

Bacterial Community in Gut Contents of the Sea Urchin *Diadema setosum* (Leske, 1778) and the Ambient Sediments from Sichang Island using Metagenomics Approaches

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ABSTRACT

Next-generation sequencing of 16S rRNA gene was applied for the study of bacterial communities in the gut contents of sea urchin, *Diadema setosum* and from the ambient sediments which were collected from Tha Wang Coast, Sichang Island, Chonburi, Thailand. Both species richness and diversity in the ambient sediments were greater than the diversity in the gut content samples. In the gut content samples, Bacteroidetes was found as the most abundant phylum whereas in the ambient sediments was predominated by Proteobacteria. The results indicated that bacterial communities between gut content and the sediments were clustered into different groups. Specific of intestine conditions and host positive selection capacity may contribute to the different bacterial community. It is somewhat surprising from this study that bacteria in genus *Fusibacter* was detected in the gut content samples which have not been reported in other species of sea urchin so far. Also, sulfate-reducing bacteria (SRB) were detected in gut content samples. This group of bacteria might play a crucial role in sulfate metabolism in their host intestine

Keywords : 16S rRNA, bacterial community, Diadema setosum

INTRODUCTION

Sea urchin *Diadema* sp. live in the marine intertidal zone. This genus is some of the most widespread, abundance and has an ecologically important role in tropical zone (Lessios et al, 2001; Muthiga and McClanahan, 2013). In Thailand, the distribution of *D. setosum* was reported to be found in both Gulf of Thailand and Andaman Sea (Putchakarn and Sonchaeng, 2004). They are marine benthic animals that can consume variety of foods with different feeding method e.g. grazing on the hard substrates and benthic sediments. It is possible that bacteria from their surrounding environment enters to sea urchin gut through their feeding (Harris, 1993). However, the studies of bacterial community in sea urchin guts have been reported to only some case of commercial species e.g. *Echinus esculentus* (Unkle, 1977), *Paracentrotus lividus* (Meziti et al, 2007), *Lytechinus variegatus* (Nelson et al, 2010) and *Strongylocentrotus intermedius* (Zhang et al, 2014). Nevertheless, there have been limited studies on the biology of bacterial symbiotic relationship in the sea urchin in genus *Diadema*. Therefore, this study will clarify the effect of food on the symbiotic bacterial community in their guts.

During the past, the bacterial communities can be identified using classical microbiology techniques e.g. screening bacteria from the gut and culture them. However, some species or bacterial taxa cannot be cultured and this could lead to some

misinterpretations of the bacterial community diversity. In order to overcome the problem of uncultured bacteria, genomics study can resolve and enhance the efficiency by using culture-independent approaches (Streit and Schmitz, 2004). Metagenomics is a method that utilizes metagenome for analysis and this can also explore the whole genome of microbial in an environment and also understand about microbial community function and interaction with each other (Streit and Schmitz, 2004). So there is more challenge to finding the novel or closely related species than single species studies (Thomas et al, 2012). Recently, metagenomics approach is widely used for microbial studies e.g. the studies of bacterial communities in human gut (Qin et al, 2010), in sediment (Mason et al, 2014) and in benthic marine ecosystem (Tangherlini et al, 2016).

The aims of this study are therefore to focus on bacterial diversity in gut content of sea urchin *Diadema setosum* and the ambient sediment as circumstantial evidence to represent the food that sea urchin consumed. Also, relatedness between these two samples group using 16S rRNA sequencing with Illumina Miseq platform. The results of bacterial diversity including the relationship between bacterial community diversities in the gut and their ambient sediments may help to understand roles of symbiont bacteria on the physiological aspects and may be lead to the further study on their roles in marine ecosystems.

METHODOLOGY

Sample collection

Three adult *Diadema setosum* (40-50 mm in sea urchin test diameter) were collected in November 2016 from Sichang Island, Chonburi province, Thailand. Samples were then rinsed with sterile seawater and further sterilized by 70% ethanol to reduce any contaminants before the dissection procedure. Each sea urchin was cut through the circumference to open the test using dissecting scissor. After that, the gut contents were transferred and kept in zip lock plastic bag and stored at -20 °C until use. In addition to the collection of gut contents, sediments (n=3) from the location around the sea urchin samples were also collected and stored at -20 °C.

