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Bacterial community structure analysis of deep-sea water and surface seawater in Japan by pyrosequencing

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Abstract

Until recent years, marine bacterial researches have been conducted mainly on surface seawater (SSW) and marine sediment, but only few studies have been undertaken on deep-sea water (DSW). Therefore, in this study, bacterial community structures of DSW and SSW collected from seven DSW pumping stations in Japan were investigated by molecular biological techniques using a 454 FLX sequencer. The result of pyrosequencing analysis showed that the bacterial diversity of DSW was considerably higher than that of SSW as we studied before. The proportions of several phyla (*Actinobacteri*a, *Chloroflex*i, *Gemmatimonadete*s, and SAR046) in DSW were higher than those in SSW. In addition, principal coordinates analysis (PCoA) based on the pyrosequencing data showed that DSW samples tended to form clusters separated from those of SSW samples. These results indicate that the bacterial community structure of DSW is different from that of SSW and there are various bacteria adapting in DSW environment, which differ from those in SSW. Therefore, DSW is believed to contain novel bacteria not found in SSW as a source for finding novel bioactive compounds.

Key Words: Deep-sea water, Bacterial community structure, Pyrosequencing

1. Introduction

Marine samples such as seawater, sediment, organisms and microorganisms have provided various bioactive compounds potential in several fields, especially new chemical agents for critical diseases. DSW is defined as seawater below compensation depth where decomposition of various organic matters is much more than production by photosynthesis. There is a few microorganisms and vertical mixing is little caused physically (Takahashi, 2005). Furthermore, DSW is one of the unique marine resources for searching of novel bioactive substances due to unique characteristics, such as low temperature, a high concentration of nutrients, high hydrostatic pressure and a low concentration of microorganisms (Nakasone and Akeda, 1999; Imada, 2013). For above reasons, much attention has been given to novel bioactive compound-producing microorganisms from DSW in recent years (Igarashi *et al.*, 2015; Harunari *et al.*, 2017). However, information about microorganisms in DSW was still limited until today. Therefore, an attempt was made to analyze the microbial community structure in DSW.

In Japan, sixteen DSW pumping stations are operating in various geographical locations now (Deep Ocean Water Applications Society: http://www.dowas.net/facilities/index.html [In Japanese]). DSW has been applied to various fields of industry such as aquaculture, agricul-

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ture (Fujita and Takahashi, 2006), the ocean thermal energy conversion system (Nakajima, 2002) and daily items such as foods, pharmaceuticals and cosmetics (Itou *et al.*, 2006), which are produced by the utilization of functional effects of DSW, such as antioxidation and atheroprotective (Takahashi, 2005).

It is well known that unculturable bacterial population constitutes 99% or more of all the bacteria living in the natural environment (Amann *et al.*, 1995; Ishida and Sugita, 2005). For this reason, pyrosequencing analysis is an effective method for investigating the bacterial community structure because big data sets can be obtained and the difference of bacterial community structures can be compared using bacterial 16S rRNA (Edwards *et al.*, 2006). Pyrosequencing analysis is also used for various fields such as environmental microbiology, and food microbiology, especially used for analyzing bacterial community structure (Margulies *et al.*, 2005).

Microbial community structure analysis using denatured gradient gel electrophoresis (DGGE) and pyrosequencing analysis has been performed in our laboratory (Terahara *et al.* 2016). The result of DGGE analysis revealed that there is a difference of bacterial community structures among the eight pumping stations of DSW and SSW in Japan. There was no relationship between horizontal variations of bacterial community structures and geographical locations from northern to southern Japan. However, only two stations of DSW and SSW (Izu-Akazawa, Toyama) were investigated by pyrosequencing analysis and the bacterial community structure was described in phylum level (Terahara *et al.*, 2016).

In this study, we clarified the difference between DSW and SSW in the bacterial community structures from the seven pumping stations in Japan by statistics and relative abundant analysis. Furthermore, we verified how bacterial community structures were different in detail and which bacteria were more abundant in DSW than in SSW.

