The Journal of Microbiology, August 2008, p. 357-363 Vol. 46, No. 4
DOI 10.1007/s12275-008-0071-9

Rapid Phylogenetic Dissection of Prokaryotic Community Structure in Tidal Flat Using Pyrosequencing

Bong-Soo Kim[1], Byung Kwon Kim[1], Jae-Hak Lee[2], Myungjin Kim[1], Young Woon Lim[3], and Jongsik Chun[1,2]*

[1]School of Biological Sciences and Institute of Microbiology and
[2]Interdisciplinary Program in Bioinformatics, Seoul National University, Seoul 151-742, Republic of Korea
[3]Division of Non-vascular Plants (Fungi/Alage), National Institute of Biological Resource, Incheon 404-708, Republic of Korea

(Received March 18, 2008 / Accepted April 30, 2008)

Dissection of prokaryotic community structure is prerequisite to understand their ecological roles. Various methods are available for such a purpose which amplification and sequencing of 16S rRNA genes gained its popularity. However, conventional methods based on Sanger sequencing technique require cloning process prior to sequencing, and are expensive and labor-intensive. We investigated prokaryotic community structure in tidal flat sediments, Korea, using pyrosequencing and a subsequent automated bioinformatic pipeline for the rapid and accurate taxonomic assignment of each amplicon. The combination of pyrosequencing and bioinformatic analysis showed that bacterial and archaeal communities were more diverse than previously reported in clone library studies. Pyrosequencing analysis revealed 21 bacterial divisions and 37 candidate divisions. Proteobacteria was the most abundant division in the bacterial community, of which Gamma- and Delta-Proteobacteria were the most abundant. Similarly, 4 archaeal divisions were found in tidal flat sediments. Euryarchaeota was the most abundant division in the archaeal sequences, which were further divided into 8 classes and 11 unclassified euryarchaeota groups. The system developed here provides a simple, in-depth and automated way of dissecting a prokaryotic community structure without extensive pretreatment such as cloning.

***Keywords*:** prokaryotic community, pyrosequencing, tidal flat, taxonomic assignment

Microbial diversity in natural and artificial environments can be accessed using molecular methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP) and pyrosequencing (Dar *et al.*, 2005; Petersen and Dahllof, 2005; Abdo *et al.*, 2006; Martinez *et al.*, 2006; Wilms *et al.*, 2006b; Huber *et al.*, 2007). Among these methods, pyrosequencing has been developed more recently and can generate hundreds of thousands of sequences in a single run (Ronaghi *et al.*, 1998; Gharizadeh *et al.*, 2002). Even though the read length of sequence is short (average 100 or 250 bp), this new method has been successfully applied to various microbiological applications: analysis of single nucleotide polymorphisms (SNPs) (Isola *et al.*, 2005), sequencing of deep mine metagenomes (Edwards *et al.*, 2006), bacterial and fungal typing (Jonasson *et al.*, 2002; Ronaghi and Elahi, 2002) and microbial community analysis of the deep sea environments (Sogin *et al.*, 2006; Huber *et al.*, 2007) and macaque gut (McKenna *et al.*, 2008).

In the present study, we applied pyrosequencing to investigate prokaryotic community structure in tidal flat sediments and compared these with the results from the previous clone library analysis. Tidal flats are characterized by high primary production rates and intense remineralization in sediments (Carling, 1982). These characteristics may enhance microbial abundance and diversity, thus high microbial heterogeneities have been reported from tidal flat sediments (Mussmann *et al.*, 2005; Wilms *et al.*, 2006b). Previously, we reported the presence of diverse prokaryotic species in tidal flat sediments in Korea using conventional clone libraries (Kim *et al.*, 2004; Kim *et al.*, 2005a). Here, we demonstrated that pyrosequencing and a subsequent bioinformatic analysis provide an accurate, cost-effective and reliable way of obtaining qualitative and quantitative information on prokaryotic community structure in natural environments.

* To whom correspondence should be addressed.
(Tel) 82-2-880-8153; (Fax) 82-2-874-8153
(E-mail) jchun@snu.ac.kr

Materials and Methods

Sample collections and PCR amplifications

We used DNA samples which were used in the previous studies (Kim *et al.*, 2004; Kim *et al.*, 2005a). The DNA was extracted from the tidal flat sediments of Dongmak, Ganghwa Island, Korea (depth 1 to 10 cm). Geochemical analyses were performed in previous studies and the concentration of SO_4^{2-} was determined by ion chromatography (DX-120, Dionex, USA).

