Korean Journal of Microbiology (2016) Vol. 52, No. 1, pp. 49-58
DOI http://dx.doi.org/10.7845/kjm.2016.6005

pISSN 0440-2413
eISSN 2383-9902

보 문

Succession of bacterial community structure during the early stage of biofilm development in the Antarctic marine environment

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남극 해양에서 생물막 생성 초기 단계의 세균 군집 구조 변화

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(Received February 11, 2016; Revised March 11, 2016; Accepted March 11, 2016)

ABSTRACT: Compared to planktonic bacterial populations, biofilms have distinct bacterial community structures and play important ecological roles in various aquatic environments. Despite their ecological importance in nature, bacterial community structure and its succession during biofilm development in the Antarctic marine environment have not been elucidated. In this study, the succession of bacterial community, particularly during the early stage of biofilm development, in the Antarctic marine environment was investigated by pyrosequencing of the 16S rRNA gene. Overall bacterial distribution in biofilms differed considerably from surrounding seawater. Relative abundance of *Gammaproteobacteria* and *Bacteroidetes* which accounted for 78.9–88.3% of bacterial community changed drastically during biofilm succession. *Gammaproteobacteria* became more abundant with proceeding succession (75.7% on day 4) and decreased to 46.1% on day 7. The relative abundance of *Bacteroidetes* showed opposite trend to *Gammaproteobacteria*, decreasing from the early days to the intermediate days and becoming more abundant in the later days. There were striking differences in the composition of major OTUs (≥ 1%) among samples during the early stages of biofilm formation. Gammaproteobacterial species increased until day 4, while members of *Bacteroidetes*, the most dominant group on day 1, decreased until day 4 and then increased again. Interestingly, *Pseudoalteromonas prydzensis* was predominant, accounting for up to 67.4% of the biofilm bacterial community and indicating its important roles in the biofilm development.

Key words: *Bacteroidetes*, *Pseudoalteromonas*, Antarctica, biofilm succession, bacterial community, pyrosequencing

A biofilm is an assemblage of microbial cells attached to a surface and encapsulated within a self-produced extracellular polymeric matrix (Donlan, 2002). Planktonic and motile bacteria transit to an aggregated biofilm on the surface by successional processes (Watnick and Kolter, 2000). Adsorption of dissolved organic molecules and primary colonization of free-living bacteria on the surface trigger the accumulation of bacteria through growth and reproduction, which modifies the characteristics of the surface and renders it suitable for subsequent colonization by secondary microorganisms (Dang and Lovell, 2000). The primary biofilm community is formed through specific and/or nonspecific interaction between initial colonizer and planktonic bacteria and different pioneer microorganisms contribute to biofilm formation in different environments (Dang and Lovell, 2000; Lee *et al*., 2008). Biofilm maturation proceeds by synergistic and/or competitive interactions among colonized species, as well as through recruitment of new species and/or loss of colonized species (Dang and Lovell, 2000).

Microbial biofilms are ubiquitous in aquatic environments

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(Sekar *et al*., 2002) and formed on a wide variety of surfaces, including medical or industrial devices, pipelines, water filtration systems, and body surfaces of marine organisms in natural aquatic systems (Gillan *et al*., 1998; Dang and Lovell, 2000; Armstrong *et al*., 2001). Biofilms are a protective mode of growth that allows microorganisms to survive in hostile or oligotrophic environments by increasing access to nutrients, allowing co-metabolic interactions with neighboring microorganisms, and protecting against toxins and antibiotics (Costerton *et al*., 1999; Dang and Lovell, 2000). In addition, biofilms play a key role in primary production, biodegradation of organic matter and environmental pollutants, and nutrient recycling in nature (Dang and Lovell, 2000). On the other hand, biofilms can be detrimental to the surfaces of man-made structures such as ships and bridges in aquatic environments (Gaylarde and Morton, 1999; Dang and Lovell, 2000; Flemming, 2002). Due to such profound importance of biofilms in ecology and industry, many studies have been performed on the bacterial communities, developmental processes, and physiology of biofilms in various aquatic environments, and methods to control biofilm formation have been developed (Gaylarde and Morton, 1999; Dang and Lovell, 2000; Armstrong *et al*., 2001; Molin and Tolker-Nielsen, 2003; Webster *et al*., 2006; Webster and Negri, 2006; Jones *et al*., 2007; Egan *et al*., 2008; Lee *et al*., 2008).