2. Material and methods

2.1 DSW and SSW sample preparation

Fig. 1 shows geographical location of seven DSW pumping stations. Information on the sampling date at each station is shown in Table 1. Two liters of DSW and

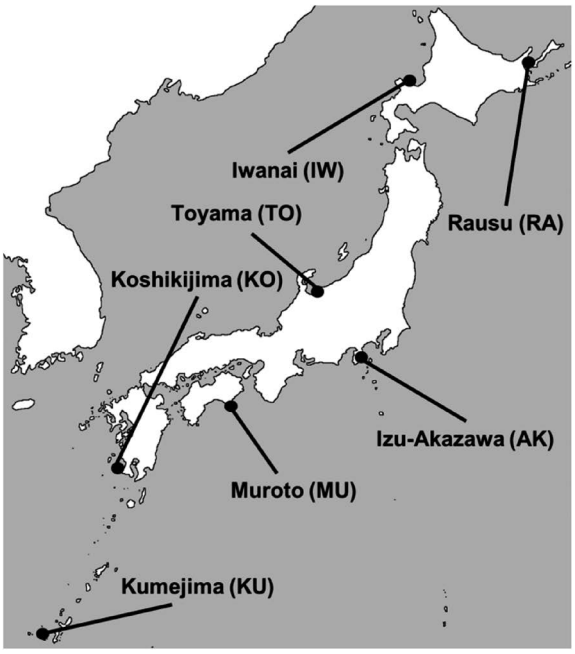

Fig. 1. Geographical location of DSW pumping stations in Japan examined in this study.

Table 1. Depth and sampling date of DSW pumping stations in Japan.

Pumping station	Depth (m)	Sampling dates
Kumejima (KU)	612	25/May/2013, 30/Aug./2013, 29/Nov./2013, 27/Feb./2014
Koshikijima (KO)	325	24/May/2013, 30/Aug./2013, 29/Nov./2013, 27/Feb./2014
Muroto (MU)	374	15/May/2013, 30/Aug./2013, 29/Nov./2013, 28/Feb./2014
Izu-Akazawa (AK)	800	15/May/2013, 29/Aug./2013, 28/Nov./2013, 27/Feb./2014
Toyama (TO)	333	28/May/2013, 29/Aug./2013, 28/Nov./2013, 27/Feb./2014
Iwanai (IW)	300	7/May/2013, 23/Aug./2013, 29/Nov./2013, 28/Feb./2014
Rausu (RA)	356	7/May/2013, 23/Aug./2013, 29/Nov./2013, 28/Feb./2014

SSW samples were firstly filtered through Nucleopore filter (Pore size 3.0 μm Track-Etch Membrane, Whatman®), and then filtrates were passed through by Nucleopore filter (Pore size 0.2 μm Track-Etch Membrane, Whatman®) using a vacuum pump (DA-30S, ULVAC KIKO). Each of the 0.2 μm filters was stored with an aseptic condition in a 2 mL tube at −20℃ until DNA extraction.

2.2 DNA extraction and PCR amplification

Bacterial DNA was extracted by cetyltrimethylammonium bromide (CTAB) and the beads-beating method. Six hundred μL of TE buffer and 20 μL of lysozyme (5 mg/mL, Wako) were added to the tube with glass beads (φ0.105–0.125 mm, AS ONE) and incubated at 37℃ for 1 h. Then, 3 μL of proteinase K (20 μg/mL, Invitrogen) and 30 μL of 10% sodium dodecyl sulfate (SDS) were added and incubated at 37℃ for 1 h. After incubation, 600 μL of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added. Bacterial cells were then crashed with glass beads using beads cell disruptor (MS-100R, TOMY) at 3,000 rpm for 30 s. After beads-beating, 100 μL of CTAB was added, mixed, and incubated at 65℃ for 10 min. The tubes were centrifuged at 5,000 × *g* for 30 min, and an aliquot of 650 μL of the supernatants was transferred to new tubes. Then, 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, 10 μL of 3*N* sodium acetate was added and then 600 μL of isopropyl alcohol was added. After removing the supernatant, DNA was dissolved in 20 μL of sterilized water and stored at −20℃. The bacterial V4 region of 16S rRNA genes was amplified using primer sets (563F 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 as follows: 2 min at 94℃; 35 cycles of 2 min at 94℃, 1 min at 53℃, 1 min at 72℃; 5 min of final extension at 72℃. The PCR products were verified by electrophoresis on 1% (w/v) agarose gels, followed by staining with ethidium bromide.

