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Distinct influence of trimethylamine *N*-oxide and high hydrostatic pressure on community structure and culturable deep-sea bacteria*

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Abstract Trimethylamine *N*-oxide (TMAO) is one of the most important nutrients for bacteria in the deep-sea environment and is capable of improving pressure tolerance of certain bacterial strains. To assess the impact of TMAO on marine microorganisms, especially those dwelling in the deep-sea environment, we analyzed the bacterial community structure of deep-sea sediments after incubated under different conditions. Enrichments at 50 MPa and 0.1 MPa revealed that TMAO imposed a greater influence on bacterial diversity and community composition at atmospheric pressure condition than that under high hydrostatic pressure (HHP). We found that pressure was the primary factor that determines the bacterial community. Meanwhile, in total, 238 bacterial strains were isolated from the enrichments, including 112 strains affiliated to 16 genera of 4 phyla from the Yap Trench and 126 strains affiliated to 11 genera of 2 phyla from the Mariana Trench. Treatment of HHP reduced both abundance and diversity of isolates, while the presence of TMAO mainly affected the diversity of isolates obtained. In addition, certain genera were isolated only when TMAO was supplemented. Taken together, we demonstrated that pressure primarily defines the bacterial community and culturable bacterial isolates. Furthermore, we showed for the first time that TMAO had distinct influences on bacterial community depending on the pressure condition. The results enriched the understanding of the significance of TMAO in bacterial adaptation to the deep-sea environment.

Keyword: deep-sea bacteria; high hydrostatic pressure (HHP); trimethylamine *N*-oxide (TMAO); community structure

1 INTRODUCTION

The deep-sea accounting for over 75% volume of the ocean is characterized by low temperature, lack of light, oligotrophic, and high hydrostatic pressure (HHP). Increasing number of studies on deep-sea bacterial isolates revealed that they evolved in multiple ways to better acclimate the deep-sea environment. For example, they prefer respiration systems with better pressure tolerance for higher efficiency of energy production under HHP conditions (Chikuma et al., 2007; Aono et al., 2010). They also tend to encode dual flagellar systems and numerous extracellular enzymes that are believed to facilitate nutrient acquisition under the oligotrophic environment (Eloe et al., 2008; Wang et al., 2008; Qin et al., 2011).

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Table 1 General information of samples involved in this study

Sampling site	Location	Longitude (E)	Latitude (N)	Depth (m)	Experiment involved
JL144	Mariana Trench	142°22.81′	10°88.78′	6 300	Enrichment and bacterial isolation
JL145	Mariana Trench	142°14.23′	11°62.86′	6 500	Bacterial isolation
JL146	Mariana Trench	141°69.50′	10°92.05′	6 700	Bacterial isolation
JL147	Mariana Trench	141°98.30′	10°96.21′	6 700	Bacterial isolation
JL150	Yap Trench	137°59.70′	8°05.08′	6 500	Enrichment and bacterial isolation
JL151	Yap Trench	137°62.93′	8°04.00′	6 580	Bacterial isolation
JL152	Yap Trench	137°84.27′	8°02.33′	6 700	Bacterial isolation

Being as an important component of dissolved organic nitrogen, trimethylamine *N*-oxide (TMAO) is widespread in marine environment and plays an important role in the biogeochemical cycle of nitrogen (Gibb and Hatton 2004; Marino et al., 2011). It is also a major precursor of methylamine in sea water, which, including trimethylamine (TMA), dimethylamine (DMA) and monomethylamine (MMA), is the main source of active atmospheric gases such as N_2O and methane (King, 1984; Montzka et al., 2011).

TMAO can be metabolized by marine microorganisms through diverse pathways. Marine *Roseobacter* clade and SAR11 clade bacteria are capable of degrading TMAO into DMA by TMAO demethylase (Tdm) and producing NH_4 and formaldehyde (Lidbury et al., 2014, 2015). Or else, it can be utilized as electron acceptor of anaerobic respiration by diverse species of marine bacteria, including *Altermonas*, *Shewanella*, *Photobacterium*, *Pseudomonas* and *Vibrio*, and the key enzyme catalyzes this reaction is the TMAO reductase (Barrett and Kwan 1985; Dos Santos et al., 1998; Dunn and Stabb, 2008). Recent studies suggested that TMAO is involved in bacterial adaptation to HHP environment as well. Several deep-sea bacteria are found encoding high-pressure inducible TMAO reductase (Vezzi et al., 2005; Zhang et al., 2016; Yin et al., 2018). Our recent study demonstrated that not only the expression of TMAO reductase but also its enzymatic activity and the efficiency of TMAO reduction were promoted under HHP conditions. More importantly, with supplementation of TMAO in the cultural medium, a piezosensitive deep-sea bacteria *Vibrio fluvialis* QY27 exhibited better pressure tolerance, suggesting important physiological role of TMAO under deep-sea environment (Yin et al., 2018).

