The first interesting finding related to the distribution of prokaryotes was the markedly high prokaryotic abundance revealed by FISH staining at depths of 281 to 312 m in the upper Wakkanai Formation. Of the more than 65% of prokaryotes identified using FISH, 61.9% and 3.5% were representatives of

the domain *Bacteria* and domain *Archaea*, respectively. As FISH targets at RNA, probability might be high that the elucidated cells are active. These high numbers of domain *Bacteria* are comparable to those found in marine surface environments, which are characterized by high photosynthetic activity (Yokokawa and Nagata, 2005). Conversely, of the other four water samples successfully analysed to date using FISH, only 6 to 14% of the total cells reacted with the probe for the domain *Bacteria*. The high values for FISH observed in HDB-6 (281 to 312 m) was also associated with a high frequency of dividing cells (FDC), suggesting high prokaryotic activity (Hagstrom *et al.*, 1979; Roussel *et al.*, 2008), which was not found at deeper depth (364 to 409 m).

The sampling depth of 281 to 312 m in HDB-6 represents the transition zone between the Koetoi and Wakkanai formations. The former is composed of diatomaceous soft mudstones (porosity ca. 65%) containing abundant fragments of siliceous diatom skeletons and siliceous sponge spicules with a uniaxial compressive strength of 5-10 MPa. The latter consists of hard shales (porosity ca. 35%) characterized by the presence of opal-CT with a uniaxial compressive strength of 10-30 MPa. Consequently, the rate of permeation of the water through the Koetoi Formation starts to decrease in the transition zone where it is thought to stagnate. This was corroborated by the fact that the pooled water flushed out at a flow rate of 3 to 10 L/min at depths of between 280 and 307 m at the time of drilling. The sample site was located within a network of cracks that developed diagonally along a south-north axis (Fig. 1).

The transition zone is characterized by gradual changes in physical properties of the strata compared to the overlying Koetoi Formation, including an increase in the effective porosity and a decrease in unit weight and elastic modulus. These changes in the characteristics of the rock contribute to lowering the water permeation rate which

leads to an accumulation of suspended, and even dissolved, materials in the water. Interestingly, a markedly high total organic carbon [TOC, measured by Shimadzu TOC 5000A (Shimadzu Corp., Japan)] concentration of 130 mg/mL was observed in this transition zone, ranging from 2 to 63 mg/mL at other depths in this and other boreholes.

Clone analysis using 16S rDNA revealed that, at depths of 281 to 312 m, the domain *Bacteria* in HDB-6 consisted exclusively of gamma-Proteobacteria while the alpha- and beta-Proteobacteria comprised 77.5% of the revealed total bacterial community in HDB-6 at 364 to 409 m (Fig. 2, Supporting Information Figure 2). Clone analysis showed that the predominant gamma-Proteobacterial population consisted of anaerobic heterotrophs with 100% similarity observed for *Pseudomonas stutzeri* (556 bp) and 99% for *Pseudomonas veronii* (776 bp) using 16S rDNA.

The second remarkable finding regarding the distribution of prokaryotes was a very high abundance of members of the domain *Archaea* in borehole HDB-4 drilled just 1.5 km northeast of HDB-6 in the same formation. In HDB-4, TDC values of 8.36×10^5 cells/ml at 224 to 233 m were the lowest of all three depths examined, while TDC values of 5.06 and 1.13×10^6 cells/ml were obtained at depths of 281 to 291 m and 475 to 482 m, respectively. The contribution of the *Archaea* to TDC increased with depth and accounted for 10 to 19% of TDC (0.86 to 5.52×10^5 cells/ml in HDB-4), while it was only approximately 3% in HDB-6. Clone analysis of *Archaea* carried out successfully in HDB-4 from 281 to 291 m revealed that the isolated clones belong to the *Euryarchaeota* in the genus of *Methanospirillum.* Some clones exhibited similarities as high as 97% with *Methanobacterium hungatei* by 16S rDNA (ca. 650 bp), which is known to form methane from long-chain alkanes (Zengler *et al.*, 1999). The Wakkanai Formation consists primarily of diatoms, in which alkanes are abundant (ten Haven *et*