Antarctic marine environments that experience seasonal advance and retreat of pack ice are usually characterized by low temperature (usually -1.8–6°C) and extreme seasonal variations in irradiance and day length (Sakshaug and Slagstad, 1991; Delille, 1996). Biofilm formation may help microorganisms adapt to the harsh Antarctic marine environment by increasing resistance to such environmental stresses and contributing to nutrient cycling. However, few studies had been performed focusing on microbial community dynamics during the biofilm formation in the different types of surfaces in Antarctic marine environments (Maki *et al*., 1990; Webster *et al*., 2006; Webster and Negri, 2006). In addition, investigating the composition and diversity of early microbial colonizers during biofilm formation in natural marine environments of Antarctica is challenging due to the practical problem of conducting experiment in cold environments. Previous studies on microbial community changes during biofilm development had been performed in natural environments of temperate areas by using fingerprinting techniques (Moss *et al*., 2006; Jones *et al*., 2007; Lee *et al*., 2008; Pohlon *et al*., 2010; Salta *et al*., 2013), which suffer from a low taxonomic resolution. Thus, application of next-generation sequencing technology that allows the better characterization of the unseen realm is required for the analysis of unexplored bacterial community of biofilm formed in Antarctic marine environments.

In this respect, the objectives of this study were to investigate i) the differences in the bacterial communities between free-living bacteria and attached bacteria, and ii) the pioneering species and bacterial diversity during the early stage of biofilm establishment by using pyrosequencing technology.

Materials and Methods

Sample collection and processing

Acryl plates (200 mm × 300 mm × 3 mm) were cleaned with dish-cleaner, washed with sterilized water, and then submerged in seawater at a depth of approximately 30–60 cm vertically in the coastal area near King Sejong Station, King George Island, Antarctica (62°13'20.67"S, 58°47'11.32"W) from January 15–21, 2007. A subset of acryl plates (40 plates at day 1 and 20 plates each day from days 2–7) was retrieved at intervals of 24 h, rinsed with 0.2-μm-filtered seawater, and harvested by scraping with a razor blade. Two-liters of seawater were collected on January 13, 2007 and microorganisms present in this sample were concentrated using 0.2-μm Sterivex filters (Millipore) to compare bacterial community with biofilm samples. Harvested samples were transported to the laboratory in Korea at -20°C and preserved at -80°C until genomic DNA extraction. Environmental conditions of seawater during the experiment are presented in Table 1. Water temperature ranged from 0.9 to 2.2°C, pH ranged from 7.72 to 7.92, and the salinity was 31.6–33.6‰ during biofilm formation (Table 1).

Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from the seawater and biofilm samples using a modified CTAB method (Stewart and Via, 1993) as follows. A biofilm sample (0.5 ml) or filtered seawater membrane was placed in 0.7 ml of extraction buffer

Table 1. Description of sampling site and SSU rRNA tag characteristics

Sample		Environmental factors			Summary of SSU rRNA tags		Bacterial diversity indices		
		Water temperature (°C)	pH	Salinity (‰)	Bacterial reads	Plastid reads	No. of OTUs	Chao1	ACE
Seawater		1.6	7.75	33.3	2309	352	170	334	313
Biofilm	Day 1	2.2	7.79	31.6	1357	2584	380	703	710
	Day 2	1.5	7.79	33.3	2142	2929	284	681	721
	Day 3	1.1	7.74	33.2	1703	1803	203	440	451
	Day 4	1.3	7.72	33.1	2665	2071	172	461	462
	Day 5	0.9	7.74	32.6	2260	2586	316	648	722
	Day 7	2.2	7.82	33.1	2613	1406	321	714	778

* Bacterial diversity indices of Chao1 and ACE were calculated for 1,357 resampled bacterial sequences from each sample.

(2% [w/v] CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl; pH 8.0, 1% (w/v) Polyphenolpyrollidine-40) with 0.2 g of glass beads and incubated at 37°C for 15 min. Lysozyme (0.4 ml of a 5 mg/ml solution) was added and incubated at 37°C for 15 min, then the mixture was shaken at 140 rpm for 1 h. Following addition of 20 µl of proteinase K (20 mg/ml) and 200 µl of 10% (w/v) sodium dodecyl sulfate, mixtures were incubated at 65°C for 1 h. Samples were centrifuged at 14,000 × *g* for 10 min at 4°C, and then the upper phase was transferred to a fresh microcentrifuge tube. Following phenol:chloroform:isoamyl alcohol (25:24:1) extraction, DNA was recovered by precipitation with 0.6 volumes of isopropanol. The quality of genomic DNA was assessed using agarose gel electrophoresis.

To amplify bacterial 16S rDNA sequences, primers 27F (5′-TGCTGCCTCCCGTAGGAGT-3′) (Lane, 1991) and 338R (5′-AGAGTTTGATCCTGGCTCAG-3′) (Field *et al*., 1997) with barcodes and adapter were used. PCR was carried out in 100-µl reaction mixtures containing 10× PCR reaction buffer, 160 µM dNTPs, 0.5 µM of each primer, approximately 10 ng of gDNA, and 2.5 units of Taq DNA polymerase (In-Sung Science). The PCR procedure included an initial denaturation step at 95°C for 5 min, 25 cycles of amplification (95°C for 0.5 min, 57°C for 0.5 min, and 72°C for 0.5 min), and a final extension step at 72°C for 7 min. Each sample was amplified in triplicate and pooled. PCR products were purified using a LaboPass purification kit (Cosmogenetech).

