

## Bacterial community analysis of sediment seep in Kagoshima Bay, Japan

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Microorganisms in the deep-sea environments such as hydrothermal vent and cold-seep regions are primary energy producers and an important community in these ecosystems. We have used 454-Pyrosequencing and 16S rDNA clone library methods to determine the diversity of bacteria in the sediment of the seep regions around the vestimentiferan tubeworm habitat at Kagoshima Bay. Taxonomic composition from both libraries suggested that 454-Pyrosequencing methods can represent more diverse groups than the conventional clone library methods. Most abundant taxa with higher folds were Proteobacteria and Bacteroidetes found in both methods. Through the 454-Pyrosequencing method, we were able to detect underrepresented taxa as well as non-detectable taxa. This analyses and comparison provide bacterial taxonomic group detection efficiency of both library types and emphasize the different uses and utilities for exploring the unknown microbial domain.

[Keywords: Kagoshima; 454-Pyrosequencing; Sedimented seep]

### Introduction

Hydrothermal vent and cold-seep macrofaunal groups such as vesicomid clams, siboglinid tubeworms, limpets, shrimps and mussels are supported by the nutrients supplied from the chemosynthetic bacteria. Bacteria, like aerobic sulfur oxidizers, sulfur reducers and methanogens, as well as anaerobic denitrifiers including mixotrophs, utilize abundant dissolved chemicals like methane, H<sub>2</sub>, H<sub>2</sub>S, NH<sub>4</sub><sup>+</sup>, Fe<sub>2</sub><sup>+</sup> and Mn<sub>2</sub><sup>+</sup> in these regions<sup>1-3</sup>. In this study, we are focusing on the bacterial diversity surrounding the siboglinid tubeworm habitat. These tubeworms usually inhabit at the sulfide-rich deep-sea hydrothermal vent and/or cold-seep environments are dependent on the nutrition provided by the chemoautotrophic endosymbionts<sup>2,3</sup>. Previous studies have shown that the tubeworms obtain their symbiotic partner by horizontal transmission process from the environment<sup>4</sup>. This study was conducted to know the sediment bacterial community structure in the regions around the vestimentiferan tubeworm habitat at Kagoshima Bay by using 454-Pyrosequencing and 16S rDNA clone library methods.

### Materials and Methods

#### *Study area and sample collection*

In this study, three sediment samples were collected from the surrounding of the tubeworm

habitat at Kagoshima Bay (KB)<sup>5</sup>. All sediment samples were collected during dives of the remotely-operated vehicle 'Hyper Dolphin' by using a core sampler. Sample KB-44 was collected from a tubeworm colony site (31°39.695'N, 130°48.062'E) of depth ~110 m and sample KB-46 from the nearby region of the tubeworm site (31° 39.700'N, 130° 48.058'E). Sediment surface at the KB-46 region was layered by thick bacterial biofilm, surrounded with numerous gas bubbles released from the seafloor. Sample KB-47 was collected from the caldera region (31° 39.749'N, 130° 46.290'E) at a depth of 203 m, where the temperature of the surrounding vent fluid was ~133 °C.

#### *Isolation and purification of genomic DNA from sediment*

Total DNA was isolated from ~ 0.5 g of each sediment samples using a Power Soil® DNA extraction kit (Mo Bio Laboratories, Inc., USA) followed by the instructions provided. Isolated DNA was further purified by a PowerClean® DNA cleanup kit and kept at -20 °C in Tris-EDTA buffer.

#### *Amplification and clone library construction of 16S rDNA gene*

Bacterial 16S rDNA gene amplification procedure was described in the previous study<sup>5</sup>. Successful PCR

products were purified by the AccuPrep® PCR Purification system (Bioneer, Korea) and then cloned with the pGEM-T easy vector (Promega, USA) and transformed into *Escherichia coli* DH5α competent cells (TaKaRa, Korea). Successfully transformed colonies were selected and plasmids with inserts were amplified by using the universal primers T7 and SP6 and confirmed by gel electrophoresis.

*454-Pyrosequencing and Sanger sequencing of 16S rDNA*

Sequence libraries were prepared from the PCR products following the GS-FLX plus library prep guide and quantified using the Picogreen assay (Victor 3). The emPCR of the purified clonal library was carried out by GS-FLX plus emPCR Kit (454 Life Sciences). To amplify bacterial 16S rDNA genes two universal primers 27F (5'-GAGTTTGATCMTGGCTCAG-3') and 518R (5'-WTTACCGCGGCTGCTGG-3') were used. Sequencing was conducted in a GS-FLX plus sequencer following the procedure described by the manufacturer.