PCR primers were designed to amplify the V3 region of 16S rRNA genes to produce amplicons suitable for pyrosequencing which allows the sequencing of approximately 100 bases from the 5' end of a DNA molecule. The designed

primer pairs were Bact363F; 5'-CAA TGG RSG VRA SYC TGA HS-3' and Bact531R; 5'-CTN YGT MTT ACC GCG GCT GC-3' for amplification of the bacterial 16S rRNA gene, and Arch339F; 5'-GGY GCA SCA GGC GCG VAW-3' and Arch523R; 5'-TMC CGC GGC KGC TGV CAS C-3' for the archaeal 16S rRNA gene. In order to reduce PCR-driven bias, we used three different *Taq* polymerases independently in each PCR reaction and reduced the number of PCR cycles (Head *et al.*, 1998; Kanagawa, 2003; Kurata *et al.*, 2004; Acinas *et al.*, 2005). PCR amplification procedures were performed as follows: 100 ng of template DNA, 1 nM of each primer, 10× buffers, dNTP, and each *Taq* polymerase to a final volume of 50 μl with sterile water. After an initial denaturation step of 5 min at 94°C, amplification reactions were performed with 25 cycles of denaturation (30 sec, 94°C), primer annealing (30 sec, temperatures were verified from 48°C to 57°C), and primer extension [30 sec, 72°C for Ex *Taq* (TaKaRa, Japan) or 67°C for Ampli *Taq* (Applied Biosystems, USA) and AccuPrime *Taq* (Invitrogen, USA)] and a final extension step of 5 min at 72°C or 67°C. The presence of PCR products was confirmed by 2% agarose gel analysis. The DNA amplicons were obtained from a total of 30 PCR reactions based on different polymerases and annealing temperatures, pooled, concentrated and purified with Qiaquick Gel Extraction Kit (QIAGEN, USA).

Pyrosequencing

To obtain bacterial and archaeal sequences in a single sequencing reaction, bacterial and archaeal PCR products were mixed. The quality of the product was assessed on a Bioanalyzer 2100 (Agilent, USA) using a DNA1000 LabChip. The sample used for pyrosequencing showed the proper size of the amplification products (approximately 20 ng) and were fed on a 454 Life Sciences sequencer (Branford, USA). The sequencing was carried out according to the manufacturer's instructions using a Genome Sequencer 20 system (Roche, Switzerland). A 454 Life Sciences PicoTiterPlate, where the pyrosequencing occurs, was masked into one zone to get approximately 10,000 sequencing reads.

Bioinformatics

To facilitate the taxonomic/phylogenetic assignment of each sequences, we developed a database, designated REF16S, containing 16S rRNA gene sequences of validly published bacterial and archaeal species (Chun *et al.*, 2007), and representatives of cyanobacteria, chloroplasts, mitochondria and environmental clones. The REF16S database contains 8,500 representative, non-redundant sequences with hierarchical taxonomic information. A bioinformatic pipeline was constructed for the automated taxonomic assignment of sequences using MySQL and JAVA under a Linux operating system. Firstly, the resultant sequences were divided into bacterial and archaeal subsets based on the presence of forward or reverse primer sequences used for amplification. Then, the primer sequences were trimmed from the sequences using the global pairwise alignment algorithm of Myers and Miller (1988). The processed sequences were subjected to a similarity-based search using the BLAST program (Altschul *et al.*, 1990) against the REP16S database. The five sequences showing the highest BLAST e-values were selected and used to align against the query sequence using the algorithm of Myers and Miller (1988). The sequence similarity values were calculated and the sequence with the highest similarity was selected for hierarchical taxonomic assignment using the information held in the REP16S database. JAVA-based versatile analysis tools for pyrosequencing data are under development and will be introduced elsewhere. The program DOTUR (Schloss and Handelsman, 2005) was employed to calculate diversity estimates, namely the abundance-based coverage estimator ACE (Chao and Lee, 1992) and species diversity estimator Chao1 (Chao, 1984) by using sampling without replacement.

Results and Discussion

Pyrosequencing and sequence analysis

PCR amplifications of 16S rRNA V3 regions were successfully achieved from tidal flat sediment DNAs. The sizes of the PCR products were 170~180 bp from primers Bact363F/Bact531R and 150~170 bp from primers Arch339F/Arch523R. A total of 10,166 sequences were obtained by pyrosequencing, where the average length was 103.52 bp. The quality score of sequences are provided by 454 Life Sciences, which is a measure of the confidence that the homopolymer length at that position is correct (Margulies *et al.*,

Table 1. The number of OTUs and richness estimates based on the DOTUR (Schloss and Handelsman, 2005)

Method	Primers	Sequences analyzed	Cluster distance					
			0.03[a]			0.05[b]		
			OTU	Chao1	ACE	OTU	Chao1	ACE
Clone library	Bacteria[c]	103	60	417	628	57	312	513
	Archaea[d]	102	32	55	65	27	42	43
Pyrosequencing	Bacteria (forward)	3,591	1,539	3,437	3,875	1,327	2,674	3,047
	Bacteria (reverse)	1,561	770	1,459	1,640	689	1,182	1,266
	Archaea (forward)	1,414	508	574	606	474	512	538
	Archaea (reverse)	741	222	278	279	212	260	259