Pyrosequencing and sequence analysis

Sequencing of 16S rDNA amplicons was performed by Macrogen using a 454 GS-FLX sequencer (Roche). Pre-processing was conducted using PyroTrimmer (Oh *et al*., 2012). Sequences were processed to remove primer, linker, and barcode sequences. The 3′ ends of sequences with low quality values were trimmed when average quality scores for a 5-bp window size were lower than 30. Sequences with ambiguous nucleotides or shorter than 180 bp were discarded. Chimeric reads were detected and discarded using the *de novo* chimera detection algorithm UCHIME (Edgar *et al*., 2011). Sequence clustering was performed by CLUSTOM (Hwang *et al*., 2013), with a 97% similarity cutoff. Taxonomic assignment was conducted for representative sequences of each cluster by EzTaxon-e database search (Kim *et al*., 2012). Sequences that were assigned to the plastid or unassigned to any domain were excluded based on the results of EzTaxon-e database search for bacterial community analyses. Low quality result for day 6 sample was excluded for the further analysis.

Statistical analysis

Diversity indices, including operational taxonomic unit (OTU) richness, Chao1, and ACE indices, were calculated from 100 sets of randomly subsampled 1,357 sequences, which is the lowest number of reads among samples. Rarefaction curves were analyzed using the MOTHUR program (v. 1.33.2) at a genetic distance of 3% (Schloss *et al*., 2009). Relatedness among samples was assessed by principal coordinates analysis (PCoA) using the weighted algorithm of Fast UniFrac (Hamady *et al*., 2009). Phylogenetic tree for fast UniFrac analysis was prepared by aligning sequences against pre-aligned SILVA

reference sequences in MOTHUR and phylogenetic tree reconstruction using a neighbor-joining algorithm with Jukes & Cantor model (Jukes and Cantor, 1969) in the MEGA 6 program (Tamura *et al*., 2013). OTU abundance data were prepared by logarithmic transformation of percent abundance + 1 and used for weighted Fast UniFrac analysis. Patterns of major OTU (> 1%) increase and decrease were assessed by Pearson correlation coefficients between time lapse and relative abundance using the R software package (www.r-project.org).

Nucleotide sequence accession numbers

Sequences obtained by pyrosequencing technology have been deposited in the Short Read Archive of the National Center for Biotechnology Information under accession numbers from SAMN03265448 to SAMN03265454.

Results

Bacterial community structure

Clustering of sequences from six biofilm samples and one seawater sample produced 1,217 bacterial OTUs, with 538 singleton OTUs. Fifty-four OTUs were shared among all biofilm samples. Among them, 29 OTUs were also shared with the seawater sample.

Among the 2,661 sequence reads from control seawater, 2,309 sequences (86.8%) were from bacteria and the remainder (13.2%) was from eukaryotic plastids (Table 1). Bacterial sequences were clustered into 170 OTUs and were assigned to 18 major bacterial taxonomic groups. *Alphaproteobacteria*, *Bacteroidetes*, and *Gammaproteobacteria* were the predominant taxonomic groups, accounting for 45.8%, 38.0%, and 12.0% of the bacterial community, respectively (Fig. 1).

The number of sequence reads from biofilm samples ranged between 3,506 and 5,071 (Table 1). Among them, bacterial sequences accounted for 34.4–65.0% of the total number of sequences obtained and they were assigned to 38 major taxonomic groups of bacteria. The most abundant taxonomic groups were *Gammaproteobacteria* (30.6–75.7%), *Bacteroidetes* (12.6–49.4%), *Alphaproteobacteria* (4.8–9.2%), *Cyanobacteria* (2.1–4.1%), *Firmicutes* (0.5–5.7%), GN02 (0.2–1.7%), *Deltaproteobacteria* (0.5–1.1%), *Actinobacteria* (0.3–1.3%), *Epsilonproteobacteria* (0.3–1.4%), and OD1 (0.1–0.7%) (Fig. 1). Relative abundance of *Gammaproteobacteria* increased from 30.6% on day 1 to 75.7% on day 4 and then decreased to 36.2% (Fig. 1). *Bacteroidetes* accounted for 49.4% of the day 1 biofilm sample, but decreased to 12.6% on day 4, and then increased to 46.1% on day 7. Abundance of *Alphaproteobacteria* ranged between 4.8% and 9.2% and showed no significant change during biofilm development. Abundance of *Cyanobacteria* ranged between 2.1% and 4.1%. *Actinobacteria* showed the highest relative abundance on day 1, accounting for 1.3% and then decreased to 0.3% on day 4. The relative abundance of *Firmicutes* was highest on day 3, accounting for 5.7% of the bacterial community. The other major taxonomic groups, which accounted for less than 1% of the total number of bacterial sequences, included *Lentisphaerae*, *Chloroflexi*, TM7, *Tenericutes*, *Planctomycetes*, TM6, *Chlorobi*, *Fibrobacteres*, *Armatimonadetes*, *Verrucomicrobia*, *Caldithrix*_p, *Gemmatimonadetes*, SAR406, *Spirochaetes*, GN04, SR1, DQ404828_p, DQ499300_p, WS3, 4P001694_p, OP3, *Deinococcus-Thermus*, and EF092200_p.