The DNA sequences from clone libraries were sequenced on an (ABI Gene Scan 3100, USA) automated sequencer by using the primer 518R. To identify bacteria and phylogenetic analysis, 16S rDNA gene sequences of ~500 bp length were acquired. Chimeric sequences from 454-Pyrosequencing and 16S rDNA clone library sequences were identified by DECIPHER (<http://decipher.cce.wisc.edu/index.html>) and removed from the analysis.

*Diversity analysis*

Biodiversity indices for sequence libraries were generated by the Ribosomal Database Project web-server (<http://rdp.cme.msu.edu>). Alpha diversity was calculated from sequence libraries through Rarefaction curves<sup>6</sup>, Shannon biodiversity index<sup>7</sup> and Chao1 species richness index<sup>8</sup> analysis. Beta diversity was calculated to estimate the resemblance between bacterial communities based on the relative OTU's abundance.

**Results**

Bacterial 16S rDNA sequences were successfully sequenced by 454-Pyrosequencing and Sanger sequencing from the clone library of the sediment samples of the seep regions around the tubeworm habitat. A total of 14,362 454-pyrosequences contained 5225 OTUs (0.03 cut-off distance threshold) and 575 clone library sequences contained 336 OTUs from all sites. Rarefaction curves and Good's coverage indicated that bacterial communities were covered by 454-sequence libraries in the range of 56 to 71 %, whereas insufficiently covered by clone libraries between 26 and 54 % (Table 1 and Fig. 1). Statistical analyses using alpha diversity calculated through the Chao 1 richness and Shannon's diversity index suggested that the KB-44 sample in the 454-Pyrosequencing method had higher bacterial diversity, however, KB-46 sample in the clone library method had higher bacterial diversity (Table 1). Both samples were collected from the area closer to the tubeworm habitat.

To compare the bacterial 16S rDNA sequencing analysis between methodologies, results of the 454-pyrosequence library method are depicted with the Sanger sequencing from the clone library as reported

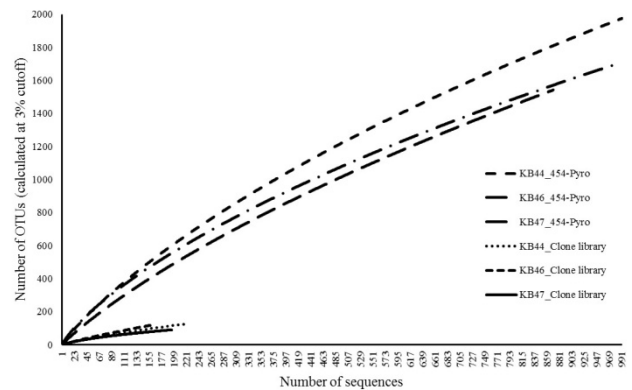


Fig. 1 — Rarefaction curves of the valid sequences of the bacterial 16S rDNA libraries from the sediments.

Table 1 — Alpha diversity and Good's coverage for valid bacterial 16S rDNA sequences generated by 454-Pyrosequencing and clone library sequencing methods. Diversity indexes were calculated with distance cut-off value 0.03 from each library.

Methods	Sample ID	Number of seq. (N)	No. of OTUs	Coverage (%)	Chao I	Shannon (H')
454 Sequencing	KB-44	4960	1977	60.1	4540	6.699
	KB-46	3480	1543	55.7	4452	6.194
	KB-47	5922	1705	71.2	3916	6.261
16S rDNA clone library sequencing	KB-44	217	125	42.4	435	4.408
	KB-46	163	121	25.8	550	4.574
	KB-47	195	90	53.8	261	4.105

previously<sup>5</sup>. The two different sequencing methods yielded a different quantitative depiction of the taxonomic composition in the sediment samples. *Proteobacteria* comprised higher than 42 % in the 454-sequencing method and higher than 51 % in the clone library sequencing method (Fig. 2). Within *Proteobacteria*, the classes *Epsilonproteobacteria* and *Deltaproteobacteria* were most abundantly observed in both methods. Class *Epsilonproteobacteria* in the region close to tubeworm habitat KB-44 was most dominant in both sequencing methods.