[a] OTU was defined at 3% difference level.
[b] OTU was defined at 5% difference level.
[c] Data obtained from Kim *et al.* (2004)
[d] Data obtained from Kim *et al.* (2005a)

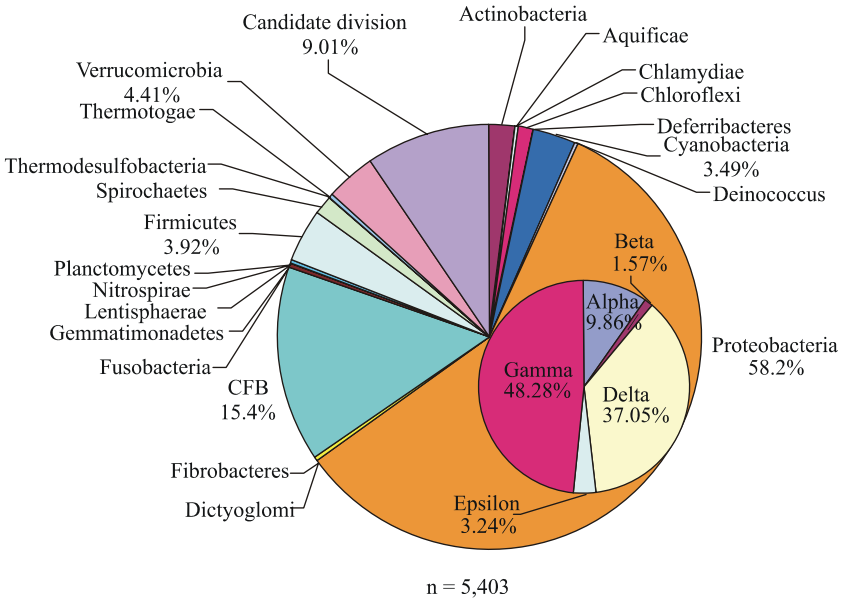

Fig. 1. Phylogenetic composition of bacterial pyrosequences in tidal flat of Dongmak. The percentage of each division was calculated and shown if it is higher than 3%. The composition and percentage of proteobacteria is shown in the sector of proteobacteria.

2005; Huse *et al.*, 2007). The average of the mean quality score was 26.01.

173 sequences (1.7% of total) containing one or more ambiguous bases (N) were excluded as these may contain high sequencing errors (Huse *et al.*, 2007). 1,282 sequences out of 9,993 did not contain amplification primer sequences of bacteria or archaea and were removed from further analysis. These sequences might be occurred when DNA capture beads attached to middle of templates in emulsion PCR step. Of the remainder, 6,065 sequences included bacterial primers and 2,646 sequences contained archaeal primers. Sequences which were shorter than 50 bp were omitted from further analysis (570 for bacteria and 11 for archaea). The remaining 8,130 sequences were analyzed by a BLAST search against REF 16S database (Chun *et al.*, 2007). Among the bacterial sequences, 251 were thought to be eukaryotic origin (mitochondria or plastids).

The taxonomic assignment of each sequence was done in

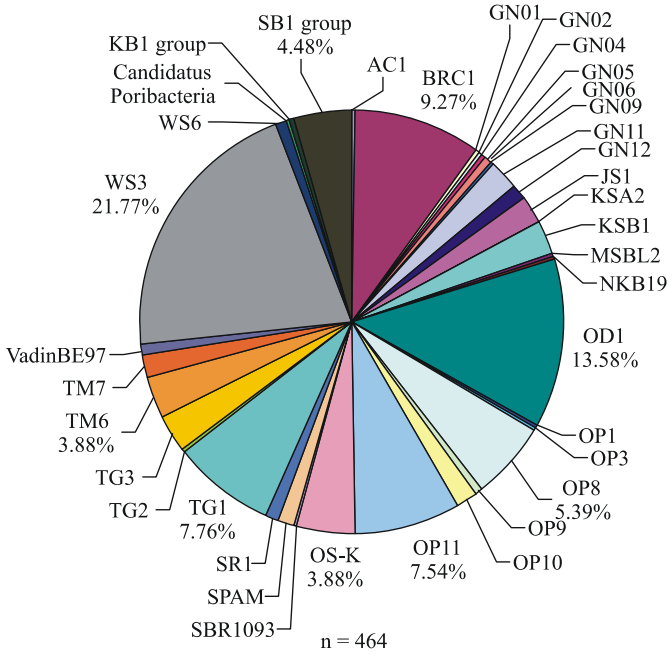

Fig. 2. Assignment of bacterial candidate divisions. The percentage of each candidate division was calculated and shown if it is higher than 3%.