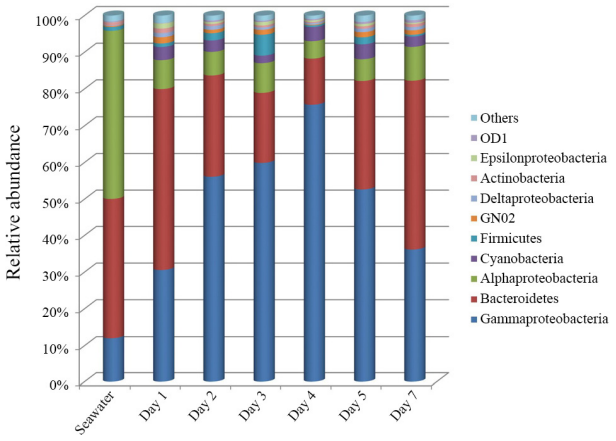

Fig. 1. Bacterial community composition at the phylum or proteobacterial class level. Others include *Betaproteobacteria*, *Fusobacteria*, *Acidobacteria*, *Lentisphaerae*, *Chloroflexi*, TM7, *Tenericutes*, *Planctomycetes*, TM6, *Proteobacteria*_uc, *Chlorobi*, *Fibrobacteres*, *Armatimonadetes*, *Verrucomicrobia*, *Caldithrix*_p, *Gemmatimonadetes*, SAR406, *Spirochaetes*, GN04, SR1, DQ404828_p, DQ499300_p, WS3, 4P001694_p, OP3, *Deinococcus-Thermus*, and EF092200_p.

PCoA results, based on weighted UniFrac distances between OTUs, showed a succession of bacterial populations during early stage of biofilm formation and the bacterial community of seawater was clearly distinguished from those of biofilms (Fig. 2).

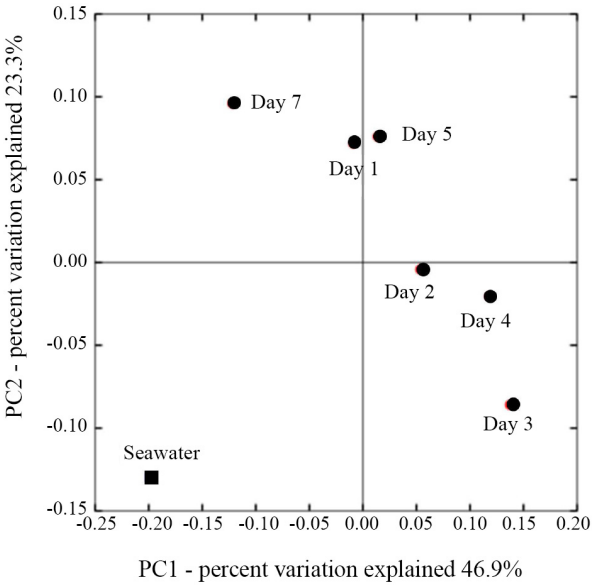

Fig. 2. Principal coordinates analysis (PCoA) of bacterial communities by weighted Fast UniFrac analysis. Filled square indicates seawater sample and filled circles indicate biofilm samples. The proportion of variance explained by ordination axes is represented on each axis.

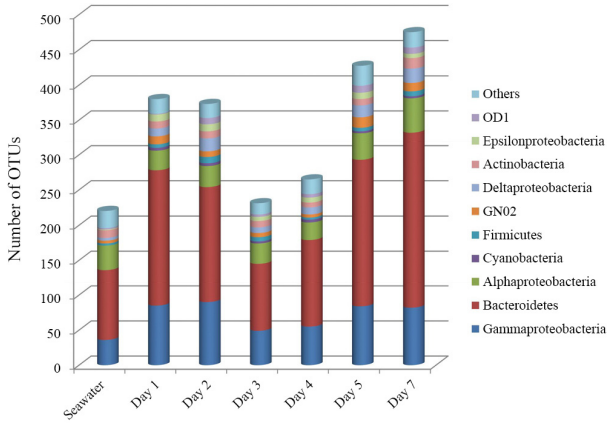

Fig. 3. Number of OTUs of each bacterial phylum or proteobacterial class. Others include *Betaproteobacteria*, *Fusobacteria*, *Acidobacteria*, *Lentisphaerae*, *Chloroflexi*, TM7, *Tenericutes*, *Planctomycetes*, TM6, *Proteobacteria*_uc, *Chlorobi*, *Fibrobacteres*, *Armatimonadetes*, *Verrucomicrobia*, *Caldithrix*_p, *Gemmatimonadetes*, SAR406, *Spirochaetes*, GN04, SR1, DQ404828_p, DQ499300_p, WS3, 4P001694_p, OP3, *Deinococcus-Thermus*, and EF092200_p.

