Comparative analysis of the gut microbial communities between two dominant amphipods from the Challenger Deep, Mariana Trench

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ABSTRACT

The gut microbiota is contributable to the adaptation and health of deep-sea organisms and has been revealed to be affected by many factors, especially the host species and diet. Here, we employed high-throughput sequencing of 16S rDNA to compare the gut microbial composition and diversity of two hadal amphipod species, Haloclinea gigas and Halice sp. MT-2017, which are predominant inhabitants in the Challenger Deep. Results showed that Tenericutes and Proteobacteria were the most abundant phyla, occupying more than 50% of total phyla, across all the specimens. At genus level, “Candidatus Hepatoplasma” was overall dominant, followed by Psychromonas in H. gigas and Psychrobacter in Halice sp. MT-2017 respectively. Notably, though two hadal amphipod species shared the same predominant symbiont of genus “Ca. Hepatoplasma”, they were classified into two different OTUs, which suggested that the colonization of symbionts were host-specific. Moreover, in terms of species relative abundance, alpha-diversity and beta-diversity, there was still a significant divergence of gut microbiota between two hadal amphipod species, which dwelled in the same extreme environment. Altogether, the results dropped a hint to the importance of symbiont genus of “Ca. Hepatoplasma” to amphipod survival in the hadal zone.

1. Introduction

The hadal zone constitutes the deepest 45% of the ocean’s vertical depth, predominately comprised of trenches with different depths expanding from 6000 m to 11,000 m. The hadal trenches are characterized by extreme environmental conditions, such as limited food supplies, high hydrostatic pressures, and temperatures as low as 1–2 °C (Jamieson et al., 2010). All these factors shaped the organisms living in the hadal zone (Jamieson, 2015). However, how hadal organisms adapt to and survive in such harsh environments has not been explained completely.

Amphipods are an important scavenging macrofauna of the deep sea and have been found in high abundance within many of the major hadal trenches, such as the Philippine Trench, the Yap Trench, and the Mariana Trench (Hessler et al., 1978; Jamieson et al., 2011). Lysiannid amphipods (H. gigas) are predominant at depths greater than 10,000 m and are widely distributed throughout the Northwest Pacific Trenches, including the Mariana Trench (France, 1993; Eustace et al., 2013). Pardalidaceae amphipods (Halice sp. MT-2017) are also common within the Challenger Deep. During our sampling cruise (Mar. 2017 and Oct. 2018), numbers of individuals were identified at several sampling sites at approximate depths of 11,000 m (Li et al., 2019).

Previous studies have shown that the gut microbiota plays a crucial role in the health of organisms, including pathogen defense, adaptation to environmental stress due to salinity changes, digestion of complex carbohydrates, and production of secondary metabolites, such as vitamin B12 (Sugita et al., 1991; Flint et al., 2008; Clemente et al., 2012; Zhang et al., 2016). An increasing body of evidences indicated the critical role of gut symbiont for organisms to adapt to the hadal environments. For examples, compared with terrestrial symbiotic Mycoplasma, the two draft genomes of Mycoplasma derived from a deep-sea Bathynomus isopod stomach exhibited a greater number of genes responsible for nutrient uptake, including sugars, amino acids, and other carbohydrates, suggesting that the symbiont may contribute to host adaption in nutrient-poor environments (Wang et al., 2016). Genome analysis of the gut symbiotic Spiroplasma from a hadal holothurian showed the presence of three cas genes and 76 clustered regularly interspaced short palindromic repeat (CRISPR) spacers, likely to benefit the Spiroplasma symbiont and then holothurian host by providing protection from external environmental viruses (He et al., 2017).

The community structure of gut symbionts was affected by many factors, such as the host genetic background, diet, and habitat variation.
Benson et al., 2010; Claesson et al., 2012; Suzuki, 2017). Recently, taxonomic analysis revealed that the gut microbiota of *H. gigas* from the Japan Trench was significantly different from that of the amphipods in the Mariana Trench (Zhang et al., 2019). Both *H. gigas* and *Halice* sp. MT-2017 are representative species of the Challenger Deep, but the gut microbiota of them has not been compared. In this study, we used high-throughput sequencing of microbial 16S rDNA to decipher composition and diversity of gut microbiota of the two hadal amphipod species, aiming to further highlight the relationships between symbionts and hosts.