2.3 Pyrosequencing analysis

The PCR products were purified using the FastGene Gel/PCR Extraction Kit (Nippon Genetics), and the concentrations were measured using an UV spectrophotometer (MALCOM). The amplicon sequences of V4 region of 16S rRNA gene were obtained and processed using a Roche 454 GS (FLX titanium) pyrosequencing platform as described previously (Nakayama *et al.*, 2015). Equal amounts (100 ng) of the purified PCR products were pooled and subsequently sequenced on a 454 Genome Sequencer FLX system using a 2/4 picotiter plate (Roche Diagnostics) with V4 FLX forward primer (sequence tag + AYTGGGYDTAAAGNG)and reverse primer(sequence tag + CCGTCAATTCMTTTRAGT). The raw sequence data were assigned to individual samples by specific barcodes (Kuczynski *et al.*, 2012). The 16S rDNA primers and barcodes were then removed to generate pair-end reads. The raw tags were then filtered and analyzed using QIIME software package (Caporaso *et al.*, 2012). Reads from all samples were quality filtered and chimera reads were excluded. For species analysis, sequences with ≥97% similarity were assigned to the same OTUs (operational taxonomic units) using Uparse (Edgar, 2013), and similarity hits below 97% were not considered for classification purpose. A representative sequence of each OTU was picked out and the taxonomic information was annotated to submit to GenBank database (https://www.ncbi.nlm.nih.gov/genbank).

2.4 Diversity analysis

Alpha diversity and beta diversity (Lozupone *et al.*, 2007) were calculated with QIIME software package. In alpha diversity analysis, we rarified the OTU table and performed Mann-Whitney U-test. Cluster analysis of beta diversity was conducted by three-dimensional principal coordinates analysis (PCoA), which was applied to visualyze the dimension of the original variables using the QIIME software package. Also, relative abundance

analysis was performed for comparing the bacterial diversity among the all samples.

3. Result

3.1 Pyrosequencing analysis

Pyrosequencing analysis using 16S rRNA gene V4 short-tag sequences was carried out to examine the bacterial community structure of samples from the seven DSW pumping stations in Japan (Kumejima: KU, Koshikijima: KO, Muroto: MU, Izu-Akazawa: AK, Toyama: TO, Iwanai: IW, Rausu: RA). The numbers of OTUs, reads, phylum, and class of each sample are shown in Table 2, except for the two samples (KO DSW in Nov., TO SSW in Feb.). There were 4,848 kinds of OTUs in total samples. Following data were acquired using OTUs100 (OTUs has over 100 reads by mothur software, Lesniewski, 2009; Suh *et al.*, 2015) for statistical analysis.

Table 2. Summary of pyrosequencing data of DSW and SSW samples.

Samples			OTUs	Reads	Number of phylum	Number of class	Samples			OTUs	Reads	Number of phylum	Number of class
May	KU	S*	771	2,989	30	63	Nov	KU	S	336	2,574	21	41
		D	1,279	29,546	39	79			D	492	3,554	25	52
	KO	S	620	4,909	36	51		KO	S	243	1,894	20	32
		D	1,112	18,923	26	68			D	N**	N	N	N
	MU	S	396	983	29	43		MU	S	91	364	13	20
		D	738	6,186	26	64			D	254	2,026	19	35
	AK	S	791	14,397	28	69		AK	S	202	3,910	19	31
		D	1,371	39,137	30	84			D	458	7,652	24	42
	TO	S	281	695	21	44		TO	S	96	377	14	22
		D	720	9,869	23	61			D	157	673	16	43
	IW	S	495	2,127	22	49		IW	S	96	347	14	22
		D	1,017	8,285	30	63			D	528	8,028	24	49
	RA	S	748	37,028	23	53		RA	S	353	13,978	21	39
		D	1,224	33,887	24	73			D	526	13,716	25	46
Aug	KU	S	1,479	15,093	33	77	Feb	KU	S	143	1,843	10	14
		D	1,028	18,010	32	76			D	231	2,487	13	20
	KO	S	756	6,710	24	60		KO	S	145	1,900	13	19
		D	1,246	70,321	29	75			D	358	7,479	15	22
	MU	S	891	30,341	29	64		MU	S	346	3,201	17	30
		D	718	6,397	34	63			D	125	655	13	21
	AK	S	790	4,868	33	59		AK	S	117	543	10	16
		D	493	2,583	31	51			D	60	195	10	15
	TO	S	787	51,464	30	53		TO	S	N	N	N	N
		D	1,285	53,790	29	71			D	403	27,512	23	26
	IW	S	795	41,915	25	58		IW	S	269	24,201	21	33
		D	1,009	13,663	29	74			D	62	162	9	20
	RA	S	711	22,241	26	54		RA	S	139	1,683	13	23
		D	1,052	9,502	28	71			D	139	725	17	28