OTU diversity change during biofilm development

Rarefaction curves and diversity indices (Chao1 and ACE) calculated from 1,357 resampled sequence reads for each sample at 97% similarity levels revealed that the biofilm samples showed a higher level of bacterial diversity than seawater (Table 1, Fig. 3, and Supplementary data Fig. S1). Bacterial OTU richness on day 1 was 703 and 710, as represented by Chao1 and ACE indices, respectively, which was almost twice of that of seawater. During biofilm formation, Chao1 and ACE values decreased until day 3 and then increased again.

The number of OTUs in the biofilm ranged from 172 to 380 (Table 1 and Fig. 3). In terms of OTU richness, the phylum *Bacteroidetes* was the largest group, followed by the classes *Gammaproteobacteria* and *Alphaproteobacteria* (Fig. 3). The number of OTUs affiliated with *Bacteroidetes* and *Gammaproteobacteria* decreased until day 4, and then increased again (Fig. 3). The number of OTUs affiliated with *Bacteroidetes* decreased from 193 on day 1 to 96 on day 3 and then increased to 250 on day 7 (Fig. 3). Similarly, the number of OTUs affiliated with *Gammaproteobacteria* decreased from 85 on day 1 to 49 on day 3. The decrease of *Bacteroidetes* OTU numbers paralleled the decrease in their relative abundance in the bacterial community. However, the relative abundance of *Gammaproteobacteria* was negatively correlated with OTU number, which may indicate the presence of major OTUs of *Gammaproteobacteria* that accounts for the high proportion in the biofilm bacterial community (Figs. 1 and 3).

Compositional change of major OTUs

Thirty-six major OTUs with 1% or higher relative abundance in at least one biofilm sample were selected to investigate the dynamics of bacterial community composition. Sequence similarities between major OTUs and their closely related type strains ranged from 83.8% to 100% (Table 2). Major OTUs were affiliated with *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Gammaproteobacteria*, and *Alphaproteobacteria*.

Correlation between abundance of major OTUs and sampling time was analyzed by Pearson correlation analysis. Negative values indicate that the OTUs are early colonizers and positive values indicate that they are late colonizer during biofilm development. The most evident pioneer species were those that are related to *Polaribacter filamentus* (96.5% sequence similarity), *Psychrobacter okhotskensis* (100%), and *Halomonas sulfidaeris* (100%) (Pearson correlation, $r = -0.93$, $r = -0.84$, $r = -0.82$, respectively and all $P < 0.05$) (Table 2). The other early colonizers mostly belonged to *Bacteroidetes* and *Proteobacteria*. The predominant late colonizers included OTUs related to *Tenacibaculum dicentrarchi* (96.5% sequence similarity),

Table 2. Heat map and taxonomic affiliation of major OTUs of biofilm. Major OTUs with 1% or higher compositions at least in one biofilm sample were selected. The color gradient from white to black indicates the lowest to highest relative abundance values