A variety of phytoplankton, marine invertebrates and fishes produce TMAO to counteract adverse effects of low temperature, osmotic stress and HHP (Yancey et al., 1982; Gillett et al., 1997; Saad-Nehme et al., 2001; Zou et al., 2002; He et al., 2009; Petrov et al., 2012). It has been reported that deep-sea fishes accumulate TMAO up to approximately 30 g per kg muscles (Yancey et al., 2014). Therefore, the abundant TMAO released from deep-sea organisms could be precious nutrient for microbes nearby, and cells utilize TMAO may acquire growth advantages over others and better survive in the nutrient deficient habitats. However, the hypothesis is based on limited studies on purified deep-sea bacterial strains, how TMAO influences the bacterial community, especially under high-pressure condition, remains unknown.

To better understand the influence of TMAO and pressure on deep-sea bacterial community, we performed enrichment experiment under different conditions using sediment samples collected from the Mariana Trench and the Yap Trench and analyzed bacterial community structure of both enrichments and culturable bacterial strains. Our results demonstrated that pressure was the primary factor in determining the bacterial community and isolates, while the effect of TMAO depended on the pressure condition. Collectively, we described for the first time the influence of TMAO to deep-sea bacterial community under different pressure conditions and our result further extended the understanding of the significance of TMAO in bacterial adaptation to the deep-sea environment.

2 MATERIAL AND METHOD

2.1 Sample collection and enrichment

Sediment samples were collected during the cruise Da Yang 38 by using the Push core carried on manned submersible Jiao Long from the Mariana Trench and the Yap Trench (Table 1). Samples were proceeded to enrichment cultivation on board immediately after collection. Approximately 2-g sediment samples were suspended with filtered in situ seawater to a final volume of 20 mL. Mixtures were then transferred into

sterilized high-pressure vessels (Feiyu Science and Technology Exploitation Co. Ltd., Nantong, China), and hydrostatic pressure was applied by injection of sterilized sea-water with a water pump (Top Industrie, France). The enrichments were incubated at atmospheric pressure (0.1 MPa) or at 50 MPa at 4°C for 40 days before the cells were collected by centrifugation. TMAO (Sigma, USA) was added to a final concentration of 0.1% (w/v) when required.

2.2 DNA extraction, 16S rRNA gene amplification and sequencing

Total DNA was extracted using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, USA) according to manufacturer's instructions. Briefly, 0.25 g (wet weight) of the enrichment samples was used for DNA extraction. DNA was finally eluted with 50 μL elution buffer supplied with Mo Bio soil DNA extraction kit. The concentrations of DNA were determined by Nanodrop 2000 (Thermo Scientific, MA, USA). The V3-V4 region of 16S rRNA gene was amplified with primers338F(5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Error-correcting barcodes were added to both forward and reverse primers. PCR amplifications were performed in 25 μL reactions with four replicates. Each reaction contained 20 ng DNA template, 0.2 mmol/L of each primer, 6 mmol/L of bovine serum albumin (TaKaRa, Japan) and 12.5 μL of 2× DreamTaq Green PCR Master Mix (Thermo Scientific, USA). Thermal cycling parameters consisted of initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 45 s, and final extension at 72°C for 10 min. The PCR products were analyzed on 2% agarose gel, and the DNA fragment of 500–600 bp was purified with Qiagen PCR purification kit (Qiagen). The Miseq sequencing was performed by Majorbio (Shanghai), and the raw sequencing data were submitted to NCBI sequence Research Archive under accession number SRP157419.

2.3 Bacterial community structure analysis

The 16S rRNA gene was analyzed using QIIME (http://qiime.org/scripts/assign_taxonomy.html) and the SILVA 128 database (http://www.arb-silva.de). Sequences with over 97% similarity were assigned to the same OTUs (Operational Taxonomic Units). Alpha and beta diversity indices were calculated using QIIME software package and distance matrices was constructed using the Bray-Curtis calculator.

2.4 Construction of phylogenetic tree of Tdm and TorA

The TMAO demethylase (Tdm) from *Ruegeria pomeroyi* DSS-3 and catalytic subunit of TMAO reductase (TorA) from *Shewanella oneidensis* MR-1 were used as the query sequence for tblast against databases of Complete genomes and Draft genomes at NCBI. The hits with *E* value of 0.0 were retrieved for the construction of the phylogenetic tree using Maximum Likelihood method in the software MEGA 5. In total 48 Tdm sequences and 50 TorA sequences are involved in the analysis.

2.5 Isolation and identification of microbes

After 50-day enrichment, 200 μL of mixture were spread on Marine Broth 2216E agar plates with or without supplementation of TMAO and incubated at 4°C. The isolates were purified by striking on plates for single colonies, and cultivated in corresponding medium at 4°C for molecular identification. Briefly, genomic DNA was extracted and the 16S rRNA gene was amplified and sequenced with 27F and 1492R primers. The 16S rRNA sequences were blasted against the EzBioCloud database (https://www.ezbiocloud.net/identify) (Yoon et al., 2017), and 97% sequence identity was applied as the thresholds for classification of species (Tindall et al., 2010). A phylogenetic tree was constructed with 16S rRNA sequences using Maximum Likelihood method in the software MEGA 5, in which 1 000 times repeated bootstrap analysis was used to test the credibility of the phylogenetic tree. The 16S rRNA genes of bacterial isolates obtained in the study were submitted to NCBI sequence Research Archive under accession number MH725320 to MH725557.