2. Materials and methods

2.1. Sampling methodology

Samples were collected from the Mariana Trench during TS09 cruise between September and October in 2018. Sampling locations were defined by sampling depths and geolocations as Location A (LA) (11°15.2821′N, 142°12.7753′E) at 10,109 m depth and Location B (LB) (11°20.2118′N, 142°13.0340′E) at 10,910 m depth. *H. gigas* specimens were collected from LA (n = 9) and LB (n = 2); *Halice* sp. MT-2017 specimens were collected from LB (n = 15). All samples were obtained with a trap bounded to the Tianya Lander. Fish and chicken carcasses wrapped in high-density mesh bags served as baits and secured inside the Niskin bottles. The baits were not released and eaten by amphipods during trapping. Once collected on-board, the specimens were immediately preserved in liquid nitrogen. After transported to the laboratory, they were stored at −80°C before used.

2.2. DNA extraction and 16S rDNA sequencing

The samples were dissected at laboratory. All the tools for dissection were sterilized. To avoid the contamination from cuticle, the samples were washed several times with milli Q water before dissection, and after cutting open from the back, the gut tissue was isolated and moved to a clean tube with another sterilized tweezers. For PCR, the reaction without template was acted as control to avoid the contamination from the PCR chemical and environment. DNA was extracted from each gut sample with the Power Soil DNA isolation kit (Mo Bio Laboratories, USA). 16S rDNA was amplified using the universal bacterial primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 802R (5′-TACNVGGGTATC- TAATCC-3′), with a random 6-nucleotide barcode added to the 5′-ends (Wang and Qian, 2009). PCR reactions were set up with a 10–20 ng genomic DNA template, 4 μl primer at 10 μM, 4 μl of 10 mM dNTP, 10 μl of 5x PCR buffer, 0.5 μl Prime STAR HS DNA Polymerase (TAKARA, Japan), and enough ddH2O to bring the volume to 50 μl. PCR was performed on a Gene Amps PCR System 9700 (PE Applied Biosystems, USA). 16S rDNA was amplified using the universal bacterial primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 802R (5′-TACNVGGGTATC- TAATCC-3′), with a random 6-nucleotide barcode added to the 5′-ends (Wang and Qian, 2009). PCR reactions were set up with a 10–20 ng genomic DNA template, 4 μl primer at 10 μM, 4 μl of 10 mM dNTP, 10 μl of 5x PCR buffer, 0.5 μl Prime STAR HS DNA Polymerase (TAKARA, Japan), and enough ddH2O to bring the volume to 50 μl. PCR was performed on a Gene Amps PCR System 9700 (PE Applied Biosystems, USA) using the following program: 98°C for 10 s; 98°C for 10 s, 48°C for 15 s, and 72°C for 30 s for 30 cycles, followed by 72°C for 5 min. PCR products were purified with a QIAEX II Gel Extraction Kit (QIAGEN, Germany) and quantified using a Pico Green dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Following the quantification, equal amounts of PCR amplicons were pooled, and paired-end sequencing was performed on an Illumina MiSeq platform.
**Fig. 2.** Phylogenetic analysis of the amphipods used in this study. The phylogenetic tree was analyzed by maximum likelihood with GTR + G model based on partial COI sequences. The COI sequence of the amphipod *Gammarus duebeni* (AY926669) was used as an outgroup. The bootstrap values were indicated near the branches of the tree.

**Fig. 3.** Phylogenetic analysis of “Ca. Hepatoplasma” from different hosts. The phylogenetic tree was analyzed by maximum likelihood with Tamura-Nei model based on partial 16S rDNA sequences. The bootstrap values were indicated on the branches of the tree. The symbionts of “Ca. Hepatoplasma” from this study were marked with bold.