DNA extraction and PCR amplification

Genomics DNA in the gut contents and sediment samples was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. The Illumina primer pair, 16S Amplicon PCR Forward Primer (5' TCGTCGGCAGC GTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 16S Amplicon PCR Reverse Primer (5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG ACTACHVGGGTATCTAATCC) was used for amplification of the V3 and V4 regions of the 16 rRNA gene (Klindworth et al, 2013). PCR amplification was set up as follow: 2X KAPA HiFi HotStart ReadyMix, 200 nM of each primer and 12 ng of genomic DNA for a total reaction volume of 25 µl. Perform PCR in a thermal cycler using the following program: initial denaturation step at 94°C for 3 min; 25 cycles of 98°C for 20 sec, 55°C for 30 sec, and 72°C for 30 sec and final extension step at 72°C for 5 min. The amplicon products were purified using AMPure XP beads and attached dual indices using the Nextera XT Index Kit, then purified using AMPure XP beads again.

Sequencing and bioinformatics analysis

Purified 16S amplicons were pooled and adjusted to a concentration of 4 nM. Cluster generation and 250 bp paired-end read sequencing were performed on an Illumina MiSeq at the Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). Bioinformatics were analysed using QIIME (v1.9.1) pipeline to process data. The pair-end sequencing reads from Illumina were de-multiplexed and stitched together, followed by trimmed low quality reads using FASTX toolkit as well as removed chimeric reads using VSEARCH. Sequences were grouped into operational taxonomic units (OTUs) and assigned taxonomic position using UCLUST algorithm as executed in USEARCH (Edgar, 2010) against the Greengenes (v13.8) database. The alignment of the sequences was performed with PyNAST (Caporaso et al, 2010)

Statistical analyses

Alpha diversity of each sample was calculated using the observed OUTs, chao1 richness estimator, Good's coverage and the Shannon-Wiener and Simpson diversity indices. Phylogeny based metric as PD whole tree was also calculated. All metrics were computed by Qiime (v1.9.1). Heatmaps and PCA plot were generated with STAMP (v2.1.3)

RESULTS AND DISCUSSION

Richness and diversity

After Illumina MiSeq sequencing platform and filtering out the low quality reads, a total of 128,664 optimized reads of the V3-V4 regions of the 16S rRNA gene were obtained from 3 samples of sea urchin's gut contents and 3 samples from the ambient sediment. The reads were grouped into the total amount of 7,528 OTUs (cutoff of $\geq 97\%$ similarity using UCLUST (Edgar, 2010)). The percentage of the sequence used for analysis was shown in Table 1, coverage percentage of all the gut content samples was over 98% while coverage percentage of sand samples was lowered, with the value in the range from 85.4% to 90.9%.

The sea urchin's gut contents samples have shown the relatively lower bacterial richness, contained only 510 to 604 OTUs, compared with the sediment samples which contained from 2667 to 3169 OTUs (Table 1). According to Shannon and Simpson's indices from table 1, bacterial diversity in the sediments have also shown higher diversity relative to the gut content bacterial communities. This corresponded with Gao et al (2014), who compared the bacteria in gut content of sea cucumber *Apostichopus japonicus* and the surrounding sediment. Conditions inside the gut such as temperature pH and oxygen level are one of the factors that could affect to the presence of bacteria in their gut (Harris, 1993).

Table 1 Sample statistics and diversity values, as determined by Qiime (v1.9.1)

Samples	Observed OTUs	Chao1	%Coverage	PD_whole tree	Shannon	Simpson
Sand 1	3169	4921	85.4	191	10.25	0.94
Sand 2	3073	4521	87.0	183	10.33	0.94
Sand 3	2667	3360	90.9	163	10.14	0.95
Gut content 1	604	670	98.9	48	7.22	0.86
Gut content 2	510	639	98.5	43	6.63	0.82
Gut content 3	649	777	98.3	50	6.12	0.78