3 RESULT AND DISCUSSION

3.1 The bacteria enriched under different conditions

To examine the effect of TMAO and pressure on microbes from deep-sea environment, sediments collected from the Mariana Trench and the Yap Trench were incubated at different conditions (Table 2), and the bacterial community was analyzed using the v3–v4 region of 16S rRNA gene as gene marker. In total, (3.35×10^4)–(1.03×10^5) sequences with 441–461 bp in length were generated for enrichments analyzed (Table 2). The Chao1 and Shannon index indicated that while most enrichments had comparable biodiversity, the community richness and diversity of

Table 2 Enrichment conditions and statistic of bacterial communities from 16S rRNA gene amplicon libraries

Enrichment[a]	Sediment sample	Pressure condition (MPa)	Supplementation of TMAO	Total clean read	No. of OTUs [b, c]	Chao1[b]	Shannon[b]
MT_H	JL144	50	No	57 038	144	129.50	1.83
MT_L	JL144	0.1	No	39 984	123	100.12	2.30
YT_H	JL150	50	No	99 476	261	180.52	1.92
YT_L	JL150	0.1	No	38 564	267	179.44	2.19
MT_HT	JL144	50	Yes	60 279	95	156.50	1.85
MT_LT	JL144	0.1	Yes	42 668	59	56.00	0.81
YT_HT	JL150	50	Yes	103 221	250	190.38	1.57
YT_LT	JL150	0.1	Yes	33 518	64	65.00	0.39

[a]: MT and YT represents sample from the Mariana Trench and the Yap Trench, respectively; H and L represents enrichment at 50 MPa and 0.1 MPa, respectively; HT and LT represent enrichment with supplementation of TMAO under corresponding pressure conditions; [b]: calculated after subsampling of 31 411 reads for bacterial samples; [c]: sequences with over 97% similarity were assigned to the same OTUs (operational taxonomic units).

two enrichments at 0.1 MPa with supplementation of TMAO (MT_LT and YT_LT) were considerably lower than the others. In most enrichments, Gammaproteobacteria was the most abundant group and accounted for over 90% of the communities, except for YT_L, in which Gammaproteobacteria and Alphaproteobacteria each constituted approximately 40% of the community.

After incubated at 50 MPa (MT_H and YT_H), *Colwellia*, *Moritella* and *Psychrobium* were the dominant populations. Other taxa representing over 1% of the communities included uncultured Oceanospirillaceae and unclassified Flavobacteriaceae in MT_H and a single genus related to S085 of the phylum Chloroflexi in YT_H (Fig.1). The observation of *Colwellia* and *Moritella* in samples collected from the Mariana Trench is consistent with previous reports, yet their proportions are considerably lower in microbial communities associated with natural deep-sea sediments (Tarn et al., 2016; Peoples et al., 2018). One possible explanation is that the microbial community structures in the enrichments altered during long incubation at 50 MPa without supplementation of nutrient and air exchanges (Peoples et al., 2019). Enrichments at atmospheric pressure conditions (MT_L and YT_L) were dominated by *Halomonas* and *Sulfitobacter*, respectively. In addition, *Psychrobacter*, *Idiomarina*, *Pseudomonas*, and *Marinobacter* were identified in both enrichments, accounting for from 5% to 25% of the communities (Fig.1).

With supplementation of TMAO, the dominant groups in MT_HT were *Moritella* (47.1%) and *Shewanella* (34.9%), while *Psychrobium* was the only taxa accounting for over 10% of the community in sample from the Yap Trench (YT_HT) (Fig.1). Apart from that, a few groups accounting for over 1% of the community were observed under this condition, including *Colwellia*, *Psychromonas*, unclassified group of Rhodobacteraceae and group MBAE14 in MT_HT and *Colwellia*, and *Oleispira* in YT_HT.

Consistent with alpha diversity analysis, the community structure of enrichments at 0.1 MPa with presence of TMAO was rather simple; the genus *Methylophaga* took up 84% and 94% of the communities of MT_LT and YT_LT, respectively. Five taxa of *Halomonas*, *Thalassospira*, *Idiomarina*, *Pseudomonas*, and *Marinobacter* together took up 14% of the communities in MT_LT while *Sulfitobacter* and *Idiomarina* together accounted for approximately 3.5% in YT_LT (Fig.1).

3.2 The influence of pressure on bacterial community

Alterations in community structure caused by different pressures have been observed repeatedly. For example, the abundance of microbes in costal sample decreased when incubated under HHP condition, meanwhile the diversity increased as the dominant Epsilonproteobacteria were replaced by Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Flavobacteria (Marietou and Bartlett, 2014). Likewise, the community structure and microbial activity of deep-sea sample differed when incubated at atmospheric pressure and in situ pressure conditions (Wannicke et al., 2015; Marietou et al., 2018).