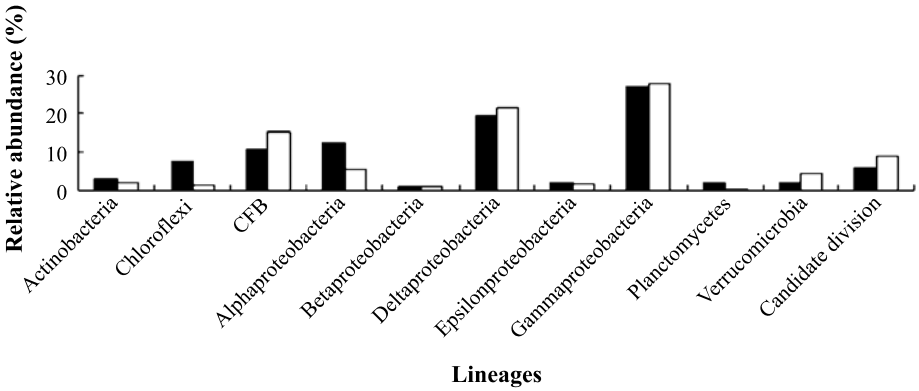

Fig. 3. Comparison of 16S rRNA gene sequences from pyrosequencing analysis and previous clone library-based analysis. The white bars represent taxonomic lineages of the clone library and the dark bars from pyrosequencing sequences. Other divisions which were detected only in pyrosequencing were removed in this figure.

an automated manner, and the results were the list of the taxonomic assignment of each amplicon and the number of amplicons in each major taxonomic rank. The statistical richness estimates of ACE and Chao1 determined by DOTUR were given in Table 1. The read length obtained from a 454 sequencer was shorter than that of amplified product and the direction of read could be both forward and reverse. Thus we divide forward and reverse sequence of prokaryotes by detect primer sequences. Our sampling of prokaryotic richness was far from complete, rarefaction curves were not shown.

Bacterial diversity in tidal flat sediments

Taxonomic/phylogenetic assignment of bacterial sequences indicated that the sample contained 21 divisions and 37 candidate divisions at the division level. Proteobacteria was the most abundant in tidal flat sediments (2,993 sequences, 58.09% of total bacteria), followed by Cytophaga-Flavobacterium-Bacteroides (CFB) group (795, 15.4%), *Verrucomicrobia* (227, 4.41%), *Firmicute* (202, 3.92%), and *Cyanobacteria* (180, 3.49%). The other 16 divisions were present only in minor components (Fig. 1). Among the Proteobacteria, Gammaproteobacteria (1,445 sequences, 48.28% of proteobacteria) was the most abundant, followed by Deltaproteobacteria (1,109, 37.05%).

464 sequences were assigned to 37 candidate divisions for which no cultural representatives are available (Fig. 2). Candidate division WS3 was the most abundant group among the candidate divisions (101 sequences, 21.77%), followed by candidate divisions OD1 (63, 13.58%), RC1 (43, 9.27%), TG1 (36, 7.76%), OP11 (35, 7.54%), SB1 group (20, 4.31%), and OS-K (18, 3.88%).

The bacterial community profile revealed by pyrosequencing was compared with those previously obtained by the clone library analysis at the division/class level (Fig. 3). The bacterial community structures from the conventional clone library and pyrosequencing analyses are similar at these high taxonomic ranks. The sequences of the previous clone library study fell into 7 divisions and 4 candidate divisions (Kim *et al.*, 2004). Among them, Gamma- and Deltaproteobacteria were the most abundant bacteria; the fact is now confirmed by in-depth investigation based on pyrosequencing. Similar results were reported from marine sediments in previous studies (Gray and Herwig, 1996; Urakawa *et al.*, 1999; Kim *et al.*, 2004).

Geochemical data of sampling sites were investigated in previous clone library study in which the concentration of SO_4^{2-} in our sediment sample was found to be 0.518 (mg/g dry soil). This concentration was about a hundredfold of that of nitrate, which suggests that sulfur may be the major electron acceptor in tidal flat sediments (Wilms *et al.*, 2006a; Wilms *et al.*, 2006b). The pH was 8.13 and the concentration of nitrate was 2.79 (μg/g dry soil). Even though Delta- and Gamma-proteobacteria are known to play important role in sulfur metabolism in marine sediments (Ravenschlag *et al.*, 1999; Kim *et al.*, 2004), community structure revealed in this study may not conclusively supports this finding, as physiology can not be assumed from phylogenetic assignment. Indeed, we found that the major component (43%) of Gamma-proteobacteria is a close neighbor to the uncultured clone MSB-2D6 (GenBank accession no. EF125435) which any physiological inference is not possible.