al., 1993). When the environments of HDB-4 (281 to 291 m) and HDB–6 (281 to 312 m) are compared at equivalent depths, the likelihood of conditions becoming increasingly anoxic with depth in HDB-4 is greater due to its thickness of Wakkanai Formation, despite the equivalent geometric depth of HDB-6 was overlaid with Koetoi Formation of less substrate density (Fig. 1). Thus, highly reductive environment develops at the same geometric depth in HDB-4 in the Wakkanai Formation, and that this accounts for the abundance of methane producing *Archaea* observed in HDB-4 (Supporting Information Figure 3). Gas analyses supported this hypothesis as methane in HDB-4 comprised between 80 to 95% of all the gas measured, which was higher than that measured in HDB-6 which varied between 75 to 80% [Groundwater and core material samples were analysed for amount and composition by the water substitution method and gas chromatography (GC-14A, Shimadzu Corp.), respectively. Gas data were corrected by assuming that oxygen was not present in groundwater].

Environments in which methane is produced may be conducive for colonization by sulphur reducing bacteria. Clone analysis showed that water from both HDB-4 and -6 contained delta-Proteobacteria that contain sulphate reducing bacteria, with samples from HDB-4 (281-291 m) showing 99% similarity to *Desulfomicrobium* and the clone from HDB-6 (281-312 m) showing a 95% similarity to *Desulfuromonas*. Since water was sampled using a double packer system, which trapped a 10-m column of water within the borehole at any given depth for analysis at the surface, whether these bacteria exhibit sulphate-reducing activity *in situ* is uncertain.

The distribution of prokaryotes and possible function are clearly constrained by the geological properties of these deep terrestrial subsurface environments. Consequently, given the potential influence of microbial activity upon the geochemistry

of a given geological setting, particularly within the context of site selection for the storage of nuclear waste, consideration of the microbial community composition and geological attributes of the sites at which they are most prevalent need to be considered.

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Figure Legends

Fig. 1. The abundance of prokaryotes in three boreholes drilled in two geological formations (A). The abundance of prokaryotes and sampling site plotted on a detailed geological map showing a minor fault drawn by tele-viewer analysis for HDB-6 (B) and HDB-4 (C). Strike and dip of each fracture were measured using a borehole tele-viewer (Acoustic Televiewer M-W Instruments, Inc., US and Electrical Micro-Imager Tool, Halliburton, US) to map the minor fault-developing zone. Drilling was conducted on a low-lying coastal plain of Quaternary alluvial and terrace deposits resting on Neogene sedimentary rocks to depths of 520 m and 620 m for HDB-4 and 6, respectively. These two boreholes were spaced within approximately 1.5 km of each other along a southwest-northeast axis. Additional drilling, HDB-17, was performed approximately 0.5 km southeast of this line to a depth of 50 m for detailed study of the Koetoi Formation. Water was trapped by sealing in double packers of 2 to 35 m in length at each depth for each borehole and pumped up through a stainless pipe which had been thoroughly rinsed with target water and water was sufficiently pumped up before sampling.

Microbial cells were collected by filtration of the sampled water from 0.7 to 50 ml and stained with acridine orange (Hobbie *et al.*, 1977). The prokaryotic cells were observed by epifluorescence microscopy with 20 microscopic fields counted per sample. In addition, samples were fixed with 4% paraformaldehyde and filtered through a Nuclepore filter (pore size, 0.2 μm; Whatman, UK). Hybridization with prokaryotes-specific probe, EUB338 (Amann *et al.*, 1990), ARC915 (Stahl and Amann, 1991) was performed and microscopic images of the cells were taken using a fluorescence

microscope (BX50, Olympus Corp., Japan) and captured with a cooled charge-coupled device camera (DP70, Olympus Corp., Japan).

Fig. 2. Composition of bacterial community revealed by clone analysis shown by relative abundance of sub-phylum. 41 and 40 clones were characterized for HDB-6 at depths of 281 to 312 m and 364 to 409 m. Groundwater samples were filtered from 100 to 500 ml with a Nuclepore filter (pore size, 0.2 μm; Whatman, UK). Cells were lysed using a bead beater (Mini-BeadBeater^{TM}, BioSpec Products, Inc., US) and prokaryotic DNA was extracted using phenol-chloroform-isoamylalcohol (Zwart *et al.*, 1998) and purified with UltraClean Soil DNA Isolation Kit (MoBio Lab Inc., US). To generate near full-length 16S rDNA clones, the extracted DNA was subjected to PCR amplification with primers 27F and 1492R (Lane, 1991). The PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen Corp., UK), before being ligated into a pCR 4Blunt-TOPO vector (Invitrogen Corp.) and transformed into competent *Escherichia coli* TOPO10 cells (Invitrogen Corp.) and clone libraries of bacterial 16S rDNAs were constructed. The sequences of the representative 16S rDNA clones were determined using a capillary DNA sequencer (CEQ2000XL DNA Analysis System, Beckman Coulter Inc., US), with the 16S rDNA-specific primer, 341F (Muyzer *et al.*, 1993), used for sequence reactions. Sequence data were aligned using the CLUSTAL W package (Thompson *et al.*, 1994), with clones having similarities of 97% or above grouped into operational taxonomic units (OTUs). The clones were homology-searched using BLAST (Altschul *et al.*, 1997) and the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/).

