Molecular characterization of microbial communities in fault-bordered aquifers in the Miocene formation of northernmost Japan

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ABSTRACT

We investigated the diversity and distribution of archaeal and bacterial 16S rRNA gene sequences in deep aquifers of mid- to late Miocene hard shale located in the northernmost region of the Japanese archipelago. A major fault in the northwest-southeast (NW-SE) direction runs across the studied area. We collected three groundwater samples from boreholes on the southwest (SW) side of the fault at depths of 296, 374 and 625 m below ground level (mbgl) and one sample from the northeast (NE) side of the fault at a depth of 458 mbgl. The groundwater samples were observed to be neutral and weakly saline. The total microbial counts after staining with acridine orange were in the order 10^5–10^6 cells per mL and 10^3 cells per mL in the aquifers to the SW and to the NE of the fault, respectively. A total of 407 archaeal and bacterial 16S rRNA gene sequences (204 and 203 sequences respectively) were determined for clone libraries constructed from all groundwater samples. Phylogenetic analyses showed that the libraries constructed from the SW aquifers were generally coherent but considerably different from those constructed from the NE aquifer. All of the archaeal clone libraries from the SW aquifers were predominated by a single sequence closely related to the archaeon *Methanoculleus chikugoensis*, and the corresponding bacterial libraries were mostly predominated by the sequences related to Bacteroidetes, Firmicutes and δ-Proteobacteria. In contrast, the libraries from the NE aquifer were dominated by uncultured environmental archaeal clones with no methanogen sequences and by β-Proteobacterial clones with no sequences related to

Bacteroidetes and δ-Proteobacteria. Hence, the possible coexistence of methanogens and sulphate-reducers in Horonobe deep borehole (HDB) on the SW side is suggested, particularly in HDB-6 (374 mbgl). Moreover, these organisms might play an important geochemical role in the groundwater obtained from the aquifers.

INTRODUCTION

Recent estimates of microbial abundance in the terrestrial and marine deep subsurface range from 10^3 to 10^6 cells per mL of water (Pedersen & Ekendahl, 1990; Pedersen, 1993; Stevens *et al*., 1993; Stevens & McKinley, 1995) or per cubic centimetre of sediment (Parkes *et al*., 1994). It has been estimated that the subsurface biosphere is the greatest reservoir of living biomass (Whitman *et al*., 1998; Parkes *et al*., 2000). In addition, molecular approaches such as 16S rRNA gene (16S rDNA) sequence analyses have revealed unique microflorae in the deep subsurface, as exemplified in terrestrial crystalline rocks (Pedersen *et al*., 1996a), sedimentary rocks (Pedersen *et al*., 1996b; Takai *et al*., 2003; Fredrickson *et al*., 2004), petroleum reservoirs (Orphan *et al*., 2000; Watanabe *et al*., 2002a, 2002b), marine sediments (Inagaki *et al*., 2003a) and subseafloor gas hydrates (Reed *et al*., 2002).

The Miocene Wakkanai Formation in northernmost Japan consists of layers of a kind of sedimentary rock that is frequently seen in the region of the archipelago alongside the Sea of Japan;

it is particularly characterized by high porosity but low permeability (Fukusawa 1987; Yamamoto *et al*., 2004). An underground research laboratory (URL) is being constructed in the unique sediment by the Japan Atomic Energy Agency (JAEA), and the survey boreholes created for the URL construction are used for geophysical, geological, hydrological and geochemical studies. Special care that the tracer content of groundwater was periodically determined at the site during pumping, is being taken to minimize and monitor contamination of the geochemical samples by the drilling fluid so that they can also be used for microbiological studies. A major fault runs across this area and may affect microbial communities via hydrogeological and biogeochemical influences, although the effect of faulting on microflorae has not been a focus in subsurface microbiology. This study reports the diversity and distribution of 16S rDNA sequences in four different groundwater samples from the Wakkanai Formation and discusses the differences in the microbial community structures on the opposite sides of the fault.

MATERIALS AND METHODS

Field site and sample collection

The studied Horonobe area is hydrologically characterized by the shore-side Sarobetsu-genya wetland, artesian wells and upland recharge areas as well as paleo-seawater trapped in deep aquifers (Yamamoto *et al*., 2004). The geologic features of this area include the Miocene and Pliocene

siliceous mudstones of the Wakkanai and Koetoi Formations, respectively; these mudstones occur typically in northernmost Japan. The Pleistocene sandstone of the Yuchi Formation overlies these two formations. Further, the Omagari Fault runs in the northwest-southeast (NW-SE) direction in the studied area (Fukusawa, 1987); however, the hydrologic influence of the fault has not been fully understood.

As part of the regional survey for JAEA's URL construction in Horonobe Town, Horonobe Deep Boreholes (HDB) have been bored from the surface. Four groundwater samples were collected from three boreholes (Table 1). The boreholes HDB-6 (total depth: 620.0 mbgl) and HDB-11 (total depth: 1020.0 mbgl) are respectively located approximately 0.5 and 1 km southwest (SW) of the Omagari Fault, while HDB-10 (total depth: 550.0 mbgl) is located 1 km northeast (NE) of the fault (Fig. 1). The groundwater samples were collected from different depths due to the different borehole sites. Therefore, in this study, we have not focussed on the systematic comparison of the vertical succession in a single borehole and the horizontal variation in a single aquifer at separate boreholes.

The boreholes were drilled using a weak bentonite fluid (ca. 5% w/v) to stabilize the borehole wall. The drilling fluid contained microbial cells at a density of $[5.6 \pm 0.8] \times 10^3$ mL^{-1} (see below).

Groundwater sampling from the boreholes involved isolating the interval to be sampled by using a single or double packer assembly (Fig. 2). Once the packers had been inflated, the drilling fluid was removed from the packed-off interval with a submersible pump. During pumping, the tracer content

was determined periodically at the site in order to estimate the contamination of the groundwater by the drilling fluid; sodium naphthionate, a fluorescent dye, was used as the tracer. Several physical and chemical parameters were also measured simultaneously. Comprehensive chemical and microbial analyses of the samples were carried out in an off-site laboratory.