Composition of bacterial communities in the gut contents and the sediments

The OTUs were classified from phylum to genus, in the gut contents samples, Bacteroidetes was presented as the most abundant phylum with a mean average of $43.54 \pm 7.05\%$. Classification in term of class categories, Bacteroidia, Clostridia, Flavobacteria and Gammaproteobacteria were shown to be dominated in the gut content community, respectively as displayed in Figure 1. At the genera level, the predominant taxa were *Fusibacter*, *Deulfotalea*, *Tindalia_Anoxynatronum* and *Robiginitalea*, besides *photobacterium*, *Vibrio*, *Ferrimonas*, *Defulovibrio* and *Desulfococcus* were also found in the gut of sea urchin. These results are contrast to compare with some previous studies of gut bacteria in sea urchin host. For example, Zhang et al. (2014) reported that intestine of *Strongylocentrotus intermedius* was dominated with *Psychronomas*, *Shewanella*, *Saccharophagus degradans* and *Nitrosomonas eutropha*, all of which were not be found in the present study. Unkles (1997) found that *Vibrio*, *Pseudomonas*, *Aeromonas* and *Flavobacterium* were predominant taxa in *Echinus esculentus*, except *Vibrio*; however, all the reported genera cannot be detected in this study. In the case of *Lytechinus variegatus*, Nelson et al (2010) found that *Vibrio*, *Pseudomonas* and *Gamma Proteobacteria* were predominated taxa whereas Hakim et al (2016) reported that the dominated taxa were *Vibrio*, *Photobacterium*, *Propionigenium* and *Ferrimonas*. According to Hakim et al (2016) study, the recent study has found similar groups of bacteria in our top 15 ranks of bacterial diversity except *Propionigenium* which can hardly be detected. The dominant taxa were rather different from across studies are in agreement with those obtained by Nelson et al (2010), which suggested that bacterial communities in sea urchin gut were strong conservation between both geographical regions and species of sea urchin.

The sediment samples were shown to have a high abundance of Proteobacteria with cover average $49.09 \pm 0.01\%$ of the reads. The most dominant classes were Gammaproteobacteria, Verrucomicrobiae and Deltaproteobacteria respectively, which Gammaproteobacteria and Deltaproteobacteria belonging to phylum Proteobacteria but the other is belonging to phylum Verrucomicrobia. At the genus level *Desulfococcus*, *Robiginitalea*, *Microcoleus* and *Vibrio* were clearly observed. Somboonna et al (2012) studied bacterial community in sediment from Sichang Island and found that Proteobacteria (56.95%), Actinobacteria and Bacteroidetes were dominated in the Tha Wang Coast (the present study sampling site). The result is rather similar to the present study as Proteobacteria is the major proportion of both studies. However, bacterial community composition probably alter depend on seasons (Harris, 1993). The further studies of temporal or seasonal effects on bacterial community are required.

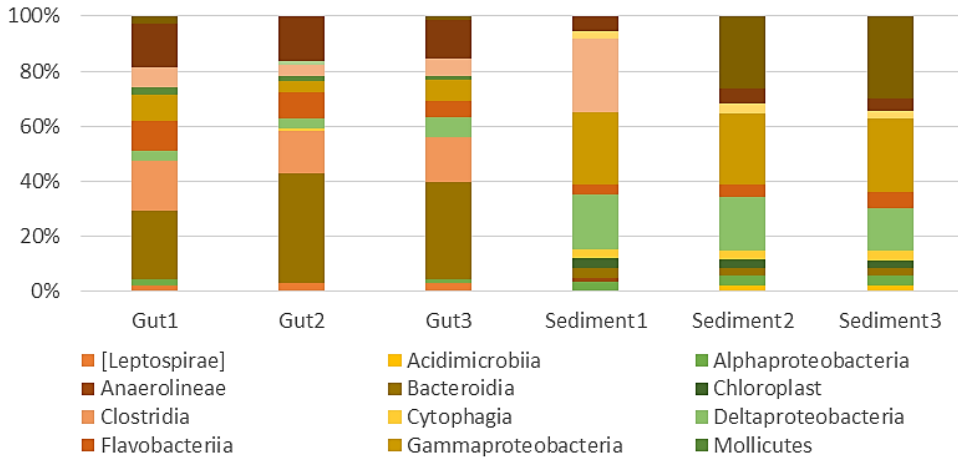


Figure 1 Bar chart of the top 10 bacterial classes found in gut and sediment samples