Despite of similarity in community structure at higher taxonomic ranks, substantial increases in the number of OTUs and statistical diversity estimates were evident in pyrosequencing over the previous clone library study (Table 1), which attributes to the significant increase in sampling size (15~35 fold) in pyrosequencing. Notable differences between two studies were found in the minor components of prokaryotic community. For instance, minor but significant number of *Firmicute* sequences were found (202, 3.92% of total bacteria) in pyrosequencing, while none was recovered in the clone library study. Various reports provide supporting evidence that *Firmicute* is present in tidal flat sediment (Yoon *et al.*, 2003; Kim *et al.*, 2005b; Kim *et al.*, 2006). Thus, it is fair to say that pyrosequencing gives a more comprehensive survey of prokaryotic structure than the conventional method. A total of 15 divisions, including *Firmicute*, were detected only in pyrosequencing data. *Acidobacteria*, Alphaproteobacteria, and *Chloroflexi* were more abundant in the clone library than in the pyrosequencing data. However, *Verrucomicrobia* and the CFB group were observed less fre-

quently in the clone library than in pyrosequencing. These discrepancies attribute to the difference in sampling size as well as the use of different primers and amplification conditions in two experiments.

A total of 37 candidate divisions were detected in pyrosequencing whereas only 4 candidate divisions (OP11, BRC1, KSB1, and WS3) were recovered in the clone library study. Candidate division WS3, the most abundant component, has been found in the methanogenic zone of a contaminated aquifer (Dojka *et al.*, 1998) and methane has been detected in anoxic-tidal flat sediments (Wilms *et al.*, 2006b). Methanogens are likely to be active in tidal flat, which explains the co-existence of WS3 division in tidal flat sediments. The WS3 division was also detected from various environments (Nesbo *et al.*, 2005; Tringe *et al.*, 2005; Ley *et al.*, 2006; Wilms *et al.*, 2006a). GN candidate divisions and several OP candidate divisions were also found in tidal flat. These bacteria were widespread in various habitats including hypersaline microbial mat, Yellowstone hot springs, Amazonian soil and bay sediment (Borneman and Triplett, 1997; Hugenholtz *et al.*, 1998; Papineau *et al.*, 2005; Ley *et al.*, 2006). Candidate divisions of OD1 (13.58% of the total candidate division sequence), TG1 (7.76%), SB1 group (4.31%), and OS-K (3.88%) were detected by pyrosequencing but not by the clone library method.

Archaeal diversity in tidal flat sediments

The all four known archaeal phyla have been detected in tidal flat sediments (Fig. 4). The Euryarchaeota was the most abundant taxon (1,628 sequences, 75.55% of total archaea), followed by Crenarchaeota (449, 20.84%), Korarchaeota (62, 2.88%), and Nanoarchaeota (16, 0.74%). A total of 8 classes and 11 unclassified euryarchaeotal groups were observed in sequences of Euryarchaeota. 515 sequences (23.9% of total archaeal sequences) belonged to candidate division 'sediment archaea-1' of Euryarchaeota, implying that the significant role of these organisms in tidal flat sediment. Pure culturing and the subsequent physiological and genomics studies are at premium need to understand ecology of tidal flat environment. Other taxa found were *Halobacteria* (245 sequences, 11.37%), *Methanomicrobia* (208, 9.65%), *Methanococci* (153, 7.1%), and *Thermoplasmata* (100, 4.64%). Among the Crenarchaeota, uncultured marine archaeal group 1 (237 sequences, 11% of total archaeal sequences) was the most abundant. The ancient archaeal group (AAG) and uncultured korarchaeota were two major components among Korarchaeota.

The presence of major amounts of Euryarchaeota and Crenarchaeota largely agrees with the previous clone library study (Kim *et al.*, 2005a), though pyrosequencing showed that Euryarchaeota was more abundant and diverse. Most of Euryarchaeota fall into methaongen and *Halobacteria* groups, implying active methanogenesis and heterotrophy in tidal flat. Methanogens consisted of *Methanococci*, *Methanobacteria*, *Methanopyri*, and *Methanomicrobia.* Both pyrosequencing and clone library studies indicated that *Methanomicrobia* is the most abundant among methanogens. The anaerobic methane oxidizing archaea (ANME) group, known to form symbiosis with sulfate reducing bacteria (SRB), were less numerous (3.53% of total archaea) than reported in the earlier studies (Kim *et al.*, 2004; Kim *et al.*, 2005a). It is likely that archaea other than the ANME are largely responsible for sulfur metabolism in tidal flat sediments.