*(S = Surface seawater; D = Deep-sea water), **No data

3.2 Relative abundance analysis

The relative abundance analysis revealed that bacterial community structure of DSW and SSW samples were mainly composed of eight major phyla, unclassified phyla and unknown phyla (Fig. 2a, 2b). There were three abundant phyla, *Bacteroidetes*, *Proteobacteria* and SAR046 (Unclassified), both in the DSW and SSW samples (Fig. 2c). *Bacteroidetes* was the most abundant and its proportion in SSW (57%) was higher than that in DSW (31%). *Bacteroidetes* in DSW was subdivided into Class *Bacteroidia* (1%), *Cytophagia* (2%), *Flavobacteriia* (28%), *Rhodothermi* (1%)and *Saprospirae* (2%). The average proportion of class *Flavobacteriia* (49%)in SSW was significantly higher than that in DSW (Fig. 2c). *Proteobacteria* was the second abundant phylum and its average proportion in DSW (20%) was higher than that in SSW (17%). *Proteobacteria* was subdivided into five classes *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Gammaproteobacteria*. Whereas *Alphaproteobacteria* (12%) showed the highest proportion among *Proteobacteria* (17%) in SSW, *Deltaproteobacteria* (9%) was abundant among *Proteobacteria* (20%) in DSW. Unclassified group (Phylum SAR046) was the third abundant phylum and its average proportion in DSW (19%) was higher than that in SSW (7%). The average abundance of phyla *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes* and *Planctomycetes* in DSW was higher than that in SSW (Fig. 2c).

3.3 Comparison of bacterial community structure of DSW and SSW samples using principal coordinates analysis

Three-dimensional principal coordinates analysis (PCoA) was performed to visualize the relationship of the bacterial communities between DSW and SSW. For verifying the difference of bacterial community structures among DSW and SSW in summer and winter seasons, PCoA was performed separately (May/Aug. and Nov./Feb., Fig. 3). As a result of the unweighted PCoA in May/Aug., the bacterial communities in the northern parts of DSW (TO D5, IW D5, IW D8, RA D5 and RA D8) and all SSW formed mixed clusters, but those in the southern parts of DSW (KU D5, KU D8, KO D5, KO D8, MU D5, MU D8, AK D5, and AK D8) and TO D8 were grouped together (Fig. 3a). Based on the unweighted PCoA in Nov./Feb., the bacterial communities of DSW and SSW samples formed two seperated clusters (Fig. 3b). Also to specify phylum which differentiates bacterial community structures of DSW and SSW, weighted Mann–Whitney *U*-test was performed using OTU100 for *p*-value ($p \leq 0.003$). The result showed that phyla *Chloroflexi* and *Planctomycetes* were key phyla differentiating bacterial community structures of DSW from SSW.

4. Discussion

The bacterial community structures of DSW and SSW from the seven pumping stations in Japan were analyzed by pyrosequencing method based on 16S rRNA gene. The result of pyrosequencing analysis shows that there is a distinct difference between the bacterial community structures of DSW and SSW through the seven stations.