The samples were collected from HDB-6, 10 and 11. The multiple piezometric logger system (MP system, Westbay Instruments, Sugar Land, Texas) was installed in HDB-6 after the drilling investigations. For microbial analyses, the groundwater samples from HDB-6 and HDB-11 were collected in a few 1-L pressure-retaining bottles by using the MP system and T-type sampler system patented by Taiseikisosekkei Co., Japan. The pressure-retaining bottle was specially developed to avoid chemical and microbial disturbance of water sample due to degassing and oxidization. The samples from HDB-10 were collected using the pumping system and by employing a hydraulic-packer test on the surface. For chemical analyses, the samples from all boreholes were collected using the same pumping system. The 1-L bottles were washed with MilliQ-purified water and autoclaved before use. There was no PCR amplification of bacterial and archaeal 16S rDNA; this was confirmed with autoclaved MilliQ water which served as a negative control.

Chemical analyses

Chemical analyses were carried out separately from the microbial analyses but by using the same

groundwater samples filtered through microfiltration (MF) membrane filters (pore size, 0.45 μm; Millipore, Billerica, Massachusetts), according to the Japanese Industrial Standards (JIS). Na^+ and K^+ were determined by flame photometry according to JIS K0101 47.1 and 47.2, respectively, using an AA-880mkII spectrophotometer (Thermo Electron, Yokohama, Japan; formerly Nippon Jarrel-Ash). NH_4^+ was determined by the indophenol blue absorption photometry according to JIS K0101 36.2 by using a U-1100 spectrophotometer (Hitachi, Tokyo, Japan). Fe^{2+} and Fe^{3+} were determined by 1,10-phenanthroline absorption photometry according to JIS K0101 60.1 by using a U-1100. Ca^{2+} and Mg^{2+} were determined by inductively coupled plasma-atomic emission spectrometry according to JIS K0101 49.3 and 50.3, respectively, using an ICAP-575mkII spectrophotometer (Thermo Electron). Anions were determined by ion chromatography according to JIS K0101 32.5, 37.2.5 and 42.4 by using a DX-320J ion chromatography system (Nippon Dionex, Osaka, Japan). Total inorganic and organic carbon concentrations were determined by combustion catalytic oxidation and non-dispersive infrared detection according to JIS K0101 20.1 by using a TOC5000A analyser (Shimadzu, Kyoto, Japan).

Total microbial count

To entrap microbial cells, 0.1–5 mL of a groundwater sample was mixed with one-tenth its volume of 0.22 μm-filtered formalin at atmospheric pressure immediately after sampling and kept in the dark

at 4°C; this mixture was filtered through MF membrane filters (pore size, 0.22 μm; Millipore). The captured cells were stained with acridine orange and counted under an Olympus BX51 epifluorescence microscope (Ghiorse & Balkwill, 1983). At least 100 view fields were examined to estimate the average microbial abundance in the groundwater sample. The total cell count in the drilling fluid was determined by the same procedure and was found to be $[5.6 \pm 0.8] \times 10^3$ cells per mL.

DNA extraction

Approximately 1 L of groundwater sample was filtered to capture microorganisms on an autoclaved MF membrane filter (pore size, 0.22 μm; Millipore) and was maintained at –80°C until DNA extraction. Bulk DNA per filter was obtained by phenol-chloroform-isoamyl alcohol extraction performed in a 2-mL sterilized tube. Pieces of a filter were placed in the tube and 700 μL of TE buffer (1 mM EDTA, pH 8.0) and 40 μL of lysozyme (50 $mg \cdot mL^{-1}$) were added to it; the tube was incubated at 37°C for 1 h. Then, 50 μL of proteinase K (20 $mg \cdot mL^{-1}$) and 100 μL of sodium dodecyl sulphate (200 $mg \cdot mL^{-1}$) were added, and the tube was incubated at 55°C for 2 h. To the lysate, 700 μL of TE-saturated phenol was added, and the mixture was vigorously vortexed for 4 min followed by centrifugation at 16000 × *g* for 3 min; this procedure was repeated twice. The supernatant that was collected after three rounds of centrifugation was washed thrice with chloroform-isoamyl

alcohol (24:1) and condensed to 100 μL with 2-butanol. Bulk DNA was precipitated from the condensate with ethanol and purified using a BD Chroma Spin+TE-1000 Column (Clontech Laboratories, Mountain View, California). Purified bulk DNA solutions of 0.3–5.0 $\mu g \cdot mL^{-1}$ with an A_{260}/A_{280} ratio of 1.1:1.7 were thus obtained. A positive control at a minimum of 100 cells per filter and a negative control resulted in positive and negative amplification, respectively, of archaeal/bacterial 16S rDNA (see below).

Amplification and cloning of 16S rDNA

Archaeal 16S rDNA sequences were amplified using the primers A25F (5′-CYGGTTGATCCTGCCRG-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3′) according to Dojka *et al.* (1998). A 20-μL reaction mixture for PCR amplification contained 1 μL of bulk DNA solution, 1.6 μL of 25 mM $MgCl_2$, 1.6 μL of each 2.5 mM dNTPs, 0.3 μL of each 0.02 mM primer, 13.1 μL of autoclaved distilled water and 0.5 U of Ex*Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Japan). The reaction mixtures were initially heated at 94°C for 12 min, followed by 30 cycles of amplification on a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA) as follows: 94°C for 1 min, 50°C for 45 s and 72°C for 2 min, with a final extension at 72°C for 12 min.

Bacterial 16S rDNA sequences were amplified using the primers B27F

(5′-AGAGTTTGATCCTGGCTCAG-3′) and U1492 R (5′-GGTTACCTTGTTACGACTT-3′) according to Lane (1991). A 20-μL reaction mixture for PCR amplification contained 1 μL of bulk DNA solution, 1.6 μL of 25 mM $MgCl_2$, 1.6 μL of each 2.5 mM dNTPs, 0.2 μL of each 0.02 mM primer, 13.3 μL of autoclaved distilled water and 0.5 U of Ex*Taq* DNA polymerase (TaKaRa Bio Inc.). The reaction mixtures were initially heated at 96°C for 2 min, 58°C for 40 s and 72°C for 40 s, followed by 27 cycles of amplification carried out on a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems) as follows: 96°C for 30 s, 58°C for 25 s and 72°C for 25 s, with a final extension at 72°C for 10 min.