Prevalent taxonomic groups, which were consistently underrepresented in a higher fold in the clone library sequencing, were *Acidobacteria*, *Actinobacteria*, *Aquificae*, *Bacteroidetes*, *Chloroflexi*, *Deferribacteres*, *Firmicutes* and *Planctomycetes*. As the next abundant phylum, *Bacteroidetes* comprised between 10 to 13 % of total sequences in the 454-sequencing libraries, whereas only between 1.4 to 1.8 % representation in the clone library sequencing (Fig. 2).

Unique representation of taxonomic groups observed in the 454-Pyrosequencing and rarely representation in the clone library sequencing methods, were *Caldisericia* (except in KB-47), candidate division ZB3, *Chlamydia* (except in KB-46), *Chrysiogenetes*, *Cloacimonetes*, *Fusobacteria*, *Gemmatimonadetes*, *Ignavibacteria* (except in KB-47) and *Latescibacteria*, *Lentisphaerae*, *Marinimicrobia*, *Parcubacteria*, *Spirochaetes*, *Thermotoga* (except in

KB-47) and *Verrucomicrobia* (except in KB-46). However, in the clone library sequencing method, unique taxonomic group was not observed (Fig. 2).

**Discussion**

The sediment bacterial community varied with distance from the tubeworm habitat. Rarefaction curves suggest that KB-47 has lower bacterial diversity than KB-44 and KB-46. Sample KB-47 was collected from the caldera region, father from the tubeworm habitat and farther from the shore.

The class *Epsilonproteobacteria* was most abundant in the sample close to the tubeworm habitat. According to the previous study in this region<sup>5</sup>, the clone library sequencing data suggest that *Epsilonproteobacteria* were related to the most prevalent deep-sea sulfur-oxidizing free-living bacteria genus *Sulfurovum* and *Sulfurimonas*<sup>9</sup> as well as symbiotic bacteria associated with hydrothermal vent and seep invertebrates such as *Lamellibrachia satsuma*, *Alvinella pompejana*<sup>10</sup>, *Shinkaia crosnieri*<sup>11</sup> and *Rimicaris exoculata*<sup>12</sup>. At site KB-46, *Deltaproteobacteria* (574 seq) showed a slightly higher abundance than *Epsilonproteobacteria* (466 seq) by the 454-sequencing method. *Deltaproteobacteria* were usually related to sulfate-reducing bacteria, associated with invertebrates such as *Olavius algarvensis* and deep-sea clams<sup>13,14</sup>, and also found as free-living bacteria of genera *Desulfosarcina*<sup>15</sup> and *Desulfobacterium*<sup>16</sup>, and related to bacteria observed in the hydrothermal vent region of Okinawa