The marine archaeal group 1, the most abundant Crenarchaeota, has been isolated from diverse marine environments (Eder *et al.*, 2002; Konneke *et al.*, 2005). The diversity

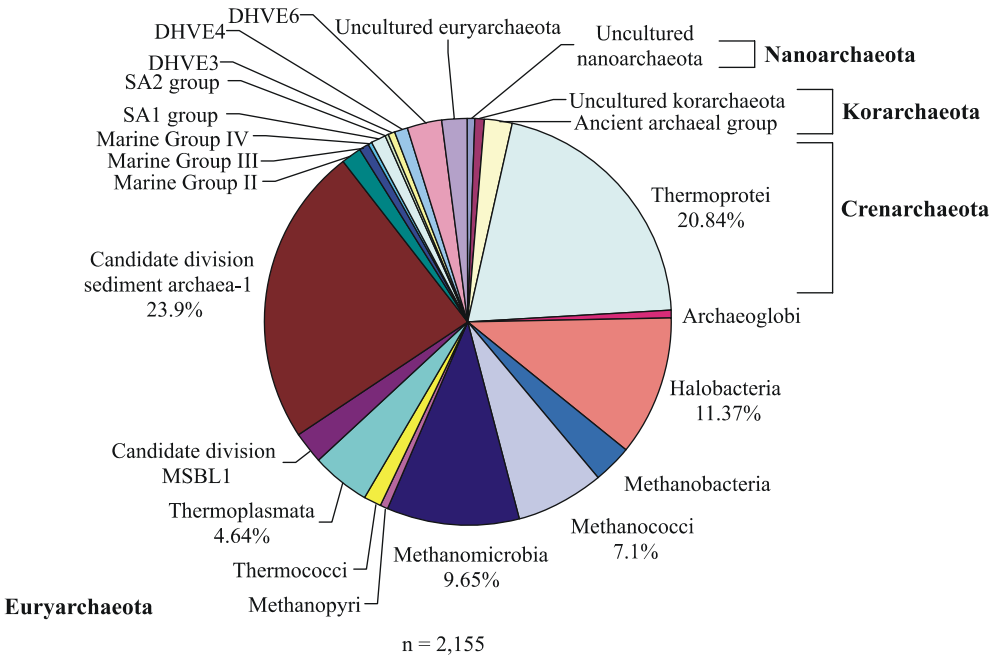

Fig. 4. Phylogenetic composition of archaeal sequences in tidal flats of Dongmak. The percentage of each archaeal group was calculated and shown if it is higher than 3%.

estimates of tidal flat (Table 1) are generally lower than those of deep sea (Huber *et al.*, 2007), which is largely due to the different sampling sizes in two studies.

Conclusions

In this study, we investigated bacterial and archaeal community structure in tidal flat sediments using pyrosequencing and a newly developed bioinformatics pipeline. A larger and in-depth amount of community information was obtained through this method. We also compared the resultant community structure with that obtained from the previous clone library-based studies (Kim *et al.*, 2004; Kim *et al.*, 2005a). The major groups of microbial community in the Dongmak tidal flat analyzed using pyrosequencing were overall similar to those based on the clone library studies. Even though pyrosequencing does not exhaustively detect all of prokaryotic cells in a sample, it can produce substantially larger number of sequences in a single sequencing run than conventional clone library method. As shown in this study, pyrosequencing is suitable to dissect community structure of very complex environment such as tidal flat sediments. Finally, the short lengths of sequences used in this study may not warrantee the identify prokaryotes at species level, but this limitation will be overcome through improved technologies.

Acknowledgements

This work was supported by Korea Ministry of Science and Technology under National Research Laboratory Program (R0A-2005-000-10110-0). Y.W. Lim was supported by Korea Research Foundation grant 2005-005-J16001.

References

Abdo, Z., U.M. Schuette, S.J. Bent, C.J. Williams, L.J. Forney, and P. Joyce. 2006. Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environ. Microbiol.* 8, 929-938.

Acinas, S.G., R. Sarma-Rupavtarm, V. Klepac-Ceraj, and M.F. Polz. 2005. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl. Environ. Microbiol.* 71, 8966-8969.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Borneman, J. and E.W. Triplett. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* 63, 2647-2653.

Carling, P.A. 1982. Temporal and spatial variation in intertidal sedimentation rates. *Sedimentology* 29, 17-23.

Chao, A. 1984. Nonparametric-estimation of the number of classes in a population. *Scand. J. Stat.* 11, 265-270.

Chao, A. and S.M. Lee. 1992. Estimating the number of classes via sample coverage. *J. Am. Stat. Assoc.* 87, 210-217.

Chun, J., J.H. Lee, Y. Jung, M. Kim, S. Kim, B.K. Kim, and Y.W. Lim. 2007. EzTaxon: A web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequence. *Int. J. Syst. Evol. Microbiol.* 57, 2259-2261.