PCR products were electrophoresed on a 0.8% agarose gel, stained with ethidium bromide to observe and excise the expected *ca.* 1500-bp bands and extracted from the gel by using a QIAquick Gel Extraction Kit (Qiagen, Valencia, California). Purified PCR products were cloned in *Escherichia coli* TOP10 cells by using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, California), and the *E. coli* transformants were selected on Luria-Bertani agar plates containing ampicillin (50 μg mL^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg mL^{-1}).

Sequencing and phylogenetic analyses

The TOPO plasmids bearing the PCR products were extracted and purified using an UltraClean Plasmid Mini Prep Kit (MO BIO Laboratories, Inc., Solana Beach, California) and were used for

sequencing on a 3730xl DNA Analyser (Applied Biosystems) with a BigDyeTerminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). In addition to M13F and M13R primers for the TOPO plasmid, the following primers were used for bidirectional sequencing: for archea, U533F (5′-GTGCCAGCMGCCGCGGTAA-3′), U907R (5′-CCGTCAATTCCTTTRAGTTT-3′) and A958R (5′-YCCGGCGTTGAMTCCAATT-3′) according to Dojka *et al*. (1998) and Pudry *et al*. (2002), and for bacteria, B530F (5′-CCAGCAGCCGCGGTAATAC-3′) and B800R (5′-TACCAGGGTATCTAATCC-3′) according to Hiraishi (1992).

The sequences were edited using the GENETYX ver. 7.0 program (Genetyx, Osaka, Japan) and chimera-checked using the Similarity Matrix program on the Ribosomal Database Project (RDP II). Sequences showing >97% similarities were grouped as one phylotype which was represented by the least deviated sequence. The representative sequences were homology-searched using the FASTA program on the DNA Data Bank of Japan (DDBJ) and aligned using the ClustalW program (Thompson *et al*., 1989). Phylogenetic trees were constructed using the TreeView program (Page, 1996) or the MEGA3.1 program package (Kumar *et al.*, 2004) based on the neighbour-joining method (NJ; Saitou & Nei, 1987) algorithms (data not shown). Sequence diversity was evaluated by the rarefaction analysis using the analytical approximation algorithm of Hurlbert (Hurlbert *et al*., 1971); this is available on the website [http://www2.biology.ualberta.ca/jbrzusto/rarefact.php].

Nucleotide sequence accession numbers

The archaeal and bacterial 16S rDNA sequences obtained in this study have been deposited in the DDBJ under the accession numbers ranging from AB237664 to AB237760 (Tables 2–4).

RESULTS AND DISCUSSION

Groundwater properties

Groundwater samples from different horizons of the Wakkanai Formation showed different physicochemical properties, as listed in Table 1. Physicochemical properties other than temperature and Eh were generally similar between the groundwaters from 458 mbgl (HDB-10) on the NE side and 625 mbgl (HDB-11) on the SW side of the fault.

The total organic carbon (TOC) concentration in the water from HDB-10 was lower than that in other boreholes; however, it is unclear whether this low TOC accounts for the total count of microbes being 10^2 times lower than that expected at other boreholes. The TOC concentrations of the groundwaters in crystalline/sandstone rocks and petroleum reservoirs are in the order 10^0–10^1 $mg \cdot L^{-1}$ and 10^1–10^2 $mg \cdot L^{-1}$, respectively (Pedersen & Ekendahl 1990; Pedersen *et al*., 1996a, 1996b; Orphan *et al*., 2000). Therefore, the TOC concentration in the Wakkanai Formation aquifers, particularly that of 130 $mg \cdot L^{-1}$ in the water at 296 mbgl (HDB-6), is comparable to that of organic-rich oil reservoir aquifers.

The total microbial counts were, however, not associated with the high TOC concentrations since they ranged from 2.7×10^3 to 1.65×10^6 cells per mL when compared with a representative range of 10^3–10^8 cells per mL in deep aquifers (Pedersen, 1993). Correlation between the TOC concentration and total microbial counts has been reported for deep terrestrial subsurface and subseafloor sediment (Pedersen & Ekendahl, 1990; Pedersen *et al*., 1996ab; Parkes *et al*., 2000). Pedersen (2001) reported that 'the only parameter which correlated with the total number was the amount of total organic carbon' and that the 'total number of microorganisms in groundwater does not correlate with any other measured parameter'. In the HDB groundwaters, the total counts did not correlate with the TOC or with other parameters which may suggest that the Wakkanai Formation has been less stable in terms of hydrology and associated biogeochemistry.

Contamination of the sampled water with the drilling fluid was shown to range from 6.2% to 8.1% (Table 1). Therefore, the microorganisms detected must be considered a mixture of indigenous and contaminating organisms; the mesophilic facultative anaerobes in particular, are from the drilling fluid. The time elapsed between drilling and sampling was different for each borehole. Moser *et. al*. (2003) suggested that given sufficient time, the population of contaminating microbes eventually reduce in boreholes. The risk of contamination was in the order HDB-6 > HDB-11 > HDB-10. However, in our study, groundwater was sampled when the tracer content decreased and stabilised. In our study, the total pumped groundwater volume from the last drilling date until sampling was

7.8–53.9 kL, while the borehole drained *ca*. 1.4 kL in Moser's study. Therefore, it can be regarded that all possible care was taken to minimize and monitor contamination in the limited time available.

Diversity of archaeal 16S rDNA

A total of 27 phylotypes based on 204 clone sequences of archaeal 16S rDNA were obtained from four groundwater samples (Table 2). Three clone libraries from HDB-6 and HDB-11 on the SW side of the Omagari Fault consisted of 163 clones, of which 151 clones (93%) were related to cultured methanogens of Euryarchaeota with greater than 98% similarity (Table 2). The phylotype HDBW-WA01 (prefix HDBW omitted hereafter) which was most closely related to *Methanoculleus chikugoensis* dominated 100% of 296-mbgl (HDB-6), 98% of the 625-mbgl (HDB-11) and 33% of the 374-mbgl (HDB-6) libraries. The second dominant phylotype was WA05 which was related to *Methanosarcina mazei* (Goe1) and comprised 31% of the library constructed from organisms at 374 mbgl (HDB-6).