From the result of relative bacterial abundance analysis, phylum *Bacteroidetes* was dominant generally in both the DSW and SSW samples. In comparison with the average proportion of dominant phylum *Bacteroidetes* in DSW, SSW showed a higher proportion of *Bacteroidetes*. Phylum *Bacteroidetes* is a Gram-negative, non-spore-forming, rod-shaped bacterium widely distributed in the entire environment (Mor and Kwon, 2015). It is reported that phylum *Bacteroidetes* is dominant also in a freshwater stream (Lee *et al.*, 2016). According to our previous study, phylum *Bacteroidetes* was also the dominant phylum in DSW. Among *Bacteroidetes*, *Flavobacteriia* was the dominant class in SSW (49%) and DSW (28%). Class *Flavobacteriia* has tendency to bloom in their environmental community when some environmental factors were optimized such as temperature and nutrient especially in SSW (Eiler and Bertilsson, 2007). Likewise, SSW

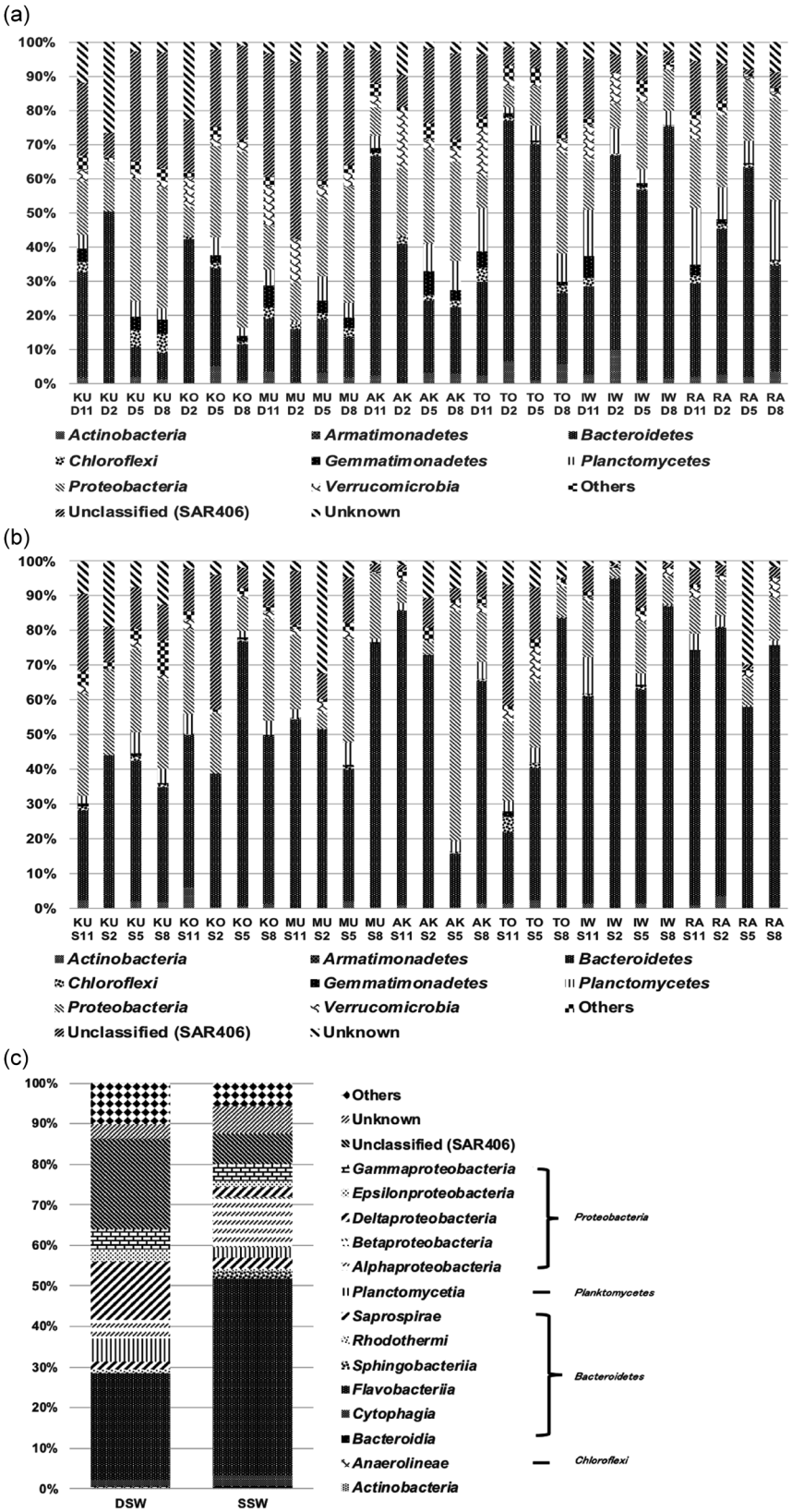

Fig. 2. Bacterial community composition in phylum level of DSW (a) and SSW (b) from seven pumping stations and the average proportion of class level of DSW and SSW (c) through all locations and seasons. Each pattern represents the percentage of the phylum or class in the total taxon tags of each sample. Symbols: DSW (D); SSW (S); Kumejima (KU); Koshikijima (KO); Muroto (MU); Izu-Akazawa (AK); Toyama (TO); Iwanai (IW); Rausu (RA). Numbers: November (11); February (2); May (5); August (8).

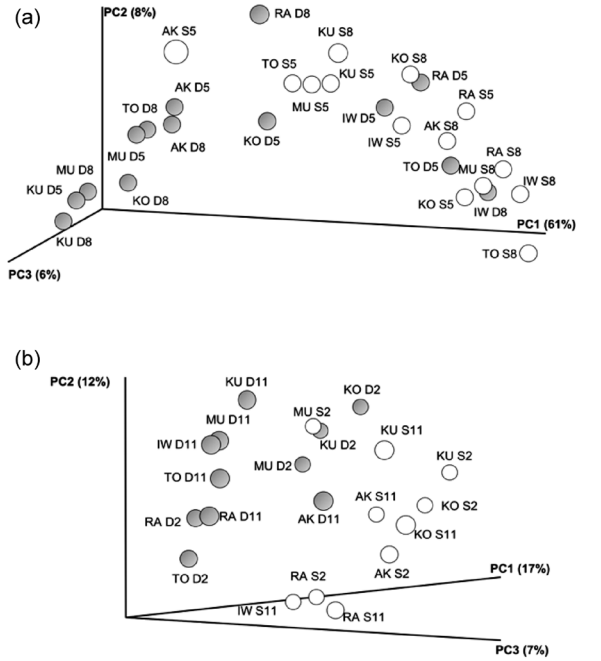