One of the remarkable results in genus level is that *Fusibacter* of the phylum Firmicutes was identified in the study. This taxon has been limited reported in sea urchin intestine so far, however, there is reported that this genus can be found in the bacterial gut study in the wild and captive-raised shrimp, *Penaeus monodon* (Chaiyapechara et al, 2012; Rungrassamee et al, 2014). *Fusibacter* was first discovered in oil producing well (Ravot et al, 1999). They are obligate anaerobic and halotolerant bacteria (Ravot et al, 2015), as well as possible that them associated with sulfate-reducing activity due to their ability to use sulfur and thiosulfate as final electron acceptors (Ravot et al, 1999; Fadhlaoui et al, 2015). In the present study, *Fusibacter* was observed in both sample groups. It was the most abundant taxa ($5.47\% \pm 1.04\%$) presented in gut content samples and also found in the sediment samples but in small amounts ($0.21\% \pm 0.36\%$). Other genera of sulfate-reducing bacteria (SRB) were also found, that are *Desulfotalea*, *Desulfovibrio* and *Desulfococcus* which belonging in Deltaproteobacteria. The presented of SRB have been reported in the digestive tract of the sea urchin, *Paracentrotus lividus* (Meziti et al, 2007). It is possible that the ambient sediments are sources of SRB in the sea urchin gut. Due to the fact that the Tha Wang Coast of Sichang Island, the sampling site of this study, have many human activities e.g. habitation, pier, shipping and fishing industries, this could lead to be one of the anthropogenic sources of accumulate sulfate in the sediments (Somboonna et al, 2012). This species of sea urchin may have an important role in terms of sulfur cycle around this area. It is recommended that further research should be undertaken in order to confirm the roles and interactions between these symbiotic bacteria and the sulfur cycle around the area of study.

Difference across gut content and sediment samples

Different composition of bacterial community between 2 sample groups was presented in this study. It is apparent from the principal component analysis (PCA) that the bacterial community in the gut content and the sediment samples were different with the total 98.7% variation (Figure 2). The gut content and sediment samples were separated by the first principal component (PC1) which is accounted for 96.5% variation. Heatmap plot was generated at the family level, gut content and sediment samples were clustered into clearly different groups (figure 3).

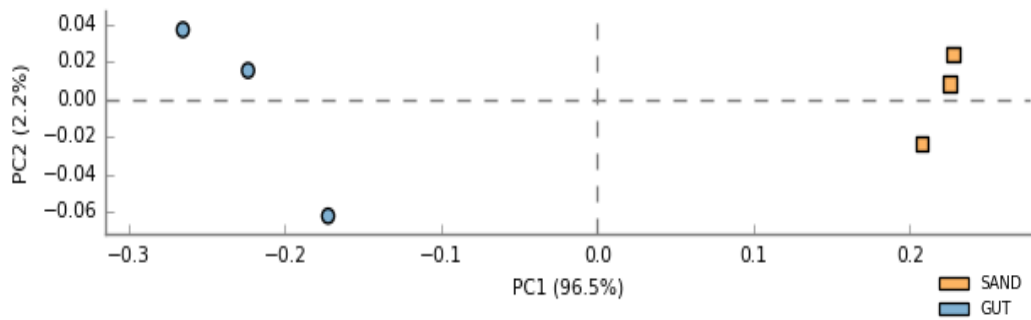


Figure 2 PCA result displayed association in the different samples produced by STAMP (v2.1.3)

Sea urchin is a marine benthic invertebrate, their grazing feeding habit is associated with hard substrate and seabed (Muthiga and McClanahan, 2013). It is also possible that bacterial community in the gut content of sea urchins is associated with their surrounding sediment. The collected sediments were only the circumstantial evidence to represent the food that consumed by the sea urchin. Therefore, bacterial community in the gut contents of sea urchins were differed from bacteria from the ambient sediment (Figure 2, Figure 3). According to the study of the relationship between the bacterial community in the gut content of sea cucumber *A. japonicus* and their surrounding sediment by Gao et al (2014), the results showed that bacterial community were rather different. The study of bacterial community between two parts of digestive tract, pharynx and esophagus as well as stomach and intestine, in sea urchin *Paracentrotus lividus* by Meziti et al (2007) also showed that bacterial community in stomach and intestine rather differed from bacteria in the environment. However, the bacterial community from pharynx and esophagus showed the similar profile to the surrounding sand and water. This may be indicated that some bacteria may enter the digestive tract of sea urchin through their feeding. However, these bacteria groups limited to the anterior part of digestive tract (pharynx and esophagus) and cannot be detected in the posterior part of digestive tract (intestine). It probably results from the specific conditions of the intestine which is quite limited to bacterial colonization (Harris, 1993). Moreover, host positive selection capacity may be one of the factors that can select some certain specific useful bacteria and expel the harmful species (Zhang et al, 2014; Hakim et al, 2015). Therefore, only some microbes from the environment can retain in their digestive tract while some ‘transient microbes’ may be ingested, passed and voided from the gut as the feces (Harris,1993). These findings could associate with the construction of the specific ‘microbiome’ in this species of sea urchin.