The methanogens in archaeal 16S rDNA libraries are known to be dominant in hot petroleum reservoirs, petroleum-contaminated aquifers and Cretaceous shale (Orphan *et al*., 2000; Watanabe *et al*., 2002a; Takai *et al*., 2003). However, the phylotype WA01 is different from the clones reported in these libraries; thus, it represents a strain indigenous to the Horonobe hydrologic area or the Wakkanai Formation. The host organisms of the methanogen-related clones may play a major

geochemical role in the Horonobe deep subsurface. However, the importance of these organisms is still a matter of speculation since the gas contents of the groundwaters have not been determined. Analysis of the dissolved gas composition and the carbon isotopic compositions of methane and dissolved inorganic carbon (DIC) might reveal the ecological impact of methanogen on the spatial carbon cycle.

In contrast, the water collected at 458 mbgl in HDB-10 on the NE side of the fault yielded a library comprising 18 phylotypes based on 41 clones which were related only to uncultured environmental clones (Table 2). It should be noted that three phylotypes (14 clones) of Crenarchaeota as well as 15 phylotypes (27 sequences) of Euryarchaeota were observed in this library, whereas only one clone of Crenarchaeota were found in other HDB libraries. HDB-10 library was characterized by numerous phylotypes related to the SAGMEG-1 cluster. This uncultured clone group had been isolated from fissure water from a deep South African gold mine (Takai *et al*., 2001). The occurrence of the deeply divergent sequence accords phylogenetic uniqueness to the HDB-10 library in contrast to the coherence between the HDB-6 and HDB-11 libraries (data not shown). This phylogenetic uniqueness may be associated with unique physiological and biogeochemical activities. However, since no archaeal strains have been cultured from the HDB boreholes, their characteristic activities remain unknown.

Diversity of bacterial 16S rDNA

A total of 70 phylotypes based on 203 clone sequences of bacterial 16S rDNA were obtained from four groundwater samples; these were affiliated with six established phyla and four clades (Tables 3 and 4). It should be noted that each library consisted mainly of unique phylotypes, suggesting that each aquifer has developed an indigenous microflora. Nevertheless, the HDB-6 and HDB-11 libraries were generally coherent at the phylum level, while they were distinctly different from the HDB-10 library, as shown in the archaeal clone libraries. This is also an example of the dissimilarity between the HDB-6–11 and HDB-10 aquifers on the SW and NE sides of the Omagari Fault, respectively.

Three clone libraries from HDB-6 and HDB-11 consisted of 148 clones that were similarly dominated by the sequences related to Firmicutes (80 clones, 54%), Bacteroidetes (27 clones, 18%) and δ-Proteobacteria (27 clones, 18%). In contrast to the similarity at the phylum level, there were differences in clone compositions among the aquifers. In particular, the 375 mbgl library (HDB-6, total 42 clones) was dominated by 20 clones related to δ-Proteobacteria and *Desulfotomaculum* sp. (Firmicutes) which suggests that sulphate-reducing bacteria may play a major geochemical role in this aquifer. Recently, it has been reported that Gram-positive sulphate-reducing bacteria such as *Desulfotomaculum* sp. may be widely distributed in the deep subsurface areas (Baker *et al*., 2003). Furthermore, it is suggested that the coexisting methanogens and sulphate reducers are common in

the deep subsurface habitats (continental and marine) (Struchtemeyer, *et al*., 2005; Moser *et al*., 2005). In this report, since the microbial community in the SW-side borehole was found to be dominated by phylotypes related to methanogen, the possibility of coexisting methanogens and sulphate-reducers in the HDB of the SW side, particularly in HDB-6 (374 mbgl), was suggested, and these organisms might play an important geochemical role in the groundwater found in these aquifers.

The HDB-10 library (total 55 clones) showed a distinct clone composition with regard to the following points: (1) dominance by 40 clones related to β-Proteobacteria; (2) unique occurrence of two phylotypes (WB01 and WB02) related to α-Proteobacteria; and (3) absence of the clones related to Bacteroidetes and δ-Proteobacteria that dominated other HDB libraries (Tables 3 and 4).

In our study, the examination of the contents of drilling fluid in HDB groundwater suggested that the HDB libraries might be contaminated by the microorganisms from the drilling fluid to some extent. Therefore, we have considered the drilling equipment contaminants (Pedersen *et. al*., 1997), the mining water contaminants (Onstott *et. al*., 2003) and the experimental contaminants in the low-biomass habitat (Tanner *et. al*., 1998) that had been described previously and have underlined the following phylotypes in the tables whose sequences might be related to those of the contaminants: WB10, WB06, WB07, WB13, WB14, WB16 in HDB-10 libraries. If the above contaminants were considered, the HDB-10 library would be the most dissimilar and diverse among

all HDBs.

Rarefaction analysis

Rarefaction curves based on the clone-to-phylotype ratios (Fig. 3) suggested that the studied HDB-6 and HDB-11 aquifers have low archaeal diversity. The archaeal HDB-6 (374 mbgl) library and the bacterial HDB-11 library also reached plateaus within the studied clone numbers, suggesting that more efforts in clone analysis would yield only a limited addition of phylotypes. In contrast, high bacterial diversity was observed in the HDB-6 aquifer (374 mbgl). The archaeal HDB-10 library and the bacterial HDB-10 and 11 libraries also do not reach plateaus, suggesting that more clone analysis would result in a more comprehensive understanding of the aquifer-specific microflorae with more phylotypes. Overall, although the HDB-6 and HDB-11 aquifers yielded phylogenetically coherent libraries, they were distinctly separated, as revealed by a diversity analysis based on the clone-phylotype ratios. The HDB-10 aquifer (458 mbgl) possibly hosts high archaeal and bacterial diversity and should be studied in more detail in the future.