In our study, the enrichments under 50 MPa and 0.1 MPa had distinct community structures. The dominant groups enriched at 50 MPa composed mainly of *Colwellia*, *Moritella* and *Psychrobium*, while *Halomonas*, *Psychrobacter*, *Idiomarina* and

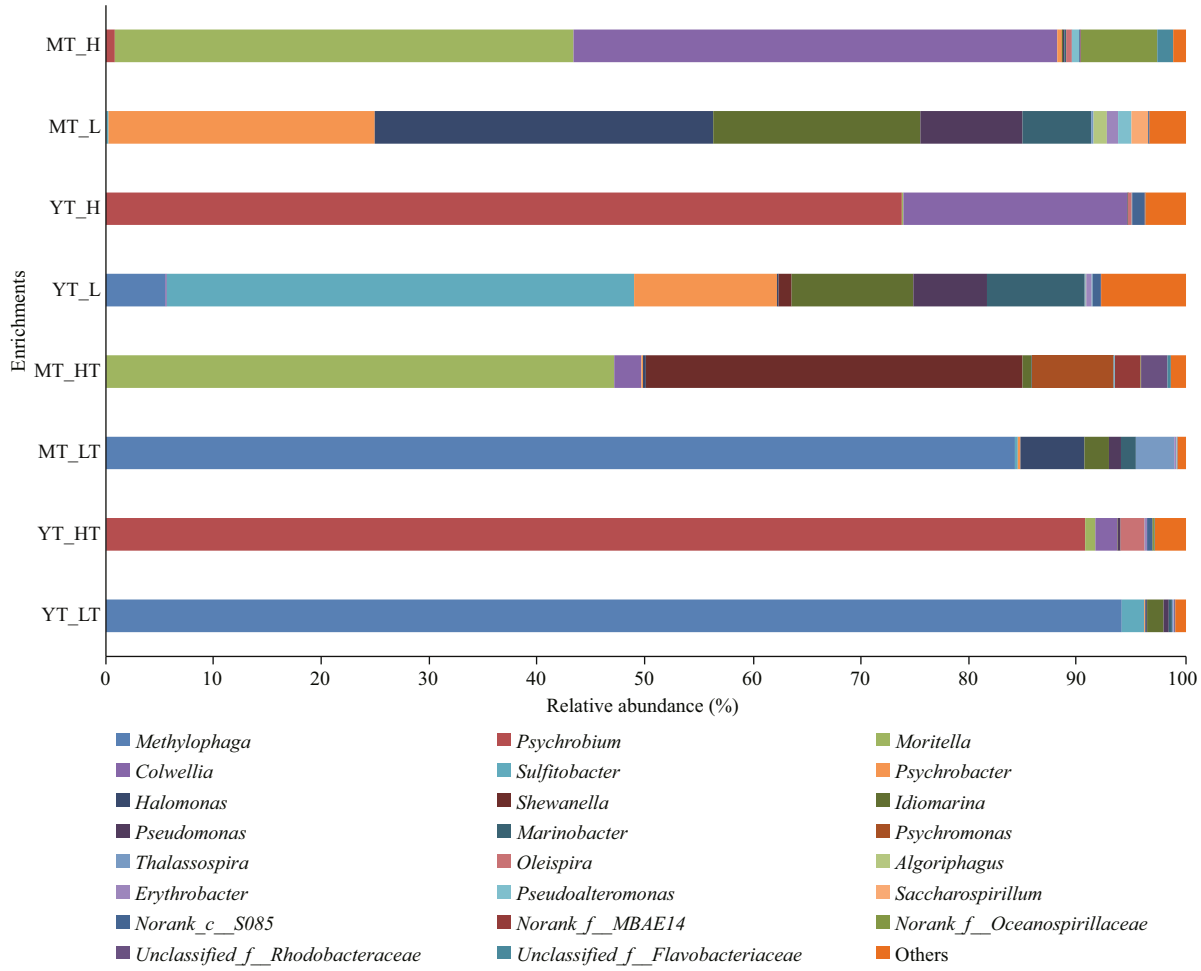

Fig.1 Relative percentage of bacterial taxa in different enrichments

The 16S rRNA gene amplicons were pyrosequenced and classified by comparing with the SILVA 128 database at genus levels. Minor group represents the sum of all genera with a proportion of less than 1% for all samples.

Pseudomonas were enriched at 0.1 MPa (Fig.1). Numerous deep-sea isolates affiliated to *Colwellia* and *Moritella* exhibited psychrophilic and piezophilic life-style, such as *Colwellia* sp. MT41, *C. marinimaniae* MTCD1 and *M. yayanosii* DB21MT-5 isolated from the Mariana trench, whose optimal growth pressure were 70 MPa, 120 MPa and 80 MPa, respectively (Yayanos et al., 1981; Kato et al., 1998; Kusube et al., 2017). The genus *Psychrobium* which is closely related to *Shewanella* was first proposed in 2014, and the type strain *P. conchae* BJ-1 isolated from gill tissue of mussel from deep-sea hydrothermal field is a psychrophilic bacterium (Nogi et al., 2014). Although the pressure tolerance of strains affiliated to this genus remains unknown, it is highly possible that the cells enriched at 50 MPa were also adapted to low temperature and HHP conditions.