Dar, S.A., J.G. Kuenen, and G. Muyzer. 2005. Nested PCR-denaturing gradient gel electrophoresis approach to determine the diversity of sulfate-reducing bacteria in complex microbial communities. *Appl. Environ. Microbiol.* 71, 2325-2330.

Dojka, M.A., P. Hugenholtz, S.K. Haack, and N.R. Pace. 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl. Environ. Microbiol.* 64, 3869-3877.

Eder, W., M. Schmidt, M. Koch, D. Garbe-Schonberg, and R. Huber. 2002. Prokaryotic phylogenetic diversity and corresponding geochemical data of the brine-seawater interface of the Shaban Deep, Red Sea. *Environ. Microbiol.* 4, 758-763.

Edwards, R.A., B. Rodriguez-Brito, L. Wegley, M. Haynes, M. Breitbart, D.M. Peterson, M.O. Saar, S. Alexander, E.C. Alexander, Jr., and F. Rohwer. 2006. Using pyrosequencing to shed light on deep mine microbial ecology. *BMC Genomics* 7, Art No. 57.

Gharizadeh, B., T. Nordstrom, A. Ahmadian, M. Ronaghi, and P. Nyren. 2002. Long-read pyrosequencing using pure 2'-deoxyadenosine-5'-O'-(1-thiotriphosphate) Sp-isomer. *Anal. Biochem.* 301, 82-90.

Gray, J.P. and R.P. Herwig. 1996. Phylogenetic analysis of the bacterial communities in marine sediments. *Appl. Environ. Microbiol.* 62, 4049-4059.

Head, I.M., J.R. Saunders, and R.W. Pickup. 1998. Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35, 1-21.

Huber, J.A., D.B. Welch, H.G. Morrison, S.M. Huse, P.R. Neal, D.A. Butterfield, and M.L. Sogin. 2007. Microbial population structures in the deep marine biosphere. *Science* 318, 97-100.

Hugenholtz, P., C. Pitulle, K.L. Hershberger, and N.R. Pace. 1998. Novel division level bacterial diversity in a Yellowstone hot spring. *J. Bacteriol.* 180, 366-376.

Huse, S.M., J.A. Huber, H.G. Morrison, M.L. Sogin, and D.M. Welch. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biol.* 8, R143.

Isola, D., M. Pardini, F. Varaine, S. Niemann, S. Rusch-Gerdes, L. Fattorini, G. Orefici, F. Meacci, C. Trappetti, M. Rinaldo Oggioni, and G. Orru. 2005. A pyrosequencing assay for rapid recognition of SNPs in *Mycobacterium tuberculosis* embB306 region. *J. Microbiol. Methods* 62, 113-120.

Jonasson, J., M. Olofsson, and H.J. Monstein. 2002. Classification, identification and subsyping of bacteria based on pyrosequencing and signature matching of 16S rDNA fragments. *PMIS* 110, 263-272.

Kanagawa, T. 2003. Bias and artifacts in multitemplate polymerase chain reactions (PCR). *J. Biosci. Bioeng.* 96, 317-323.

Kim, B.S., H.M. Oh, H. Kang, S.S. Pack, and J. Chun. 2004. Remarkable bacterial diversity in the tidal flat sediment as revealed by 16S rDNA analysis. *J. Microbiol. Biotechnol.* 14, 205-211.

Kim, B.S., H.M. Oh, H. Kang, and J. Chun. 2005a. Archaeal diversity in tidal flat sediment as revealed by 16S rDNA analysis. *J. Microbiol.* 43, 144-151.

Kim, I.G., M.H. Lee, S.Y. Jung, J.J. Song, T.K. Oh, and J.H. Yoon. 2005b. *Exiguobacterium aestuarii* sp. nov. and *Exiguobacterium marinum* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int. J. Syst. Evol. Microbiol.* 55, 885-889.

Kim, S., H. Jeong, S. Kim, and J. Chun. 2006. *Clostridium ganghwense* sp. nov., isolated from tidal flat sediment. *Int. J. Syst. Evol. Microbiol.* 56, 691-693.

Konneke, M., A.E. Bernhard, J.R. De La Torre, C.B. Walker, J.B. Waterbury, and D.A. Stahl. 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437, 543-546.

Kurata, S., T. Kanagawa, Y. Magariyama, K. Takatsu, K. Yamada, T. Yokomaku, and Y. Kamagata. 2004. Reevaluation and reduction of a PCR bias caused by reannealing of templates.

Appl. Environ. Microbiol. 70, 7545-7549.

Ley, R.E., J.K. Harris, J. Wilcox, J.R. Spear, S.R. Miller, B.M. Bebout, J.A. Maresca, D.A. Bryant, M.L. Sogin, and N.R. Pace. 2006. Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat. *Appl. Environ. Microbiol.* 72, 3685-3695.