CONCLUSION

The four aquifers yielded distinct 16S rDNA clone libraries, suggesting that the aquifers are well confined and have developed specific microflora with indigenous species, even if the contaminants

from the drilling fluid are considered The possible coexistence of methanogens and sulphate-reducers in HDB on the SW side is suggested, particularly in HDB-6 (374 mbgl). Moreover, these organisms might play an important geochemical role in the groundwater obtained from the aquifers. The archaeal and bacterial libraries of the HDB-10 aquifer (458 mbgl) were the most dissimilar and diverse of all; this uniqueness of the aquifer is ascribed to the following factors: (1) location of the aquifer on the NE side of the Omagari Fault; (2) aquifer horizon in the Wakkanai Formation; or (3) sample collection of pumped decompressed water. However, more systematic surveys are required to confirm the influence of faulting on the hydrology and geochemistry of the region which in turn affect microbial communities developed in associated aquifers.

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Table 1 Physicochemical parameters and total cell counts of deep groundwater samples collected from the Horonobe deep boreholes (HDB), northernmost Japan, in reference to the Omagari Fault.

	SW side of the Omagari Fault			NE side
	HDB-6		HDB-11	HDB-10
Depth median (mbgl[a])	297[c]/296 [d]	387[c]/374[d]	625[c,d]	458[c,d]
Depth range (mbgl)	281.0–312.0[c] 288.7–303.0[d]	364.0–409.0[c] 362.4–385.7[d]	606.0–644.1[c,d]	445.8–469.8[c,d]
Drilling date [e]	2003/10/6	2003/11/28	2005/1/26	2004/12/8
Sampling date	2003/10/16 [c] 2005/1/15 [d]	2003/12/17 [c] 2005/1/17 [d]	2005/3/30 [c,d]	2004/12/20 [c,d]
Water temperature (ºC)	22.4	25.6	35.5	30.5
pH	7.27	6.87	6.8	6.77
EC ($mS \cdot m^{-1}$)	1199	1966	3010	2510
Eh (mV) Pt-electrode	11	–52.6	–60	–22.4
Na^+ ($mg \cdot L^{-1}$)	2460	4220	6600	5000
K^+ ($mg \cdot L^{-1}$)	77	110	140	160
NH_4^+ ($mg \cdot L^{-1}$)	94	140	200	220
Ca^{2+} ($mg \cdot L^{-1}$)	69	140	250	97
Mg^{2+} ($mg \cdot L^{-1}$)	43	110	170	140
Fe^{2+} ($mg \cdot L^{-1}$)	0.03	0.04	2.3	2.1
Fe^{3+} ($mg \cdot L^{-1}$)	<0.05	<0.05	<0.05	0.07
Cl^- ($mg \cdot L^{-1}$)	3050	6300	10000	8100
NO_3^- ($mg \cdot L^{-1}$)	0.1	0.1	0.1	0.5
SO_4^{2-} ($mg \cdot L^{-1}$)	5.9	0.2	<0.2	0.4
HCO_3^- ($mg \cdot L^{-1}$)	41	41	2200	1100
Total inorganic carbon ($mg \cdot L^{-1}$)	390	510	450	320
Total organic carbon ($mg \cdot L^{-1}$)	130	63	31	20
Total cell count ($\times 10^5$ cells/mL)	8.3 ± 0.6	16.5 ± 1.2	3.2 ± 0.5	0.03 ± 0.005
Drilling fluid contamination (%)	8.1 [c] 6.2 [d]	6.2 [c] 6.8 [d]	8.9 [c,d]	7.5 [c,d]
Pumped groundwater volume from drilling date to the sampling date (kL)	26.5 [c] 29.5 [d]	53.8 [c] 53.9 [d]	17.6 [c,d]	7.8 [c,d]

a, metre below ground level

b, calculated from 5.6×10^3 cells/mL × contamination (%)

c, for chemical analyses

d, for microbial analyses

e, before sampling date

Table 2 Clone number distributions and phylogenetic affiliations of archaeal phylotypes from groundwaters in deep subsurface boreholes.

	Phylotype (HDBW-)	No. of clones				Total	Accession no.	Putative affiliation	Closest sequence (>97% similarity)	Similarity (%)
		HDB-6		HDB-11	HDB-10					
		296[a]	374[b]	625[c]	458[d]					
	WA01	54	18	53		125	AB237734	*Methanomicrobiales*	*Methanoculleus chikugoensis* MG62 (AB038795)	98.28
	WA02		4	1		5	AB237735	*Methanomicrobiales*	*Methanoculleus thermophilus* DSM 2832 (AJ862839)	98.21
	WA03		2			2	AB237736	*Methanomicrobiales*	*Methanocorpusculum parvum* DSM 3828 (AY260435)	99.85
	WA04		2			2	AB237737	*Methanomicrobiales*	*Methanoculleus palmolei* (Y16382)	99.36
	WA05		17			17	AB237738	*Methanosarcinales*	*Methanosarcina mazei* Goe1 (AE013359)	98.72
	WA06		1			1	AB237739	*Methanosarcinales*		
	WA07		7			7	AB237740	*Methanobacteriales*		
Euryarchaeota	WA08				1	1	AB237741	SAGMEG-1[e]		
Euryarchaeota	WA09				1	1	AB237742	SAGMEG-1		
Euryarchaeota	WA10				2	2	AB237743	SAGMEG-1		
Euryarchaeota	WA11				1	1	AB237744	SAGMEG-1		
Euryarchaeota	WA12				1	1	AB237745	SAGMEG-1		
Euryarchaeota	WA13				3	3	AB237746	SAGMEG-1		
Euryarchaeota	WA14				1	1	AB237747	SAGMEG-1		
Euryarchaeota	WA15				1	1	AB237748	TMEG[f]		
Euryarchaeota	WA16		3			3	AB237749	TMEG		
Euryarchaeota	WA17				1	1	AB237750	TMEG		
Euryarchaeota	WA18				1	1	AB237751	TMEG		
Euryarchaeota	WA19				3	3	AB237752	TMEG		
Euryarchaeota	WA20				2	2	AB237753	DSEG[g]		
Euryarchaeota	WA21				7	7	AB237754	DSEG		
Euryarchaeota	WA22				1	1	AB237755	MEG[h]		
Euryarchaeota	WA23				1	1	AB237756	Unaffiliated		
	Sub-total	54	54	54	27	189				
Crenarchaeota	WA24		1			1	AB237757	TMCG[i]		
Crenarchaeota	WA25				5	5	AB237758	TMCG		
Crenarchaeota	WA26				3	3	AB237759	TMCG		
Crenarchaeota	WA27				6	6	AB237760	TMCG		
	Sub-total	0	1	0	14	15				
	Archeal total	54	55	54	41	204				