On the contrary, the taxa dominant in the enrichments at 0.1 MPa were mostly bacteria ubiquitous in marine environment (Tamegai et al., 1997; Gaboyer et al., 2014; Tarn et al., 2016). Although certain isolates of these taxa seemed acclimated to HHP (Kaye and Baross, 2004; Follonier et al., 2013; Gaboyer et al., 2014), the groups detected in our experiments are probably those adapted to atmospheric pressure condition as they were mainly identified in enrichments at 0.1 MPa, and further investigation of these groups will be needed to better understand their physiological characteristics.

3.3 The influence of TMAO on bacterial community

Our previous study demonstrated that pressure tolerance of certain deep-sea bacteria can be improved by the presence of TMAO (Yin et al., 2018), which possibly bring these species more competitiveness in deep-sea environment. In this study, the influence of TMAO on bacterial community under different pressure conditions was examined. Under HHP

condition, the proportion of *Shewanella* and *Psychrobium* raised at the expense of *Colwellia* in MT_HT and YT_HT, respectively (Fig.1). Addition of TMAO also led to the increase of numerous minor taxa, such as *Halomonas*, *Idiomarina*, *Psychromonas* and clade MBAE14 in sample from the Mariana Trench (MT_HT) and *Pseudomonas*, *Oleispira*, *Erythrobacter* and *Alcanivorax* in sample collected from the Yap Trench (YT_HT). It is notable that samples from the two trenches responded differently to TMAO under HHP condition. In contrast, both communities enriched with addition of TMAO at 0.1 MPa (MT_LT and YT_LT) were dominated by *Methylophaga*. Therefore, it appears that supplementation of TMAO imposed distinct influence on bacterial community under different pressure conditions, and the changes of community composition caused by supplementation of TMAO at 0.1 MPa (diverse groups replaced by sole taxa of *Methylophaga*) was greater compared to that at 50 MPa (*Moritella* and *Psychrobium* remained dominant group).

The taxa enriched with supplementation of TMAO were presumed capable of utilizing TMAO for growth. To test this hypothesis, we analyzed the occurrence of gene markers of TMAO metabolism in the representative genomes available on NCBI. The BLASTN analyses were performed using TorA (catalytic subunit of TMAO reductase) from *Shewanella oneidensis* MR-1 (NP_716855) and Tdm (TMAO demethylase) from *Ruegeria pomeroyi* DSS-3 (AAV94849) as query sequences. We identified homologues of TorA in the sequenced genomes of *Moritella*, *Psychromonas*, *Oleiospira*, and *Shewanella*, and Tdm in *Idiomarina*, *Methylophaga* and *Pseudomonas*, while *Halomonas* and *Thalassospira* were shown encoding both enzymes, suggesting that strains from these genera are capable of TMAO metabolism (Fig.2). It is possibly the reason why their abundance increased in the presence of TMAO. It should be noted that the genomic survey was performed on the genus level, that is, orthologs of TorA and Tdm were searched in multiple reference genomes from each genus. The result is considered as positive if ortholog has been identified in at least one genome. Considering the possible differences between species and strains, the reference genomes may not authentically reflect the physiological characteristic of cells enriched in our study, thus analysis of *torA* and *tdm* by pyrosequencing or metagenomic sequencing will be needed for better understanding of bacterial TMAO metabolism in deep-sea environment in the future.

Despite of the fact that numerous taxa were found encoding genes involved in TMAO metabolism, both enrichments at 0.1 MPa (MT_LT and YT_LT) were dominated by *Methylophaga*. It has been reported that when methanol and/or TMAO were present, obligate methylotroph, especially *Methylophaga*, were enriched in the seawater (Dinasquet et al., 2018). In addition, strains belong to this genus were also known capable of utilizing diverse C1-compounds including MMA, DMA and TMA for growth (Doronina et al., 2003; Kim et al., 2007; Boden 2012; Villeneuve et al., 2013). Therefore, not only the TMAO supplemented but also metabolites of TMAO produced by other microorganisms are potential substrate of *Methylophaga* and further promote its growth over other species, especially during the long-time incubation under oligotrophic condition.

The influence of TMAO on bacterial community under high-pressure condition was not as significant as under ambient pressure condition, yet the abundance of certain genera increased by the presence of TMAO. Analysis of functional genes indicated that all these taxa are capable to utilize TMAO for growth, which might be the reason of their enrichment. Yet, we cannot exclude the possibility that TMAO facilitated their growth as a piezolyte. It has been reported that TMAO could maintain function of protein under high-pressure condition, possibly by binding to the surface of a protein and stabilizing its structure (Yancey et al., 2001; Petrov et al., 2012). TMAO could easily reach the extracellular fraction and get into periplasmic fraction by diffusion, it is thus reasonable to assume that proteins located there can benefit from the piezolyte in all organisms. However, according to current knowledge, TMAO can only be transported into cytoplasm in certain species encoding specific transporter (Raymond et al., 2002; Li et al., 2015) and protect cytoplamsic proteins from the adverse effect of HHP, and these organisms might eventually exhibit higher pressure tolerance.