**Fig. 4.** Alpha-diversity of gut microbiota based on operational taxonomic unit (OTU). Shannon and Simpson indexes were displayed by box plots. Significant differences between species were determined through two-tailed student’s t-test. p-value less than 0.05 was deemed significance.
Fig. 5. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering of samples. UPGMA clustering was calculated with unweighted UniFrac distance. MG: *H. gigas* from individuals; MH: *Halice* sp. MT-2017 from three individuals.

Fig. 6. Gut microbial communities between two amphipod species. (A) Results of Non-metric Multidimensional Scaling (NMDS; unweighted UniFrac distance) for *H. gigas* and *Halice* sp. MT-2017 gut bacterial communities. (B) Permutational multivariate analysis of variance (PERMANOVA) and ANOSIM tests based on unweighted UniFrac. p-value less than 0.05 was determined to be significant. The $R^2$ represents the ratio of variability between original data and the grouped data.

2.3. Data analysis

Sequences were analyzed using the QIIME software package (Kuczynski et al., 2011). Briefly, the low-quality sequences were filtered according to the previously described criteria by utilizing QIIME quality filters (Gill et al., 2006; Chen and Jiang, 2014), and then raw sequencing reads were assigned to respective samples according to the assigned unique barcodes. Paired-end reads were assembled by FLASH (Tanjä and Salzberg, 2011). Chimera sequences were then detected with the UCHIME algorithm and the effective tags were identified (Edgar et al., 2011). Remaining high-quality sequences were clustered into OTUs with 97% sequence identity to generate the OTU table with UCLUST (Edgar, 2010). OTUs comprising the OTU table containing less than 0.001% of total sequences across all samples were discarded. Representative sequences for the OTUs were selected and blasted against the SILVA database to obtain taxonomic information. In order to normalize the variations in sequencing depth across the samples, the reads were subsampled to a depth of 25,360 reads/sample, occupying 90% of the minimum sequencing depth of all the samples. The data were then averaged and rarefied rounded. In order to more deeply penetrate the data, microbial diversities between groups and relative abundances of the taxa at the phylum and genus levels were statistically measured using the parametric test of two-tailed Mann-Whitney U test.

Data analysis was performed using the QIIME and R packages (v3.2.0) (Caporaso et al., 2010). For alpha-diversity, OTU-level alpha-diversity indices, such as Shannon diversity index and Simpson index, were calculated using the rarefied OTU table in QIIME. Differences between indices among groups were determined using two-tailed student’s t-test, which $p$ values were adjusted by Holm-Bonferroni correction, and eventually visualized through box plots. QIIME calculations of both weighted and unweighted UniFrac were used as described previously to quantify phylogenetic measures of beta-diversity (Lozupone and Knight, 2005; Lozupone et al., 2007). Unweighted UniFrac for UPGMA clustering and NMDS were used in this study (Ramette, 2007). The significance of microbial structure variations among groups were assessed by PERMANOVA and ANOSIM using the R “vegan” package (Warton et al., 2012). Co-occurrence analysis was performed by calculating Spearman’s rank correlations between the predominant genera, correlations with |RHO| > 0.6 and $p$ < 0.01 were visualized as a co-occurrence network using Cytoscape (Shannon et al., 2003).