a, 288.7–303.0 metre below ground level (mbgl); b, 362.4–385.7 mbgl; c, 606.0–644.1 mbgl; d, 445.8–469.9 mbgl

e, South Africa gold mine Euryarchaeotic group-1 (Takai *et al*., 2001)

f, Terrestrial miscellaneous Euryarchaeotic group (Dojka *et al*., 1998; Chin *et al*., 1999; Eder *et al*., 1999)

g, Deep-sea Euryarchaeotic group (Eder *et. al*., 1999; Takai & Horikoshi, 1999; Takai *et al*., 2001)

h, Miscellaneous Euryarchaeotic group

i, Terrestrial miscellaneous Crenarchaeotic group (Barns *et al*., 1994, 1996; Hershberger *et al*., 1996; Jurgens *et al*., 1997; Chandler *et al*., 1998)

Table 3 Clone number distributions and phylogenetic affiliations of proteobacterial phylotypes from groundwaters in deep subsurface boreholes.

Phylotypes (HDBW-)	No. of clones				Total	Accession no.	Putative affiliation	Closest sequence (>97% similarity)	Similarity (%)
	HDB-6		HDB-11	HDB-10					
	296[a]	374[b]	625[c]	458[d]					
WB01				1	1	AB237664	α-*proteobacteria*		
WB02				1	1	AB237665	α-*proteobacteria*		
WB03				10	10	AB237666	β-*Proteobacteria*	*Acidovorax defluvii* (Y18616)	99.38
WB04	6				6	AB237667	β-*Proteobacteria*	Bacterium ROME315Asa (AY998136)	99.45
WB05				1	1	AB237668	β-*Proteobacteria*	*Burkholderia fungorum* W566 (AJ544691)	99.93
WB06				1	1	AB237669	β-*Proteobacteria*	Comamonadaceae bacterium SB1 (AJ606333)	99.24
WB07				2	2	AB237670	β-*Proteobacteria*	*Comamonas testosteroni* SB4. (AJ606336)	99.93
WB08				1	1	AB237671	β-*Proteobacteria*	*Dechloromonas denitrificans* ED1T (AJ318917)	98.22
WB09	1				1	AB237672	β-*Proteobacteria*		
WB10				2	2	AB237673	β-*Proteobacteria*	*Oxalobacteraceae* bacterium MWH73 (AJ556800)	99.51
WB11				5	5	AB237674	β-*Proteobacteria*		
WB12				1	1	AB237675	β-*Proteobacteria*	Clone NE83 (AJ575698)	99.45
WB13				1	1	AB237676	β-*Proteobacteria*	Clone Gitt-GS-139 (AJ582191)	100.00
WB14				1	1	AB237677	β-*Proteobacteria*	*Variovorax* sp. KS2D-23 (AB196432)	97.60
WB15				1	1	AB237678	β-*Proteobacteria*	Clone cMM319-20 (AJ536817)	98.28
WB16				14	14	AB237679	β-*Proteobacteria*	*Pseudomonas lanceolata* (AB021390)	99.59
WB17				1	1	AB237680	γ-*Proteobacteria*	Clone LB-P (AF538773)	98.43
WB18	1	1			2	AB237681	γ-*Proteobacteria*	*Pseudomonas stutzeri* 28a42 (AJ312165)	99.45
WB19				1	1	AB237682	γ-*Proteobacteria*		
WB20		1			1	AB237683	δ-*Proteobacteria*		
WB21		3			3	AB237684	δ-*Proteobacteria*		
WB22		3			3	AB237685	δ-*Proteobacteria*		
WB23		4			4	AB237686	δ-*Proteobacteria*	*Desulfomicrobium baculatum* DSM2555 (AJ277895)	99.66
WB24	3				3	AB237687	δ-*Proteobacteria*	*Desulfovibrio oryzae* (AF273083)	99.59
WB25	4	1			5	AB237688	δ-*Proteobacteria*	Clone B4 [*Desulfovibrio*] (AJ133797)	98.78
WB26	1				1	AB237689	δ-*Proteobacteria*		
WB27			3		3	AB237690	δ-*Proteobacteria*		
WB28	1				1	AB237691	δ-*Proteobacteria*		
WB29		2			2	AB237692	δ-*Proteobacteria*	Sulphate-reducing bacterium R-PropA1 (AJ012591)	98.41
WB30	1				1	AB237693	δ-*Proteobacteria*	Clone SJA-63 (AJ009471)	97.85
WB31				2	2	AB237694	ε-*Proteobacteria*	*Campylobacter* sp. (L14632)	98.39
WB32		1			1	AB237695	ε-*Proteobacteria*		
WB33	1				1	AB237696	Unaffiliated		
Sub-total	19	16	3	46	84				
Bacterial total	50	42	56	55	203				

a, 288.7–303.0 metre below ground level (mbgl); b, 362.4–385.7 mbgl; c, 606.0–644.1 mbgl; d, 445.8–469.9 mbgl
Phylotypes that are underlined are suspected common contaminants.

Table 4 Clone number distributions and phylogenetic affiliations of non-proteobacterial phylotypes from groundwaters in deep subsurface boreholes.