OTU ID	Day						Average†	Pearson correlation coefficients	Closest known species§			
	1	2	3	4	5	7			Phylum	Species name	Accession no.	Similarity (%)
OTU 41	2.10	2.09	1.17	0.82	0.90	0.38	1.25	-0.93*	*Bacteroidetes*	*Polaribacterfilamentus*	U73726	96.5
OTU 22	1.11	1.31	1.22	0.60	0.80	0.46	0.92	-0.84*	*Gammaproteobacteria*	*Psychrobacterokhotskensis*	AB094794	100.0
OTU 14	1.69	2.99	2.00	0.68	0.62	0.04	1.34	-0.82*	*Gammaproteobacteria*	*Halomonas sulfidaeris*	AF212204	100.0
OTU 51	1.33	0.61	0.41	0.11	0.31	0.11	0.48	-0.81	*Alphaproteobacteira*	*Pelagibacterubique*	CP000084	98.6
OTU 87	1.62	0.51	0.35	0.30	0.18	0.04	0.50	-0.80	*Cyanobacteria*	*Synechococcus rubescens*	AF317076	83.8
OTU 40	3.39	0.93	0.65	0.34	0.39	0.23	0.99	-0.74	*Bacteroidetes*	*Polaribacter irgensii*	AAOG01000005	96.0
OTU 26	1.92	3.03	2.58	1.01	1.33	0.96	1.81	-0.71	*Bacteroidetes*	*Ulvibacter litoralis*	AY243096	91.8
OTU 91	1.69	0.22	0.36	0.23	0.18	0.04	0.45	-0.70	*Gammaproteobacteria*	*Pseudomonas sabulinigri*	EU143352	100.0
OTU 48	1.33	3.50	2.47	0.64	0.66	0.19	1.46	-0.68	*Gammaproteobacteria*	*Pseudoalteromonastranslucida*	AY040230	100.0
OTU 58	0.52	1.31	1.29	0.30	0.27	0.04	0.62	-0.65	*Bacteroidetes*	*Mesonia algae*	AF536383	99.6
OTU 366	7.44	0.09	0.00	0.00	0.00	0.00	1.26	-0.61	*Bacteroidetes*	*Flavobacterium degerlachei*	AJ557886	99.6
OTU 402	1.11	0.00	0.00	0.00	0.00	0.00	0.18	-0.60	*Gammaproteobacteria*	*Pseudomonas abietaniphila*	AJ011504	100.0
OTU 279	1.55	0.00	0.00	0.00	0.09	0.00	0.27	-0.59	*Gammaproteobacteria*	*Shewanellaarctica*	GU564402	100.0
OTU 62	0.88	1.21	1.76	0.60	0.53	0.54	0.92	-0.55	*Alphaproteobacteira*	*Erythrobacter citreus*	AF118020	100.0
OTU 80	0.37	0.05	1.06	0.08	0.09	0.04	0.28	-0.35	*Gammaproteobacteria*	*Psychrosphaera saromensis*	AB545807	100.0
OTU 49	0.66	1.54	0.29	0.38	1.55	0.19	0.77	-0.26	*Firmicutes*	*Lactobacillus delbrueckii*	AY050172	99.6
OTU 7	1.77	1.49	0.47	0.41	2.04	1.00	1.20	-0.19	*Gammaproteobacteria*	*Colwellia demingiae*	U85845	100.0
OTU 373	0.00	0.00	4.99	0.00	0.00	0.00	0.83	-0.15	*Firmicutes*	*Bacillus muralis*	AJ628748	100.0
OTU 109	0.00	0.33	1.23	0.15	0.09	0.15	0.33	-0.14	*Gammaproteobacteria*	*Oceaniserpentilla haliotis*	AM747817	97.8
OTU 86	0.96	1.59	0.70	1.80	1.06	1.07	1.20	-0.03	*Gammaproteobacteria*	*Marinobacter vinifirmus*	DQ235263	99.6
OTU 13	1.95	2.05	1.25	0.45	1.07	2.42	1.53	0.02	*Bacteroidetes*	*Polaribacter irgensii*	AAOG01000005	97.8
OTU 1	5.53	33.74	44.16	67.43	38.89	26.71	36.08	0.29	*Gammaproteobacteria*	*Pseudoalteromonas prydzensis*	U85855	100.0
OTU 15	0.96	0.56	0.06	0.11	0.84	1.22	0.63	0.33	*Gammaproteobacteria*	*Cocleimonas flava*	AB495251	94.5
OTU 11	0.44	0.40	1.58	0.43	0.52	1.40	0.80	0.44	*Alphaproteobacteira*	*Octadecabacter jejudonensis*	KF515220	98.7
OTU 8	1.77	1.35	0.41	1.20	2.17	2.60	1.58	0.56	*Bacteroidetes*	*Lewinella persica*	ARDG01000103	84.5
OTU 4	1.55	1.46	1.53	1.85	1.31	2.30	1.67	0.63	*Alphaproteobacteira*	*Loktanella tamlensis*	DQ533556	97.4
OTU 29	0.81	2.00	1.59	3.08	3.41	2.31	2.20	0.65	*Cyanobacteria*	*Synechococcus elongatus*	CP000100	92.9
OTU 38	0.22	0.51	0.18	0.34	1.24	0.80	0.55	0.65	*Bacteroidetes*	*Aquimarina addita*	HM475137	96.1
OTU 57	0.07	0.05	0.00	0.04	0.04	1.10	0.22	0.74	*Bacteroidetes*	*Polaribacter irgensii*	AAOG01000005	97.5
OTU 45	0.00	0.09	0.47	0.11	0.13	2.10	0.49	0.77	*Bacteroidetes*	*Dokdonia genika*	AB198086	97.9
OTU 12	0.66	0.61	0.59	0.45	1.55	1.76	0.94	0.80	*Bacteroidetes*	*Lewinella cohaerens*	ARBR01000027	85.1
OTU 31	0.15	0.33	0.00	0.08	0.77	3.31	0.77	0.81	*Bacteroidetes*	*Polaribacter irgensii*	AAOG01000005	98.7
OTU 215	0.00	0.00	0.00	0.08	0.22	2.10	0.40	0.81	*Bacteroidetes*	*Bizionia paragorgiae*	AY651070	93.9
OTU 39	0.07	0.19	0.00	0.08	0.49	1.11	0.32	0.85*	*Bacteroidetes*	*Tenacibaculum dicentrarchi*	FN545354	96.5
OTU 35	0.15	0.33	0.23	0.15	1.24	3.14	0.87	0.86*	*Bacteroidetes*	*Algibacter mikhailovii*	AM491809	92.3
OTU 20	0.15	0.09	0.18	0.23	0.45	1.04	0.36	0.90*	*Bacteroidetes*	*Polaribacter butkevichii*	AY189722	95.7

§denotes closest known species retrieved by EzTaxon-e database search (Kim *et al.*, 2012). †denotes the mean relative abundance of each OTU in the biofilm for 7 days. * represents statistical significance with a *P*-value less than 0.05.