Margulies, M., M. Egholm, W.E. Altman, S. Attiya, J.S. Bader, L.A. Bemben, J. Berka, M.S. Braverman, Y.J. Chen, Z. Chen, S.B. Dewell, L. Du, J.M. Fierro, X.V. Gomes, B.C. Godwin, W. He, S. Helgesen, C.H. Ho, G.P. Irzyk, S.C. Jando, M.L. Alenquer, T.P. Jarvie, K.B. Jirage, J.B. Kim, J.R. Knight, J.R. Lanza, J.H. Leamon, S.M. Lefkowitz, M. Lei, J. Li, K.L. Lohman, H. Lu, V.B. Makhijani, K.E. McDade, M.P. McKenna, E.W. Myers, E. Nickerson, J.R. Nobile, R. Plant, B.P. Puc, M.T. Ronan, G.T. Roth, G.J. Sarkis, J.F. Simons, J.W. Simpson, M. Srinivasan, K.R. Tartaro, A. Tomasz, K.A. Vogt, G.A. Volkmer, S.H. Wang, Y. Wang, M.P. Weiner, P. Yu, R.F. Begley, and J.M. Rothberg. 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437, 376-380.

Martinez, R.J., H.J. Mills, S. Story, and P.A. Sobecky. 2006. Prokaryotic diversity and metabolically active microbial populations in sediments from an active mud volcano in the Gulf of Mexico. *Environ. Microbiol.* 8, 1783-1796.

McKenna, P., C. Hoffmann, N. Minkah, P.A. Pyone, A. Lackner, Z. Liu, C.A. Lozupone, M. Hamady, R. Knight, and F.D. Bushman. 2008. The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis. *PLoS Pathog.* 4, e20.

Mussmann, M., K. Ishii, R. Rabus, and R. Amann. 2005. Diversity and vertical distribution of cultured and uncultured Deltaproteobacteria in an intertidal mud flat of the Wadden Sea. *Environ Microbiol.* 7, 405-418.

Myers, E.W. and W. Miller. 1988. Optimal alignments in linear space. *Comput. Appl. Biosci.* 4, 11-17.

Nesbo, C.L., Y. Boucher, M. Dlutek, and W.F. Doolittle. 2005. Lateral gene transfer and phylogenetic assignment of environmental fosmid clones. *Environ. Microbiol.* 7, 2011-2026.

Papineau, D., J.J. Walker, S.J. Mojzsis, and N.R. Pace. 2005. Composition and structure of microbial communities from stromatolites of Hamelin Pool in Shark Bay, Western Australia. *Appl. Environ. Microbiol.* 71, 4822-4832.

Petersen, D.G. and I. Dahllof. 2005. Improvements for comparative analysis of changes in diversity of microbial communities using internal standards in PCR-DGGE. *FEMS Microbiol. Ecol.* 53, 339-348.

Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. *Appl. Environ. Microbiol.* 65, 3982-3989.

Ronaghi, M., M. Uhlen, and P. Nyren. 1998. A sequencing method based on real-time pyrophosphate. *Science* 281, 363-365.

Ronaghi, M. and E. Elahi. 2002. Pyrosequencing for microbial typing. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 782, 67-72.

Schloss, P.D. and J. Handelsman. 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* 71, 1501-1506.

Sogin, M.L., H.G. Morrison, J.A. Huber, D.M. Welch, S.M. Huse, P.R. Neal, J.M. Arrieta, and G.J. Herndl. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc. Natl. Acad. Sci. USA* 103, 12115-12120.

Tringe, S.G., C. Von Mering, A. Kobayashi, A.A. Salamov, K. Chen, H.W. Chang, M. Podar, J.M. Short, E.J. Mathur, J.C. Detter, P. Bork, P. Hugenholtz, and E.M. Rubin. 2005. Comparative metagenomics of microbial communities. *Science* 308, 554-557.

Urakawa, H., K. Kita-Tsukamoto, and K. Ohwada. 1999. Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology* 145, 3305-3315.

Wilms, R., B. Kopke, H. Sass, T.S. Chang, H. Cypionka, and B. Engelen. 2006a. Deep biosphere-related bacteria within the subsurface of tidal flat sediments. *Environ. Microbiol.* 8, 709-719.

Wilms, R., H. Sass, B. Kopke, J. Koster, H. Cypionka, and B. Engelen. 2006b. Specific bacterial, archaeal, and eukaryotic communities in tidal-flat sediments along a vertical profile of several meters. *Appl. Environ. Microbiol.* 72, 2756-2764.

Yoon, J.H., I.G. Kim, K.H. Kang, T.K. Oh, and Y.H. Park. 2003. *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the Yellow Sea in Korea. *Int. J. Syst. Evol. Microbiol.* 53, 1297-1303.