3.4 The determinant factor of bacterial community in enrichments under different conditions

A hierarchical clustering tree was constructed using OTU-based metrics. It demonstrated that enrichments were clustered in two main groups correlated with incubation pressures (Fig.3). The group 1 contained enrichments at 0.1 MPa and group 2 comprised enrichments at 50 MPa. It suggested that

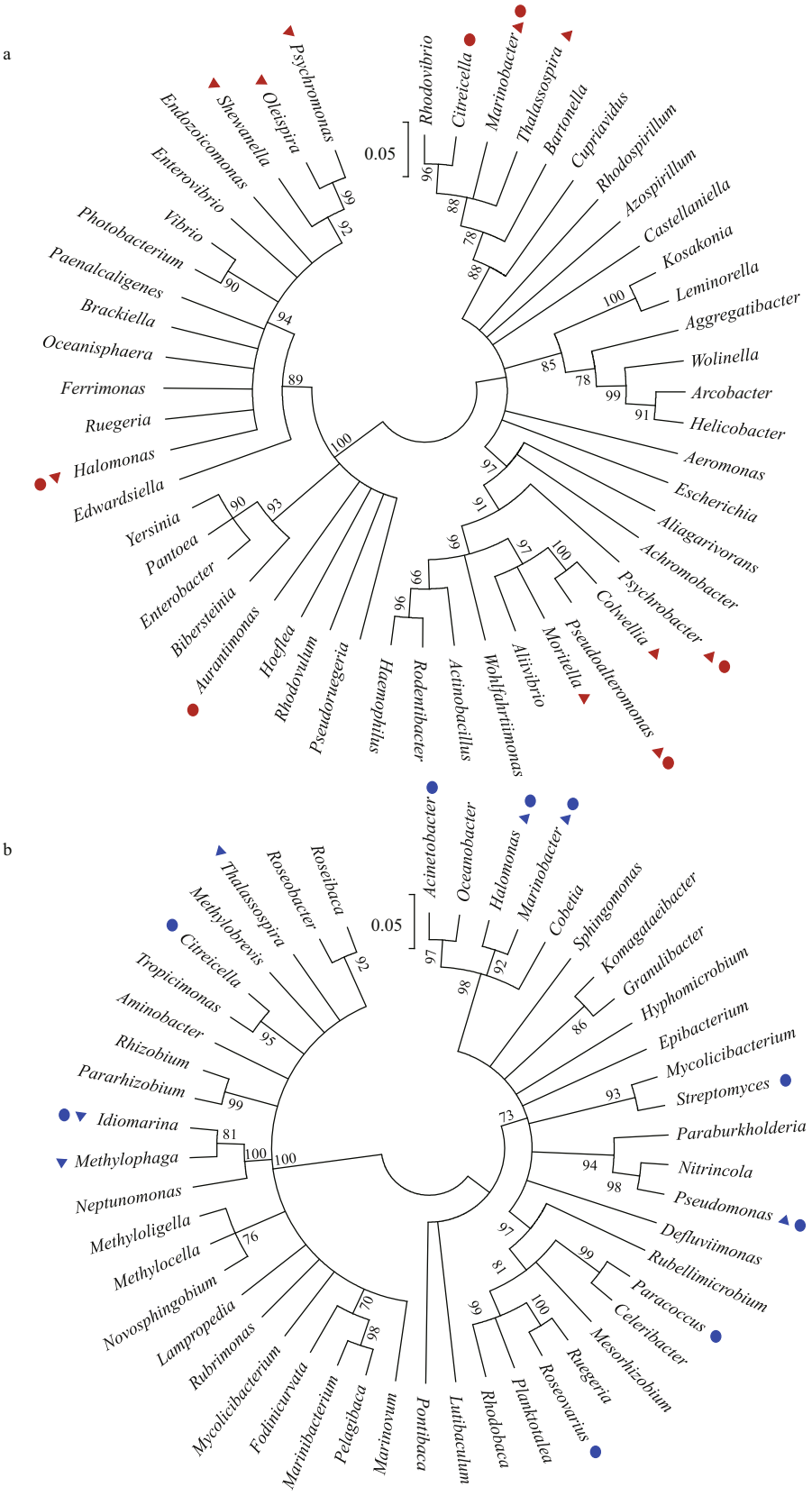

Fig.2 Phylogenetic analysis of catalytic subunit of TMAO reductase (TorA) and TMAO demethylase (Tdm)

The phylogenetic tree of TorA (a) and Tdm (b) was constructed using 50 TorA sequences and 48 Tdm sequences. Bootstrap values (1 000 replicates) greater than 70% are shown. The triangles represent genera enriched by supplementation of TMAO in the enrichment study, and the circles indicate genera of culturable bacteria enriched by TMAO.