Algibacter mikhailovii (92.3%), and *Polaribacter butkevichii* (95.7%) (Pearson correlation, r = 0.85, r = 0.86, r = 0.90, respectively and all $P < 0.05$) (Table 2). The other late colonizers mostly belonged to *Bacteroidetes*. The most prominent result from this analysis was that one major OTU, identified as *Pseudoalteromonas prydzensis*, made up 67.4% of the bacterial community. It accounted for 5.5% on day 1, but colonized the biofilm very rapidly, making up 33.7% of the bacterial

community on day 2, and 44.2% on day 3. Maximum abundance of *Pseudoalteromonas prydzensis* (67.4%) was obtained on day 4, after which the abundance decreased.

Discussion

Bacterial communities of biofilm formed in natural environments such as river or seawater have been analyzed in previous studies using molecular techniques such as denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism, and fluorescence *in situ* hybridization (FISH) or cloning of small-subunit rRNA genes (Donlan, 2002; Araya *et al*., 2003; Lyautey *et al*., 2005; Moss *et al*., 2006; Webster and Negri, 2006; Jones *et al*., 2007; Lee *et al*., 2008). However, microbial diversity studies based on clone libraries or fingerprint analysis of 16S rRNA sequences could lead to underestimation of microbial diversity. Complex interactions between the bacterial populations in the biofilm could be greatly simplified because of a limited number of clones or short DNA fragments in fingerprint analysis (Watnick and Kolter, 2002; Moss *et al*., 2006; Widmer *et al*., 2006; Hong *et al*., 2010). In contrast, the 454 pyrosequencing approach applied in this study allowed the taxonomic assignment of each OTU down to the genus level, which enabled us to analyze the biofilm bacterial community with a finer taxonomic resolution. In addition, more than 1,357 bacterial sequence reads from each biofilm sample led to recovery of 1,155 bacterial OTUs and identification of 38 major taxonomic groups of bacteria (Table 1 and Fig. 1).

In this study, a seawater sample was included as a control to recognize enriched species in biofilm. *Alphaproteobacteria*, *Bacteroidetes*, and *Gammaproteobacteria* were predominant, composing 95.8% of the bacterial community in seawater, consistent with previous work showing that these three lineages are the dominant bacterial groups in the Southern Ocean (Abell and Bowman, 2005). The structures of the bacterial communities in the seawater and biofilms were quite different, as proposed in previous studies (Jones *et al*., 2007; Lee *et al*., 2008). In particular, *Gammaproteobacteria* accounted for 12.0% of the bacterial community in seawater, whereas this class composed 30.6–75.7% of the bacterial community during biofilm development. In contrast, *Alphaproteobacteria* accounted for 45.8% of the community in the seawater, but only a relatively low proportion of *Alphaproteobacteria* were found in the biofilm (4.8–9.2%) (Fig. 1). The species richness estimators (Chao1 and ACE) showed that biofilm had a greater level of diversity than seawater. The greater bacterial diversity in biofilm might have resulted from heterogeneous niches in the solid matrix formed by exopolysaccharides (EPSs), compared with continuously mixing seawater (Decho, 2000; Wang *et al*., 2013).

Biofilm development in the Antarctic seawater was led by bacterial species belonged to *Gammaproteobacteria* and *Bacteroidetes*. Members of *Bacteroidetes* were the dominant components of the biofilm in the early stage, then gammaproteobacterial species became dominant from days 2 to 5. *Bacteroidetes* increased in abundance after day 4 and became the dominant component of the biofilm at day 7. This bacterial community structure and pattern of succession was quite different from the results reported in previous studies, where *Alphaproteobacteria* were the dominant component of the early stage of biofilms in marine environments (Dang and Lovell, 2000; Araya *et al*., 2003; Moss *et al*., 2006; Webster and Negri, 2006; Jones *et al*., 2007; Lee *et al*., 2008). This discrepancy might be partly explained by the physicochemical properties of the surface used for biofilm formation, biofilm formation period, sampling interval, seasons, and anthropogenic impact. However, it may also be explained by the occurrence of the dominant OTU, the gammaproteobacterium *Pseudoalteromonas prydzensis*, which accounted for up to 67.4% of the bacterial community. The contribution of other gammaproteobacterial species was relatively small, accounting for 8.3–25.1% of the bacterial communities during biofilm development. This indicates that *Pseudoalteromonas prydzensis* which was firstly isolated from Antarctic sea ice plays significant roles in biofilm formation (Bowman, 1998). Previously, *Pseudoalteromonas* species were known to influence biofilm formation in various niches (Bowman, 2007). The production of toxins, bacteriolytic substances, and other enzymes by many *Pseudoalteromonas* species may assist in their competition for nutrients and space, as well as in protection against predators grazing at surfaces during biofilm development (Holmström and Kjelleberg, 1999). In addition, EPSs, commonly produced by *Pseudoalteromonas* species, can control bacterial attachment and serve as antibacterial