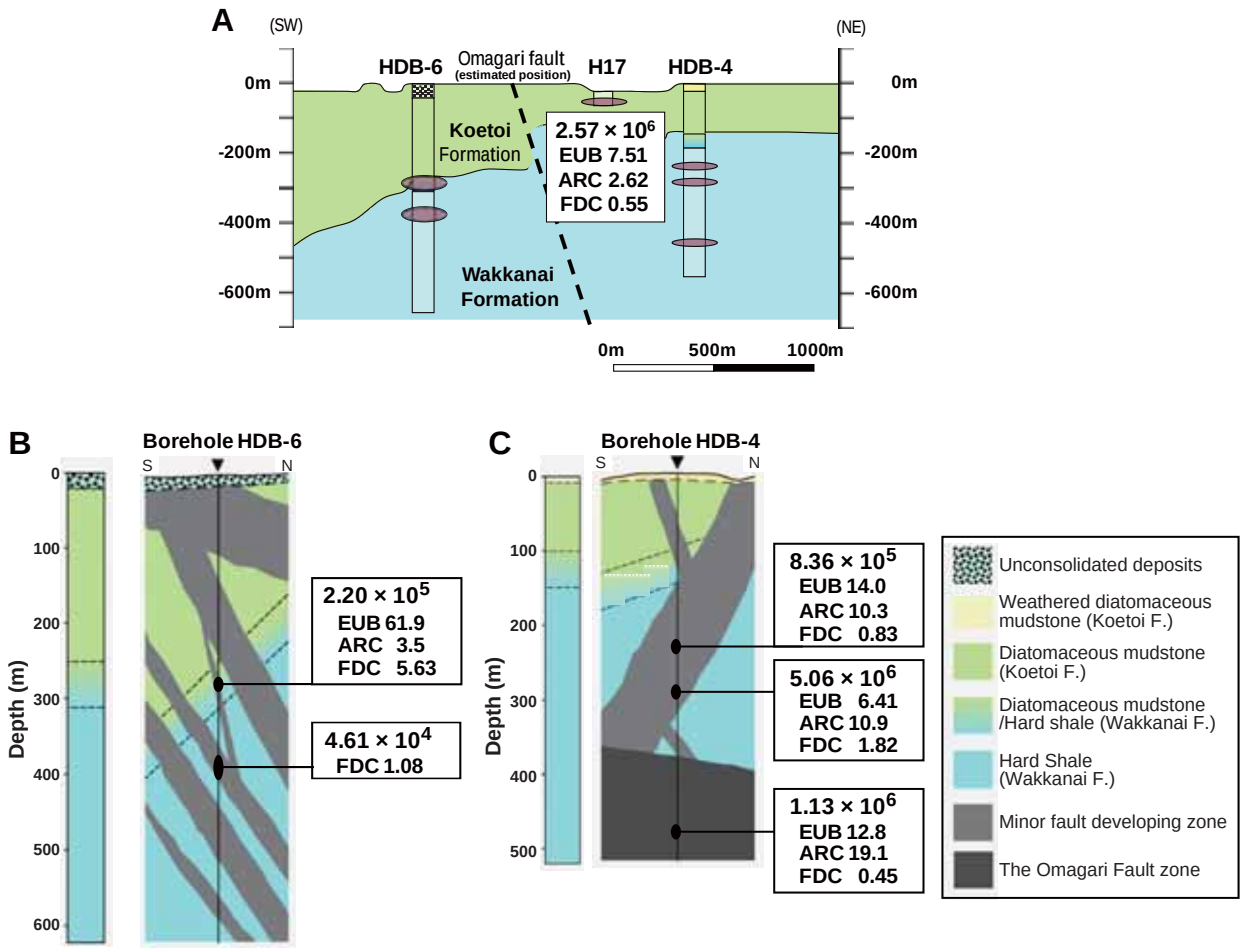

A
(SW)
(NE)
HDB-6
Omagari fault
(estimated position)
H17
HDB-4
0m
-200m
-400m
-600m
Koetoi
Formation
2.57 × 10^6
EUB 7.51
ARC 2.62
FDC 0.55
Wakkanai
Formation
0m
500m
1000m
B
Borehole HDB-6
S
N
Depth (m)
0
100
200
300
400
500
600
2.20 × 10^5
EUB 61.9
ARC 3.5
FDC 5.63
4.61 × 10^4
FDC 1.08
C
Borehole HDB-4
8.36 × 10^5
EUB 14.0
ARC 10.3
FDC 0.83