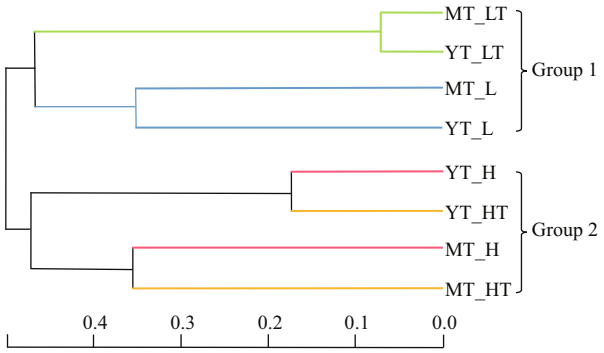

Fig.3 Clustering analysis tree of the bacterial community structure of enrichments under different conditions

The red lines represent enrichments under HHP condition without TMAO, blue lines represent enrichments under atmospheric condition without TMAO, yellow lines represent enrichments under HHP condition with addition of TMAO, green lines represent enrichments under atmospheric condition with addition of TMAO.

pressure had greater influences on bacterial community structure over other factors such as the site of sampling and the composition of media. Remarkably, enrichments under the same condition (MT_L and YT_L / MT_LT and YT_LT) were grouped together within group 1, while the two lineages in group 2 each consisted of sample from the same sampling site (MT_H and MT_HT / YT_H and YT_HT). It indicated that the determinants of bacterial community were distinct under different pressure conditions. The addition of TMAO was the dominant factor under atmospheric pressure condition while the community structure was possibly determined by its original composition under high-pressure condition.

It is well known that almost every aspect of bacterial cells, from the composition of molecules to cellular structure and complicated cellular process, are influenced by alteration of pressure (Bartlett, 2002). Compared with shallow water species, deep-sea microorganisms adapted to HHP conditions have distinct amino acid composition, ratio of polyunsaturated fatty acids, gene expression profile and metabolic pathways (Vezzi et al., 2005; Chikuma et al., 2007; Campanaro et al., 2008; Oger and Jebbar, 2010). Likewise, lowered pressure is stressful to those dwell in the deep-sea environment, whose energy generation, protein production and cellular activity decreased under atmospheric pressure condition (Wannicke et al., 2015; Peoples et al., 2018). Differences in tolerance to HHP can thus be reflected in the changing of community structure under different pressure conditions, as those with higher pressure tolerance were selected against other microbes under HHP condition and vice versa.

As indicated by genomic analysis, the dominant groups enriched at 50 MPa (*Moritella* and *Colwellia*) were capable of TMAO metabolism, thus addition of TMAO simply provided them an alternative substrate but hardly modified the community composition under HHP condition. On the contrary, merely one-tenth cells in trench samples are active under atmospheric pressure condition (Peoples et al., 2018). When incubated at 0.1 MPa, the activity of piezotolerant and piezophilic microbes decreased and these populations were eventually replaced by species better adapted to lower pressure conditions. Therefore, the nutrient available, such as TMAO in this experiment, might play a significant role during the reconstruction of the community. The observation that most genera enriched by supplementation of TMAO encoded *torA* and/or *tdm* perfectly supported this hypothesis.

3.5 The culturable bacteria isolated under different conditions

To further ascertain the physiological characteristic of microbes enriched under different conditions, sediment samples were incubated under different conditions as in the enrichment assay before plated on agar plates of corresponding medium for bacterial isolation. Four samples collected at the Mariana Trench and 3 samples at the Yap Trench were involved in this experiment (Table 1). Phylogenetic analyses revealed that the 238 strains isolated belonged to 36 species of 20 genera, 4 phyla (Fig.4). Over 80% of the isolates were affiliated to Proteobacteria (206 isolates), which was dominated by Gammaproteobacteria (180 isolates, 87.4% of Proteobacteria) and the remaining belonged to Alphaproteobacteria (26 isolates, 12.6% of Proteobacteria). In addition, 3 isolates were affiliated to Actinobacteria, four isolates were Bacteroidetes and 25 isolates were Firmicutes.

In general, strains isolated from the Yap Trench demonstrated higher biodiversity. In total 126 strains affiliated to 11 genera of 2 phyla were isolated from Mariana Trench, while 112 strains from 16 genera of 4 phyla were isolated from samples collected at the Yap Trench (Fig.5). Isolates related to the genera of *Halomonas*, *Pesudoaltermonas*, and *Psychrobacter* were obtained from both trenches. It is consistent with the work by L. M. Peoples et al., in which isolates of *Pseudoalteromonas*, *Pseudomonas*, *Halomonas*, and *Shewanella* were obtained from the sediment of the Kermadec and the Mariana Trench

