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Bacterial community structures of deep-sea water investigated by molecular biological techniques

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ABSTRACT

The aim of the present study was to investigate the bacterial community structures of deep-sea water (DSW) and surface seawater (SSW) samples in Japan by molecular biological techniques. DGGE analyses and pyrosequencing analysis revealed that bacterial community structures of DSW were diverse and differed from those of SSW. This is the first report on the horizontal variation of bacterial community structures of DSW throughout Japan. In addition, pyrosequencing analysis revealed that the number of phyla in DSW was larger than that in SSW, and specific phyla, such as *Firmicutes* and *Planctomycetes*, were characterized by a higher proportion of the bacterial community structure in DSW than in SSW. Taken together, these results indicate that a variety of bacteria that are specifically adapted to the DSW environments can be expected to be found in DSW, and DSW would thus be a potential resource for novel or unique microorganisms and compounds.

1. Introduction

Deep-sea water (DSW) is generally defined as the seawater below a depth of 200 m, where the sunlight required for photosynthesis does not penetrate. As of February 2014, 16 pumping stations for DSW in various geographical locations surrounding Japan are operating (Deep Ocean Water Applications Society: http://www.dowas.net/facilities/index.html [In Japanese]). DSW has some unique characteristics, such as low temperature, a high concentration of minerals and nutrients, and good quality, being pathogen-free and stable (Nakasone and Akeda 1999). DSW has been applied to aquaculture, the food industry, and agriculture, and used for medical treatment (Nakasone and Akeda 1999; Hataguchi et al. 2005; Katsuda et al. 2008; Hwang et al. 2009).

DSW has also been used for the cultivation of microorganisms, such as micro-algae (Nakasone and Akeda 1999) and phytoplankton (Ikeda et al. 2002); however, less attention was paid to DSW as a resource for microorganisms because DSW contains a low concentration of microorganisms. In fact, we found that the viable bacterial count in DSW was approximately 1000 times lower than that in SSW at the DSW pumping station of Izu-Akazawa in Shizuoka Prefecture, Japan (Imada 2013). However, in recent years, the interest in marine microorganisms producing bioactive compounds has been growing. To our knowledge, antibiotics have been found from the culture broth of actinomycetes and diatoms isolated from DSW of Toyama Bay, Japan (Furumai et al. 2002; Lee et al. 2006). DSW would thus be a potential resource for microorganisms producing bioactive compounds; however, only a few reports are available concerning the microorganisms in DSW. In addition, there is no report on the horizontal variation of bacterial community structures in DSW pumped from various geographical locations throughout Japan.

Molecular biological techniques are required to investigate the microbial diversity because uncultivated bacteria constitute 99% or more of the total bacteria (Amann et al. 1995). Genetic fingerprinting techniques, which are molecular biological techniques, are suitable for comparative analysis of a large number of samples. Denaturing gradient gel electrophoresis (DGGE) using the 16S rRNA gene is a genetic fingerprinting technique and has been used in microbial ecology (Muyzer et al. 1993). DGGE analysis was already utilized to examine the differences in actinomycetal community structures between seawater and freshwater (Yoshida et al. 2008). Additionally, pyrosequencing analysis has recently been recognized as the effective technique to investigate microbial diversity because extremely large datasets can be obtained and used for better understanding the diversity (Binladen et al. 2007).

The present study aimed to investigate bacterial community structures of DSW and SSW samples in Japan firstly by DGGE analyses. The investigation was conducted on the seawaters from eight selected pumping stations from Hokkaido through Okinawa Prefecture, Japan. We secondly used nonmetric multidimensional scaling (NMDS) analysis to visualize differences among the bacterial community structures.

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Fig. 1. Geographical locations of DSW pumping stations in Japan where we collected DSW and SSW samples in this study.

Combined with the results of DGGE and NMDS methods, pyrosequencing analysis was additionally used to investigate the bacterial community structures of DSW and SSW samples to find more supporting evidences.

2. Materials and methods

2.1. DSW and SSW samples

Geographical locations of DSW pumping stations where we collected DSW and SSW samples in this study are shown in Fig. 1. For PCR-DGGE analysis, DSW and SSW samples were collected from eight pumping stations in different areas in Japan. Information regarding the eight pumping stations and the sampling dates are shown in Table 1. For pyrosequencing analysis, a total of five seawater samples were examined. DSW and SSW samples were collected from the pumping stations in Izu-Akazawa (Izu) in March 2012 and from the pumping stations in Toyama in the Sea of Japan, which is opposite to the station in Izu in the Pacific Ocean, in April 2013. In addition, a SSW sample was collected from Tokyo Bay in June 2012. To collect bacteria, 2 l of each sample was passed through a Nuclepore filter (3.0 μm) and then filtered onto a Nuclepore filter (0.2 μm). Each of the 0.2 μm filters was stored in a 2-ml tube at −20 °C until DNA extraction.