Fig. 3. Principal coordinates analysis (PCoA) of bacterial community structure in DSW and SSW from seven pumping stations. PCoA was performed separately in May/Aug. (a) and Nov./Feb. (b). The PC2 axis of Fig. 3b was rotated for visualization. Symbols: closed circle (DSW); open circle (SSW); Kumejima (KU); Koshikijima (KO); Muroto (MU); Izu-Akazawa (AK); Toyama (TO); Iwanai (IW); Rausu (RA). Numbers: May (5); Aug. (8); Nov. (11), Feb. (2).

sampled of IW in February (95%) and August (88%) showed blooming of *Flavobacteriia*. However, some DSW samples of IW also showed *Flavobacteriia* blooming in August (73%) and May (69%) and it is difficult to explain the blooming with only the environmental factors of DSW.

The present study showed that phylum *Proteobacteria* was predominant in both DSW and SSW through all the stations, and this result was also consistent with our previous study (Terahara *et al.*, 2016). In the case of surface seawater, phylum *Proteobacteria* is the dominant bacterium (Suh *et al.*, 2015; Walsh *et al.*, 2016). Dominant phyla in deep-sea sediment were *Proteobacteria* followed by *Chloroflexi* and *Planctomycetes* in order (Zhang *et al.*, 2015; Ceraquera *et al.*, 2015). These phyla also appeared in DSW, although their abundances were lower compared with those in deep-sea sediment. In SSW, the abundances of these phyla were smaller than those in DSW. From these reports concerning the relative abundance analysis in deep-sea sediment and SSW, it is considered that there is a difference of dominant phylum between DSW, SSW and deep-sea sediment.