components that enhance the survival of other organisms that live in the vicinity of the producer strains (Holmström and Kjelleberg, 1999). Moreover, EPSs can act as protective barriers against external environmental stress, enhance nutrient uptake, and reduce the diffusion of some substances to and from cells, conferring an ecological advantage for formation of stable biofilms (Holmström and Kjelleberg, 1999). It is likely that these characteristics of *Pseudoalteromonas* species, represented by the production of EPSs, largely influenced the bacterial communities during the formation of biofilm.

In contrast to *Gammaproteobacteria*, there was no single dominant *Bacteroidetes* OTU. Instead, many *Bacteroidetes* species, especially members of the family *Flavobacteriaceae*, contributed to biofilm formation. OTUs related with *Polaribacter filamentus*, *Polaribacter irgensii*, *Mesonia algae*, and *Flavobacterium degerlachei* were representative early colonizers, while OTUs related with *Tenacibaculum dicentrarchi*, *Algibacter mikhailovii*, and *Polaribacter butkevichii* were late colonizers of the Antarctic marine biofilm. These results are well consistent with previous studies where many *Flavobacteriaceae* strains were isolated from the biofilm or marine organisms such as urchin and sponge. They produced EPSs, indicating involvement in biofilm formation (Nichols *et al*., 2005; Kwon *et al*., 2006; Lau *et al*., 2006; Lee *et al*., 2006; Nedashkovskaya *et al*., 2009; Vandecandelaere *et al*., 2010).

Taken together, the bacterial community composition in Antarctic marine biofilms changed drastically during the early stage of biofilm formation. Pioneer species were different from those of temperate marine environments and they did not remain high in abundance over time. These findings support the views that bacterial community composition of biofilm is niche-specific and influenced by interactions with the surrounding environment. *Pseudoalteromonas prydzensis* played a significant role in the maturation of biofilm and this may result from its known characteristics of EPS production and ability to compete for nutrients and space. As a pioneering study of the bacterial community structure in Antarctic marine environment, further study on the mechanism of microbial succession within a biofilm will provide insight into the ecological roles of them.

적 요

부유 세균의 군집과 구별되는 생물막내 세균 군집은 다양한 수생태계에서 중요한 생태학적 역할을 수행한다. 자연계에서 생물막이 생태학적으로 중요함에도 불구하고, 남극 해양 환경에서 생물막 형성 과정 동안의 세균 군집 구조와 그들의 변화에 대한 연구는 수행되지 않았다. 본 연구에서, 남극 해양 환경에서 생물막 형성 초기 단계에서의 세균 군집 구조 변화를 16S rRNA 유전자의 pyrosequencing을 통해 수행하였다. 생물막 내 전반적인 세균 군집은 주변의 해수의 군집과 매우 달랐다. 전체 세균 군집의 78.8%에서 88.3%를 차지한 *Gammaproteobacteria*와 *Bacteroidetes*의 상대적 풍부도는 생물막의 형성에 따라 급격하게 변하였다. *Gammaproteobacteria*는 생물막 형성 진행에 따라 증가하다가 (4일째에 75.7%), 7일째에 46.1%로 감소하였다. 반면, *Bacteroidetes*는 초기에서 중기로 갈수록 감소하다가 다시 증가하는 양상을 보이며, *Gammaproteobacteria*와 반대의 변화 양상을 나타내었다. 생물막 형성의 초기 과정에 우점 하는 OTU (>1%)들의 변화 양상은 시기에 따라 뚜렷한 차이를 보였다. *Gammaproteobacteria*에 속하는 종의 경우, 4일째까지 증가한 반면, 첫째날 가장 우점 하였던 문인 *Bacteroidetes*에 속하는 종은 4일째까지 감소한 후, 다시 증가하는 양상을 보였다. 흥미롭게, *Pseudoalteromonas prydzensis*가 67.4%를 차지하며 우점 하였는데, 이는 생물막 형성에 이 종이 중요한 역할을 수행함을 시사하는 것으로 보인다.

Acknowledgements

We thank Ji Young Jung (Korea Polar Research Institute) for statistical analysis using the R software package. This work was supported by Grants PE07050 and PE15020 from the Korea Polar Research Institute.

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