5.06 × 10^6
EUB 6.41
ARC 10.9
FDC 1.82
1.13 × 10^6
EUB 12.8
ARC 19.1
FDC 0.45
Unconsolidated deposits
Weathered diatomaceous mudstone (Koetoi F.)
Diatomaceous mudstone (Koetoi F.)
Diatomaceous mudstone /Hard shale (Wakkanai F.)
Hard Shale (Wakkanai F.)
Minor fault developing zone
The Omagari Fault zone

Fig. 1.

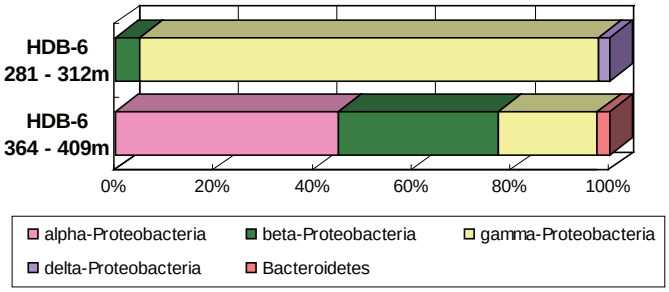
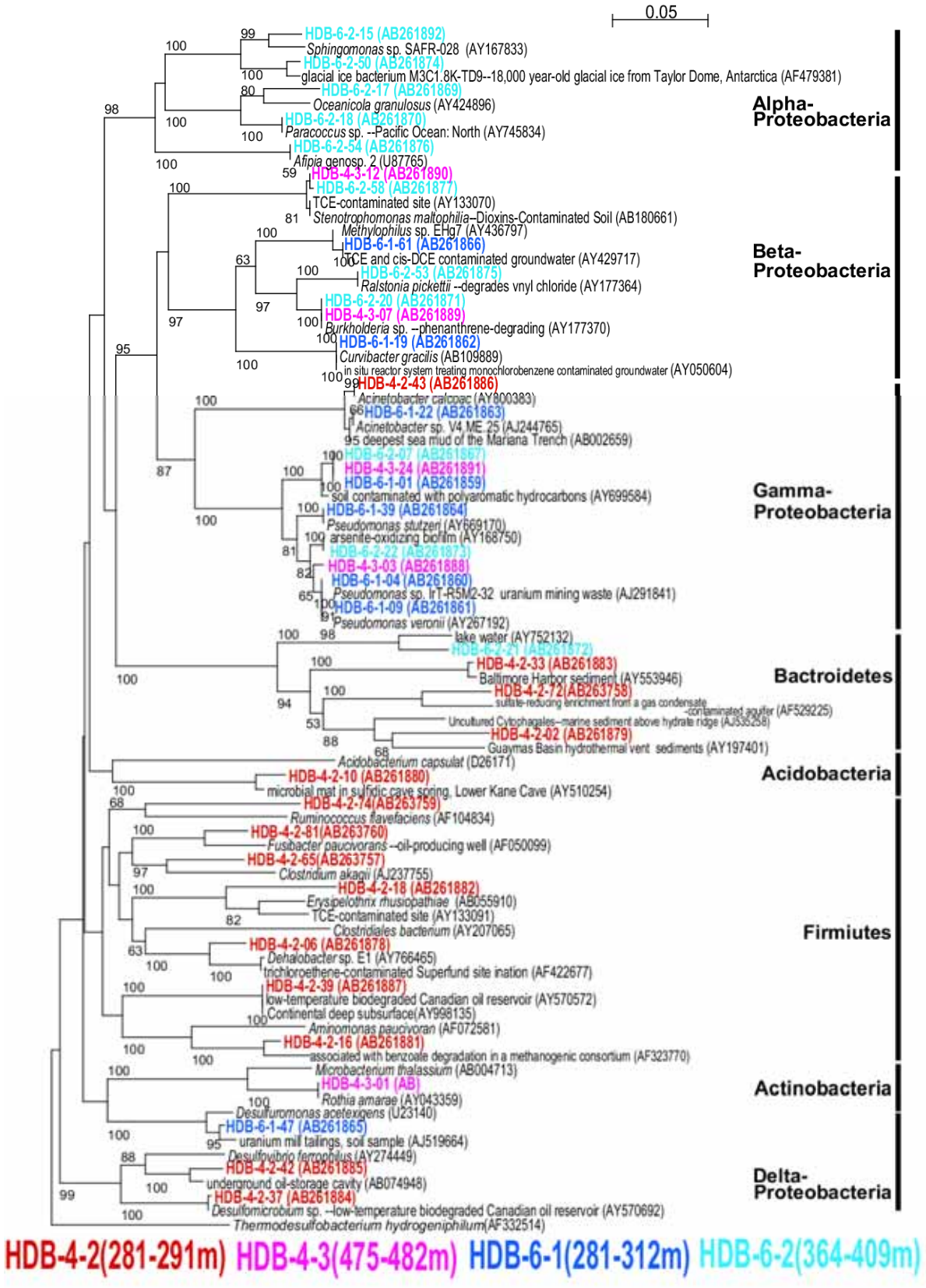