2.2. DNA extraction

Bacterial DNA was extracted by cetyltrimethylammonium bromide (CTAB) and the beads-beating method. Briefly, 600 μl of TE buffer and 20 μl of lysozyme (5 mg/ml, Wako) were added to the tube and incubated at 37 °C for 1 h. Subsequently, 3 μl of proteinase K (20 μg/ml, Invitrogen) and 30 μl of 10% sodium dodecyl sulfate (SDS) were added and incubated at 37 °C for 1 h. After incubation, 600 μl of phenol/chloroform/isoamyl alcohol (25:24:1) was added. Bacterial cells were then smashed with glass beads by a beads cell disruptor (MS-100R, TOMY) at 2000 rpm for 15 s. After beads-beating, 100 μl of CTAB was added, mixed, and incubated at 65 °C for 10 min. The tubes were centrifuged at 5000 ×*g* for 30 min, and 650 μl of the supernatants was transferred to new tubes. Subsequently, 30 μl of 10% SDS and 600 μl of chloroform/isoamyl alcohol (24:1) were added and centrifuged at 20,000 ×*g* for 20 min. After the supernatants were transferred to new tubes, DNA was purified by isopropyl alcohol precipitation, dissolved in 20 μl of sterilized water, and stored at −20 °C.

2.3. PCR-DGGE

Bacterial 16S rRNA genes were amplified using universal primer sets (GC-341F (Muyzer et al. 1993) and 907R (Muyzer et al. 1995)). Each PCR mixture (total of 25 μl) contained 2 × GoTaq ®Green Master Mix (Promega), 0.1 μM each primer, and 2.0 μl of extracted DNA. Amplification was performed under the following profile: 2 min at 94 °C; 45 cycles of 2 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C; 30 min of final extension at 72 °C. The PCR products were verified by electrophoresis on 1% (w/v) agarose gels, followed by staining with ethidium bromide.

DGGE was performed as previously described (Yoshida et al. 2008) with minor modifications. Briefly, approximately 200 ng of each PCR product was analyzed on a 8% polyacrylamide gel containing gradients of 20–65% denaturants (7 M urea and 40% deionized formamide were considered to be 100% denaturant). Electrophoresis was run for 18 h at 100 V at 60 °C in 0.5 × TAE buffer by the D-code System (Bio-Rad Laboratories) and Power Pac Basic (Bio-Rad Laboratories). Subsequently, the gel was stained with SYBR Gold (Invitrogen) for 30 min, and the gel image was taken by the Molecular Imager FX Pro System (Bio-Rad Laboratories). Furthermore, nonmetric multidimensional scaling (NMDS) analysis with SPSS 13.0 (SPSS Japan Inc., IBM) was performed using a distance matrix constructed from DGGE banding patterns (absence and presence of the bands) to visualize the similarities among the community structures. MDS analysis is a statistical technique which generates a two-dimensional map with artificial x-axis and y-axis where each DGGE banding pattern is placed as one point and distances between points represent the similarity of the community structures.

2.4. Pyrosequencing analysis

The V4 region of 16S rRNA genes was amplified using V4 FLX forward primer (sequence tag + AYTGGGYDTAAAGNG) and reverse primer (sequence tag + CCGTCAATTCMTTTRAGT) as shown at the RDP website (http://pyro.cme.msu.edu/pyro/help.jsp). Primers used in this study are shown in Table 2. Each PCR mixture (in a total of 50 μl) contained 2 × GoTaq ®Green Master Mix (Promega), 0.2 μM each of primers, and 2.0 μl of extracted DNA. Amplification was performed under the following profile: 2 min at 94 °C; 35 cycles of 50 s at 94 °C, 30 s at 40 °C, 60 s at 72 °C; 5 min of final extension at 72 °C (Claesson et al. 2010). The PCR products were verified by electrophoresis on

Table 1
DSW pumping stations from the southern to northern part of Japan and sampling dates.

Pumping station	Prefecture	Intake depth (m)	Sampling dates
Kumejima (Ku)	Okinawa	612	24/May/2010, 24/August/2010, 24/November/2010, 2/March/2011
Muroto (Mu)	Kochi	374	26/May/2010, 25/August/2010, 24/November/2010, 27/February/2011
Suruga (Su)	Shizuoka	397	24/May/2010, 30/August/2010, 25/November/2010, 3/March/2011
Izu-Akazawa (Izu)	Shizuoka	800	26/May/2010, 24/August/2010, 24/November/2010, 24/February/2011
Oshima (Os)	Tokyo	512	25/May/2010, 23/August/2010, 25/November/2010, 28/February/2011
Sado (Sa)	Niigata	332	27/May/2010, 26/August/2010, 26/November/2010, 27/February/2011
Iwanai (Iw)	Hokkaido	300	7/July/2010, 25/August/2010, 6/December/2010, 9/March/2011
Rausu (Ra)	Hokkaido	356	31/May/2010, 13/September/2010, 25/November/2010, 2/March/2011

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