Phylotypes (HDBW-)	No. of clones				Total	Accession no.	Putative affiliation	Closest sequence (>97% similarity)	Similarity (%)
	HDB-6		HDB-11	HDB-10					
	296[a]	374[b]	625[c]	458[d]					
WB34	1				1	AB237697	Bacteroidetes		
WB35	8	2			10	AB237698	Bacteroidetes		
WB36			3		3	AB237699	Bacteroidetes	*Bacteroides* sp. strain Z4 (AY949860)	99.86
WB37			2		2	AB237700	Bacteroidetes	Clone 054E07 B DI P58 (CR933218)	98.54
WB38	4				4	AB237701	Bacteroidetes		
WB39	1				1	AB237702	Bacteroidetes		
WB40		4			4	AB237703	Bacteroidetes		
WB41		1			1	AB237704	Bacteroidetes		
WB42		1			1	AB237705	Bacteroidetes		
WB43	1				1	AB237706	Firmicutes	*Nostocoida limicola* I	99.80
WB44			1		1	AB237707	Firmicutes		
WB45			1		1	AB237708	Firmicutes		
WB46	12	2		4	18	AB237709	Firmicutes	*Acetobacterium paludosum* (X96958)	97.48
WB47		1			1	AB237710	Firmicutes		
WB48	1				1	AB237711	Firmicutes	Bacterium TC8 (AB118593)	97.30
WB49			4		4	AB237712	Firmicutes		
WB50			2		2	AB237713	Firmicutes	*Clostridium propionicum* DSM 1682 (X77841)	98.29
WB51				2	2	AB237714	Firmicutes	*Clostridium* sp. (X95274)	97.14
WB52		1			1	AB237715	Firmicutes		
WB53		5			5	AB237716	Firmicutes	*Desulfotomaculum* sp. 175 (AF295656)	99.42
WB54		1			1	AB237717	Firmicutes		
WB55		1			1	AB237718	Firmicutes		
WB56			4		4	AB237719	Firmicutes	*Sedimentibacter* sp. C7 (AY766466)	99.79
WB57	1				1	AB237720	Firmicutes		
WB58		1			1	AB237721	Firmicutes	Clone IA-23 (AJ488074)	98.68
WB59	1				1	AB237722	Firmicutes		
WB60		1			1	AB237723	Firmicutes	Clone SHA-33 (AJ249104)	97.87
WB61		1	9		10	AB237724	Firmicutes		
WB62	1				1	AB237725	Firmicutes		
WB63			27		27	AB237726	Firmicutes		
WB64		1			1	AB237727	Firmicutes		
WB65				1	1	AB237728	Cenibacterium	Bacterium H12 (AY345556)	97.86
WB66		1			1	AB237729	Actinobacteria	Actinobacteria strain PB90-5 (AJ229241)	97.86
WB67		1			1	AB237730	Spirochaetes		
WB68				1	1	AB237731	Unaffiliated		
WB69				1	1	AB237732	Unaffiliated		
WB70		1			1	AB237733	Unaffiliated		
Sub-total	31	26	53	9	119				
Bacterial total	50	42	56	55	203				

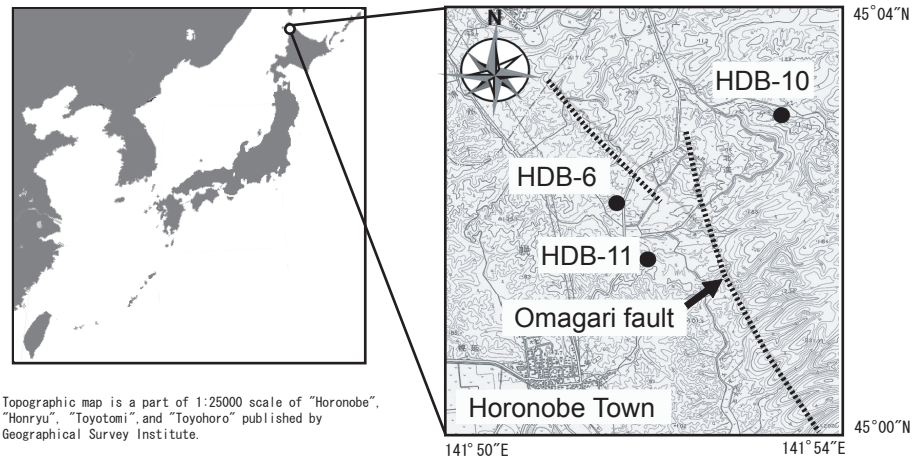
a, 288.7–303.0 metre below ground level (mbgl); b, 362.4–385.7 mbgl; c, 606.0–644.1 mbgl; d, 445.8–469.9 mbgl

FIGURE LEGENDS

Fig. 1 Location of boreholes HDB-6, HDB-10 and HDB-11 in the Omagari Fault in the Horonobe area.

Fig. 2 Groundwater sampling from the boreholes involved isolating the interval to be sampled by using packer assembly

Fig. 3 Rarefaction curves for archaeal and bacterial phylotypes occurring in four Horonobe aquifers.



Topographic map is a part of 1:25000 scale of "Horonobe", "Honryu", "Toyotomi", and "Toyohoro" published by Geographical Survey Institute.