2.4. Phylogenetic analyses

Amplification of the mitochondrial cytochrome c oxidase subunit I (COI) with CrustDF1 (5′-GGTCWACAYTACAAAGATCGYTTG-3′) and CrustDR1 (5′-TAAACCTYCAGGTAGACCAAAAYCA-3′) primers and Prime STAR DNA High Fidelity Polymerase (TAKARA BIO INC., Japan) were performed with gut template DNA from the samples (Knox et al., 2012). PCR was performed using the method mentioned above. After completion, the amplified PCR products were sequenced in both directions on a 3730xl DNA Analyzer using BigDye BGI TECH SOLUTIONS (BEIJING LIUHE Co., Ltd, Beijing, China). Sequences were manually verified and the assembly of both strands was completed with DNAMAN v7. The ampipod species was identified by blasting the partial COI sequences against NCBI databases. The phylogenetic tree for the 26 ampipod individuals was also constructed based on COI nucleotides sequences. *Gammarus duebeni* (Genbank ID: AY926669) was used as an outgroup. Sequence alignments were generated using Clustal W (Larkin et al., 2007). Gblocks 0.91b was used to remove the poorly aligned regions (Castresana, 2000). Based on the well-aligned sequences with 653 nucleotides in length, maximum likelihood method was adopted to construct phylogenetic tree with RaxmlGUI (Silvestro and Michalak, 2012) using the GTR + G model as recommended by jModelTest (Darriba et al., 2012). The node stability of the maximum likelihood tree was assessed with 1000 bootstrap replicates.
16S rDNA partial sequences of the dominant symbionts were compared in NCBI databases by blasting, and 12 closely related sequences were downloaded. Phylogenetic analysis for the symbionts was performed with an alignment of 429 nucleotides in length. Sequence alignments were generated using Clustal W, and then imported into MEGA X software for phylogenetic analysis using the Maximum Likelihood method with a bootstrap replication number of 1000 (Kumar et al., 2018).

2.5. Data accession

All raw sequences were deposited in the NCBI Sequence Read Archive under accession number SRR8517657.

3. Results

3.1. Composition of amphipod gut microbiota

Raw data of 16S rDNA V3–V4 amplicons were obtained. After quality and chimera filtration, 1,453,597 high-quality reads were produced for 26 amphipod individuals (Table S1). The rarefaction curves of Halice sp. MT-2017 samples were saturated and H. gigas samples were close to the plateau, indicating an adequate sequencing depth for analyzing the microbiota community structure (Fig. S1). A total of 1786 OTUs were clustered at the level of 97% similarity (Table S1). The OTUs from two amphipod species were classified into 7 bacterial phyla: Tenericutes, Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Cyanobacteria, and Tectomicrobia. Unclassified bacteria were relatively in a low percentage (H. gigas 0.4–2.7%; Halice sp. MT-2017 0.04–3.3%) (Fig. S2). At phylum level, Tenericutes and Proteobacteria together (H. gigas 87.3–99.6%; Halice sp. MT-2017 42.6–91.3%) represented disproportionately the largest groups in the gut microbial composition (Fig. S2). Firmicutes were significantly more enriched (Mann-Whitney U test; p < 0.05) in Halice sp. MT-2017 (8.4–51.7%) than in H. gigas (0.1–10.3%) (Fig. S2). Tenericutes and Proteobacteria were more abundant in H. gigas when compared to those in Halice sp. MT-2017, but the differences were not significant (Mann-Whitney U test; p > 0.1).

At genus level, 57 genera were detected across all analyzed samples. Tenericutes, the dominant phylum, was exclusively composed of “Ca. Hepatoplasma”. The majority of Proteobacteria were classified as Psychromonas and Psychrobacter (Fig. 1). Overall, “Ca. Hepatoplasma” was the most prevalent genus of all hadal amphipod samples. Except two samples, “Ca. Hepatoplasma” accounted for 27.6–93.4% and 45.6–74.7% for H. gigas and Halice sp. MT-2017, respectively, with no significant variation between species (Mann-Whitney U test; p = 0.937) (Fig. 1). Psychromonas was quite rare in Halice sp. MT-2017, only accounting for 0.09–0.19%. In contrast, the percentage of Psychrobacter was high in Halice sp. MT-2017, spanning between 11.6 and 43.5% (Fig. 1). With statistical tests, Psychromonas was significantly enriched in H. gigas (Mann-Whitney U test; p < 0.05), as well as Psychrobacter in Halice sp. MT-2017 (Mann-Whitney U test; p < 0.05).

3.2. 2Phylogenetic analyses

Based on COI sequences, the similarity between H. gigas individuals reached over 99%, so was to Halice sp. MT-2017. However, subgroups were still formed for both H. gigas and Halice sp. MT-2017 in the phylogenetic analysis with small genetic divergences (Fig. 2).