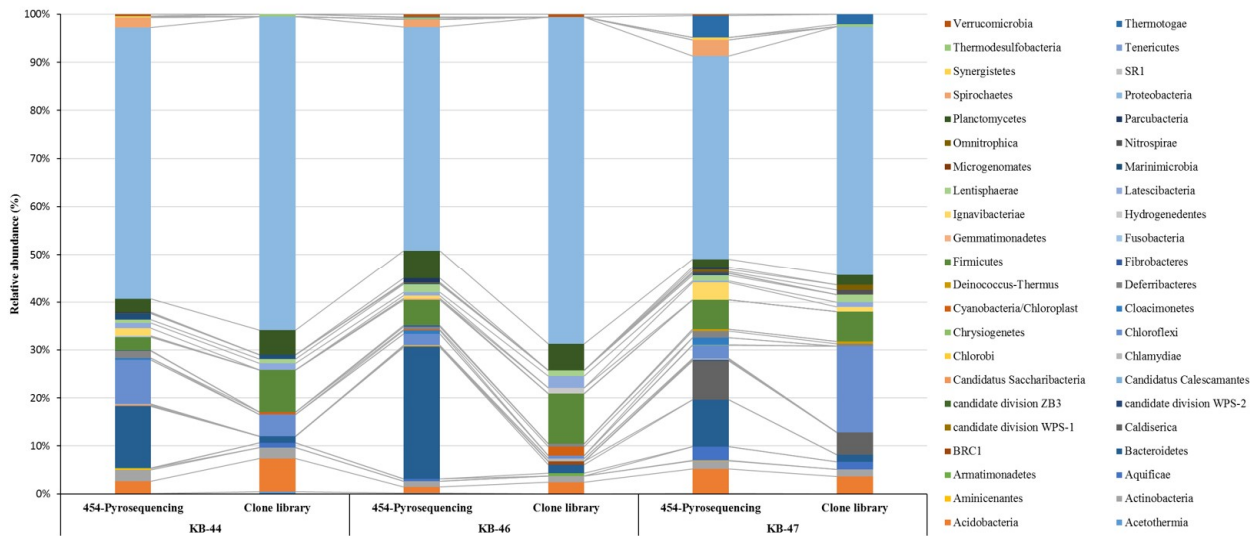


Fig. 2 — The relative abundance of bacterial 16S rDNA sequences in phylum-level generated by 454-Pyrosequencing and clone library sequencing.

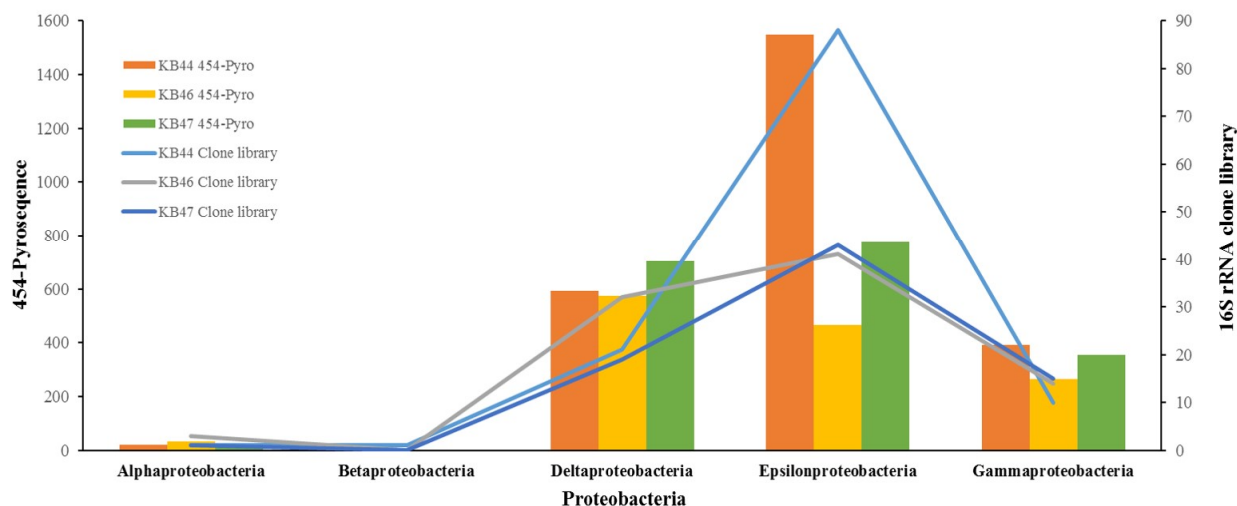


Fig. 3 — Comparison of Proteobacteria (class level) community structure generated by 454-Pyrosequencing and clone library sequencing.

Trough<sup>17</sup>. *Gammaproteobacteria* representation in the 454-Pyrosequencing methods from all sites was more than 25 fold higher than the clone library sequencing method (Fig. 3). *Gammaproteobacteria* were usually found as symbiotic partners of the vent and seep invertebrates such as *L. satsuma*<sup>5</sup> and *Bathymodiolus azoricus*<sup>18</sup>, and other free-living bacteria such as *Methylococcaceae*<sup>19</sup>, *Thiopfundum*<sup>20</sup> and *Pseudomonas*<sup>21</sup>. *Bacteroidetes* usually found in the gastrointestinal tract of animals, however, also found in the gill and intestine of the hydrothermal vent crab *Austinograea* sp.<sup>22</sup>.

The taxa observed uniquely by the 454-Pyrosequencing method may be due to the low-GC content, well studied by Danhorn *et al.* (2012)<sup>23</sup>. This study explained the efficiency of both sequencing methods to detect the bacterial taxonomic groups in a natural environment. The 454-Pyrosequencing method can detect a wide range of taxa than the clone library sequencing method. However, the abundance of most dominant Phylum *Proteobacteria* was found in both methods.

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