Fig. 1

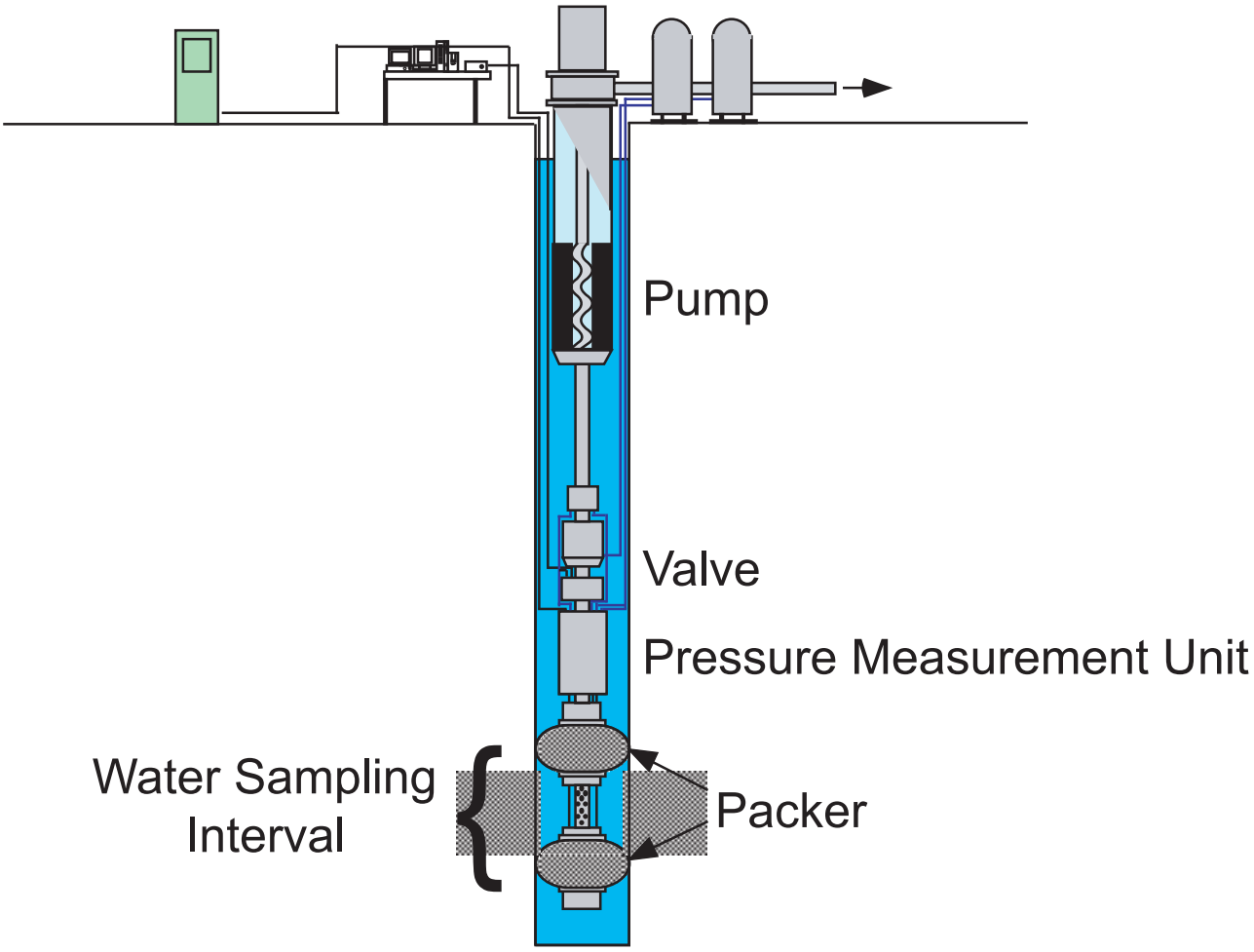

Pump
Valve
Pressure Measurement Unit
Water Sampling Interval
Packer

Fig.2

Fig. 3

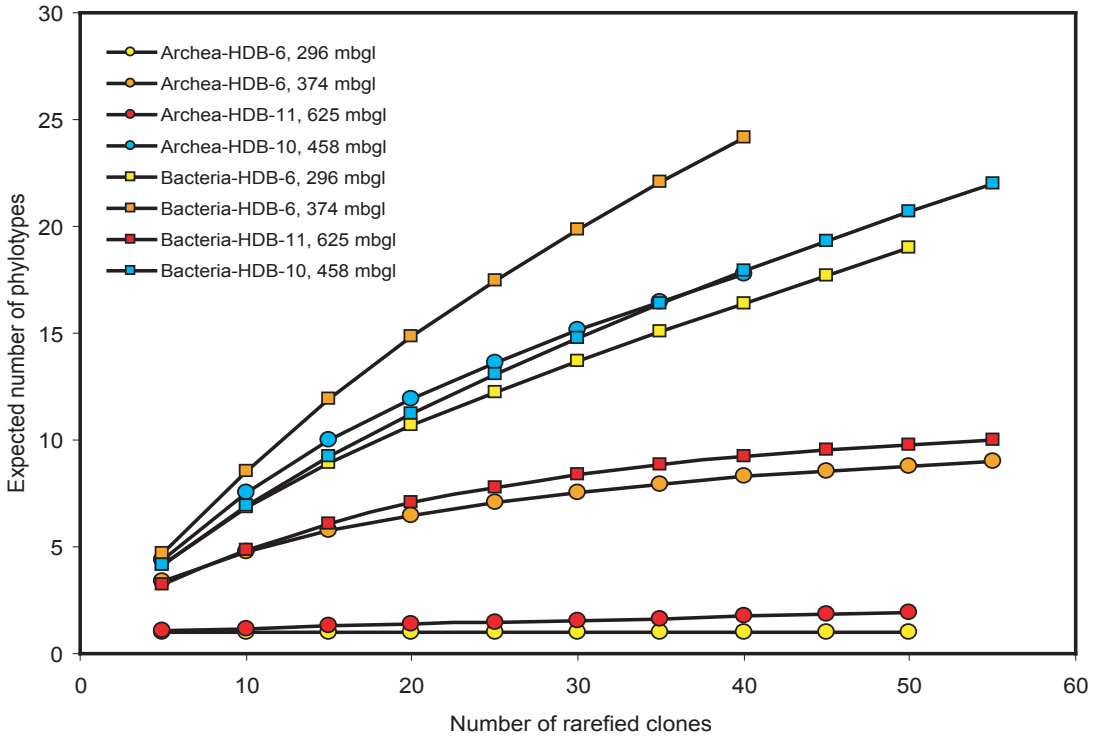

Archea-HDB-6, 296 mbgl
Archea-HDB-6, 374 mbgl
Archea-HDB-11, 625 mbgl
Archea-HDB-10, 458 mbgl
Bacteria-HDB-6, 296 mbgl
Bacteria-HDB-6, 374 mbgl
Bacteria-HDB-11, 625 mbgl
Bacteria-HDB-10, 458 mbgl
Expected number of phylotypes
Number of rarefied clones
0
5
10
15
20
25
30
0
10
20
30
40
50
60

Fig. 3