The dominant genus “Ca. Hepatoplasma” was classified into different OTUs. OTU1560 was the most abundant OTU (> 80%) in H. gigas, while Halice sp. MT-2017 was predominated by OTU22828 (> 90%). With respect to the 16S rDNA phylogenetic tree, the sequences for “Ca. Hepatoplasma” from the hadal amphipods clustered together and formed a sister clade approximate to the Mollicutes symbionts from the gut of hydrothermal vent shrimp Rimicaris exoculata. The hadal amphipod symbionts “Ca. Hepatoplasma” were also separated from those associated with other host species in the tree, such as “Ca. Hepatoplasma” previously isolated from terrestrial isopods (Fig. 3).

3.3. Divergence of gut microbial communities

A total of 1786 OTUs were identified across all hadal amphipod samples, of which 322 were shared by both species and the rest were unique to Halice sp. MT-2017 (n = 1354) or H. gigas (n = 110) (Fig. S3). In order to assess species-specific variations of the gut microbiota, alpha-diversity was measured at OTU level. The Shannon and Simpson indexes of the gut microbial communities of H. gigas samples were significantly higher than those of Halice sp. MT-2017 samples (p < 0.01) (Fig. 4).

The structural distinctiveness of gut microbial communities between amphipod species was determined through beta-diversity analysis at the genus level. Composition and similarity of gut microbiota were analyzed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA; unweighted UniFrac distance) and Non-metric Multidimensional Scaling (NMDS; unweighted UniFrac distance). Results showed that there was no significant difference (p > 0.05) between H. gigas individuals from location A and location B for gut microbial community (Table S2), though MG10 and MG11 (H. gigas from location B) clustered together, and apart from the H. gigas from the location A (Figs. 5 and 6A). Furthermore, NMDS and UPGMA clustering plots could clearly separate the samples in accordance with host genetic background (Figs. 5 and 6A). Permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) paired with unweighted UniFrac distance were consistent with the observations in species-specific variation (p < 0.01) (Fig. 6B). In sum, the beta-diversity measurements indicated the presence of significantly distinct gut microbiota (p < 0.01) in H. gigas and Halice sp. MT-2017.

4. Discussion

Amphipods are important scavengers that feed on decaying animal and plant materials, poor in nutrients but relatively rich in refractory compounds, filling an important niche within deep-sea ecosystems (Jamieson, 2015). Typically, symbiotic relationships between a host and its respective gut microbiota could translate into host organism dietary habits and facilitate the nutrient digestion (Colman et al., 2012). The presence of gut bacteria in amphipods potentially contribute to the utilization of energy and nutrition, as gut bacteria are known to enhance the digestion of refractory organic compounds (Schwarz et al., 1976; Deming et al., 1981). In the present study, the gut microbial compositions of the co-dwelling hadal amphipods were relatively constant intra-species and dominated by “Ca. Hepatoplasma”. Previously, “Ca. Hepatoplasma” was reported as a symbiont in the midgut glands of terrestrial isopods, with the results suggesting functions to be related with cellulase production for leaf litter degradation. This is a beneficial relationship for the host in low-nutrient conditions (Wang et al., 2004; Fraune and Zimmer, 2008). The hadal amphipod H. gigas, collected from the Challenger Deep, has been reported to contain a unique cellulase with potential contribution to digestion of wood debris on the seafloor (Kobayashi et al., 2012). The dominant symbiont “Ca. Hepatoplasma” may be related to the nutrition supplement, though another major microbe Psychromonas also existed in H. gigas gut, which was thought to be contributed to pressure adaptation (Zhang et al., 2019). Presently there is no direct evidence to demonstrate that “Ca. Hepatoplasma” is the producer for cellulose production and its detailed roles with its hadal amphipods remains to be analyzed.