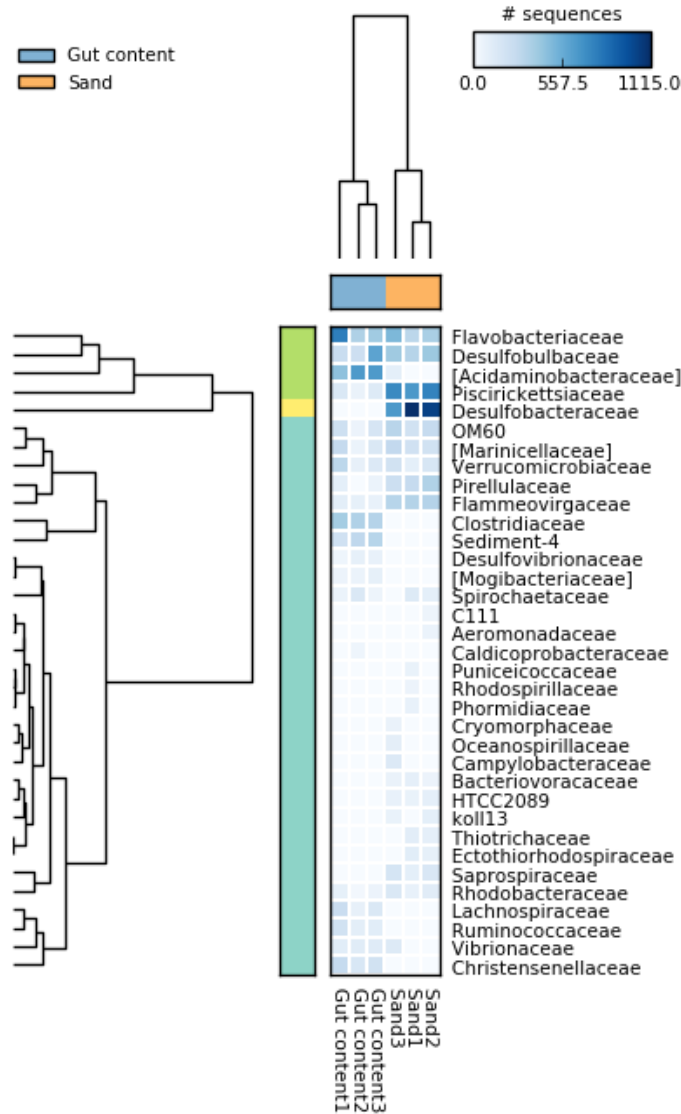


Figure 3 Heatmap generate at family level as determined using STAMP (v2.1.3)

CONCLUSION

The results indicated that bacterial community in the sediment have higher species richness and diversity compared with the sea urchin gut content. Also, the bacterial community between the sediment and the gut content samples are rather different. Specific of intestine conditions and host positive selection capacity may contribute to the different bacterial community. SRB group, *Desulfotalea*, *Desulfovibrio*, *Desulfococcus* and *Fusibacter* were also found in the present study. Contrary to the previous studies in the sea urchin, the *Fusibacter* was detected and determined as the dominant genus. This group of bacteria might have the responsibilities for sulfate metabolism in their host intestine and might also have an important role in sulfur cycle in the marine intertidal ecosystems. Further studies need to be carried out in order to validate the metabolism and ecological roles of these symbiotic bacterial taxa in their ecosystems.

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