HDB-6
281 - 312m
HDB-6
364 - 409m
0%
20%
40%
60%
80%
100%
alpha-Proteobacteria
beta-Proteobacteria
gamma-Proteobacteria
delta-Proteobacteria
Bacteroidetes

Fig. 2.

Supporting Information, Kenji Kato et al.

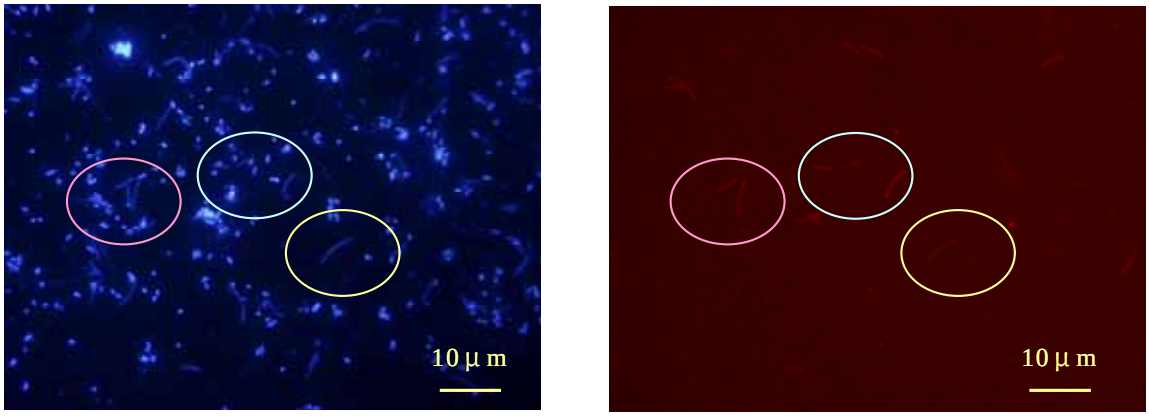
Supporting Information Figure 1. View of study site in Horonobe, Hokkaido. A drilling tower is shown at the center of the picture.



Supporting Information Figure 2. Phylogenetic tree based on 16S rRNA gene of the clones detected for the groundwater of HDB-4 (281 to 291m and 475 to 482m), HDB-6 (281 to 312m and 364 to 409m) indicating phylogenetic relationships between members of some lineages. Numbers at nodes refer to bootstrap values. Only those above 50% are shown. The sequence of *Aquifex pyrophilus* was used as an outgroup to root the tree.

Scale bar = 5% nucleotide substitution. Phylogenetic trees were produced using the neighbor-joining algorithm (Saitou and Nei, 1987) of the NJ plot program (Perriere and Gouy, 1996).

a b



Supporting Information Figure 3. Large bacteria with ca. 10 µm taken from HDB-4 (281 to 312m) were shown to be *Methanomicrobiales* group by FISH, using a probe of EURY 496 (Jurgens *et al.*, 2000). (a: DAPI staining and b: Rodaim-labeled)

Supporting References

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