Unclassified group (phylum SAR406), which was renamed *Marinimicrobia* (Rinke *et al.*, 2013) as an uncultured bacteria group, was the third dominant phylum in both DSW and SSW samples. The present study elucidated that the unclassified group in DSW was especially abundant in the southern parts (KU, KO, MU, and AK) of Japan in May and August, compared with that in the northern parts. However, this group remains to be cultured and reclassified as a new phylum in the future study.

From the result of the weighted Mann–Whitney *U*-test, it was elucidated that *Chloroflexi*, and *Planctomycetes* were the key microorganisms that distinguish the difference of bacterial community structures in both the DSW and SSW samples. DSW was higher than SSW in the abundance of *Chloroflexi* and *Planctomycetes*. During this study, *Anaerolineae* was the only class of phylum *Chloroflexi*, and *Phycisphaerae* and *Planktomycetia* were the classes of phylum *Planctomycetes* (Fig. 2(c)). The proportion of *Planctomycetia* was higher in DSW (6%) than that of SSW (2%). Previous studies showed that *Chloroflexi* was distributed widely in various hydrothermal sediment samples (Flores *et al.*, 2012; Fry *et al.*, 2008). However, up to now, only a few *Chloroflexi* from the deep-sea has been cultured (Imachi *et al.*, 2014; Fullerton and Moyer, 2016). Also, investigation of genetic properties, metabolic pathway and characteristics of *Chloroflexi* are still underway. In addition, phylum *Planctomycetes* represent an attractive group because they have unique features such as intracellular compartmentalization and the lack of peptidoglycan in their cell walls. *Planctomycetes* were initially classified as eukaryotes (Gimesi, 1924) and later re-classified as bacteria (Hirsch, 1972). However, the diversity of this phylum represents an unexploited enigma. *Planctomycetes* are also distributed in deep-sea

ecosystem including hydrothermal vents (Campbell *et al.*, 2013). This phylum includes members that conduct sulfur reduction (Elshaed *et al.*, 2007) and anammox (Chistoserdova *et al.*, 2004) coupled with the assimilation of inorganic carbon. As mentioned above, it seems that these two phyla which were more abundant in DSW than in SSW are originated from the bottom of the sea.

The PCoA result of the bacterial community structures in Nov./Feb. showed that there are two separated clusters formed in the DSW and SSW samples (Fig. 3b). However, in May/Aug., the bacterial community structures of southern DSW samples (KU D 5, KU D8, KO D5, KO D8, MU D5, MU D8, AK D5, and AK D8)including TO D8 formed a single separated cluster from others (Fig. 3a). This difference might have occurred due to the enhanced Tsushima Crurrent and Soya Warm Current in spring and summer (Fukuoka, 1957; Takizawa, 1982), because the DSW samples of RA, IW, TO in May/Aug. were located at similar positions with all the SSW samples on the coordinates. It seems that these DSW samples were influenced by suspended particles in the Tsushima Crurrent and Soya Warm Current from the south. On the other hand, the southern DSW in May/Aug. which is not affected by SSW is expected to have a unique bacterial community structure formed by environmental characteristics.

In the present study, we reported the comparative analysis of the bacterial community structure and the diversity in DSW and SSW at the seven stations of Japan using high-throughput sequencing techniques. Through the comparative method, we verified the difference of the bacterial community structures of DSW and SSW in the seven stations and seasonal change. Through the relative abundance analysis, SSW showed frequent bacterial blooming compared with DSW which had a tendency to show the consistent bacterial community composition. Phyla *Chloroflexi* and *Planctomycetes* are more abundant in DSW than those in SSW and the bacterial communities of DSW are different from those of epipelagic seawaters. Furthermore, the percentage of Unclassified group (phylum SAR406) was higher than that in SSW in all the samples. In conclusion, bacterial community structures of DSW differed from those of SSW and it is expected that there are many unexplored bacteria in DSW. Therefore, DSW would be an attractive resource for searching new useful microorganisms as exemplified in this study.

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