In the present study, “Ca. Hepatoplasma” was demonstrated to be predominant gut symbiotic bacterium of H. gigas and Halice sp. MT-2017, both common species of the Challenger Deep; however, previously Psychromonas CDPI was reported to dominate in H. gigas gut,
from the Challenger Deep (Zhang et al., 2018). Here, we found that *Psychromonas* was the second most abundant dominant bacterium in hadal amphipods. *Psychromonas* is psychrophilic and widely distributed in deep-sea environments. It has been isolated from various environments, including the Antarctic sea ice, the Japan Trench and the sediment of Mariana Trench (Mountfort et al., 1998; Nogi et al., 2002; Amato et al., 2016; Cui et al., 2019). It is therefore likely that *Psychromonas* came from external habitat environments of hadal amphipods resulting in relationships driven by food supply dynamics, as horizontal transmission could also influence the structure of animal gut microbiota (Mikaelyan et al., 2015). On the other hand, both “Ca. Hepatoplasma” and *Psychromonas* were the dominant symbionts in *H. gigas*. The co-occurrence patterns in gut microbiota of *H. gigas* and *Halice sp.* MT-2017, as demonstrated by phylogenetic molecular networks (pMENs), revealed a significant negative correlation (r < 0.01) between the gut microbiota of both *Ca. Hepatoplasma* and *Psychromonas*. Both intrinsic and environmental factors could affect the gut microbial communities and alter the relationships between the members of the microbial community. The negative co-occurrence relationship can be formed by the complex interactions among community members, such as resource competition, functional similarity and metabolite release (Faust et al., 2012; Hacquard et al., 2015). This co-occurrence symbiotic relationship could also arise from direct changes of external environmental conditions, such as energy sources (Duperron et al., 2011). It is thus of interest to explore the underlying reasons of the co-occurrence relationships between microbial inhabitants. Nonetheless, these gut microbial interactions were based on 16S rDNA sequencing, the actual ecological relationships existing between symbiotic microbes might need further biologically informative analysis. Understanding the molecular interactions of “Ca. Hepatoplasma”, *Psychromonas* and their hadal amphipod hosts still awaits more efforts.

Previous studies have indicated the critical factors that affect animal gut microbiota, including age (Yatsunenko et al., 2012), health (Sekirov et al., 2013) and habitat environment (Amato et al., 2013). Especially, host species and diet are the most important factors in shaping and shifting gut microbial communities (Goodrich et al., 2014; Hird et al., 2015). Although dominant gut microorganisms were similar between the two hadal amphipod species, there were significant differences between the two species in detail. Besides, though genus “Ca. Hepatoplasma” was detected as dominant component of the gut microbiota not only in *H. gigas* but also *Halice sp.* MT-2017, it was classified into two abundant OTUs and was distinguishable clearly between two amphipod host species. These data suggested that host species could be a determining factor of hadal gut microbiota and further implied a host-specific association between these two cohabitating hadal amphipods and “Ca. Hepatoplasma”. The current study was just related to hadal amphipod species, more species and specimens were required for further examination to investigate the relationship between host species, diets and gut symbions.

The following website shows a detailed internal structure of a typical Gammaridean amphipod. [http://reefkeeping.com/issues/2004-09/rs/index.php](http://reefkeeping.com/issues/2004-09/rs/index.php). The midgut runs along throughout most of the body. The caeca arise from the front of the midgut and lies along the midgut, which located near the mid ventral. The hindgut arises from the end of the midgut and extends forward over the midgut caeca and gonads. With a microsensor, the midgut of *Pachnoda ephippia* (Coleoptera: Scarabaeidae) larvae was revealed to have an extreme alkalinity with a pH value > 10, between the second and third crown of midgut caeca (Lemke et al., 2003). Additional studies revealed that its intestine contained a different markedly gut microbiota between midgut and hindgut (Egert et al., 2003). However, due to the difficulty to separate gut compartments of small body size of *Halice sp.* MT-2017, the samples containing the midgut and hindgut parts were used for sequencing together in this study. Consequently, the spatial distribution of microbial community in midgut and hindgut of hadal amphipods could not be distinguished and more information about the structure and function of the gut microbial community were unable to know yet at presence.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dsr.2019.103081.

### References