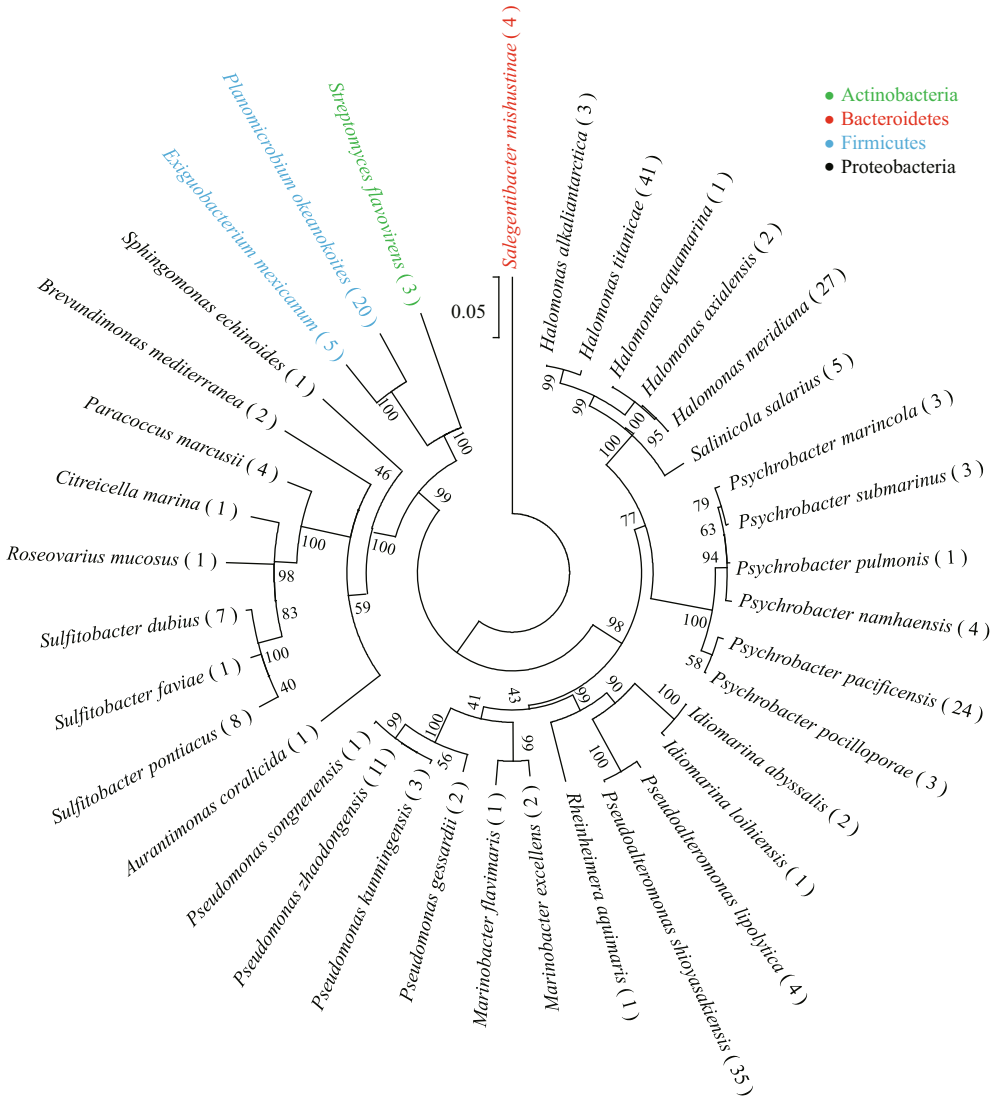

Fig.4 Phylogenetic tree of bacteria isolated from the Mariana Trench and the Yap Trench in this study

The phylogenetic tree was constructed based on 16S rRNA gene sequences of isolated obtained from the Mariana Trench and the Yap Trench. Numbers on the nodes represent bootstrap values based on 1 000 times replicates and values over 40% were presented. Green characters represent strains of Actinobacteria, red ones are from Bacteroidetes, blue ones are from Firmicutes and black characters represent strains of Proteobacteria. Number in braces indicates number of isolates obtained in this study.

(Peoples et al., 2019). In addition, three genera of *Idiomarina*, *Rheinheimera* and *Sphingomonas* and eight genera of *Marinobacter*, *Brevundimonas*, *Aurantimonas*, *Citreicella*, *Paracoccus*, *Roseovarius*, *Salegentibacter*, and *Streptomyces* were isolated only from the Mariana Trench and Yap Trench, respectively. Therefore, consistent with the result of bacterial community analysis of enrichment, the composition of culturable microbes also suggested distinct habitant in the two trenches. However, we cannot exclude the existence of these groups in natural environment although they have not been isolated in this study, due to the limited number of the experimental conditions and isolates analyzed.

Comparison between samples pre-incubated under 0.1 MPa and 50 MPa revealed that the abundance and diversity of isolates were greatly reduced after treatment of HHP. In total 153 strains affiliated to 17 genera were isolated in samples incubated at 0.1 MPa, whereas only 85 strains affiliated to 10 genera were obtained in samples incubated at 50 MPa (Table 3). Considering the isolation was performed by plating under atmospheric pressure condition, the growth of piezo-tolerant and piezophilic cells that might have been enriched during pre-incubation at 50 MPa could be restrained and thus reduced the number and type of isolates obtained. This could also be one of the reasons why no strain of dominant genera in the enrichments

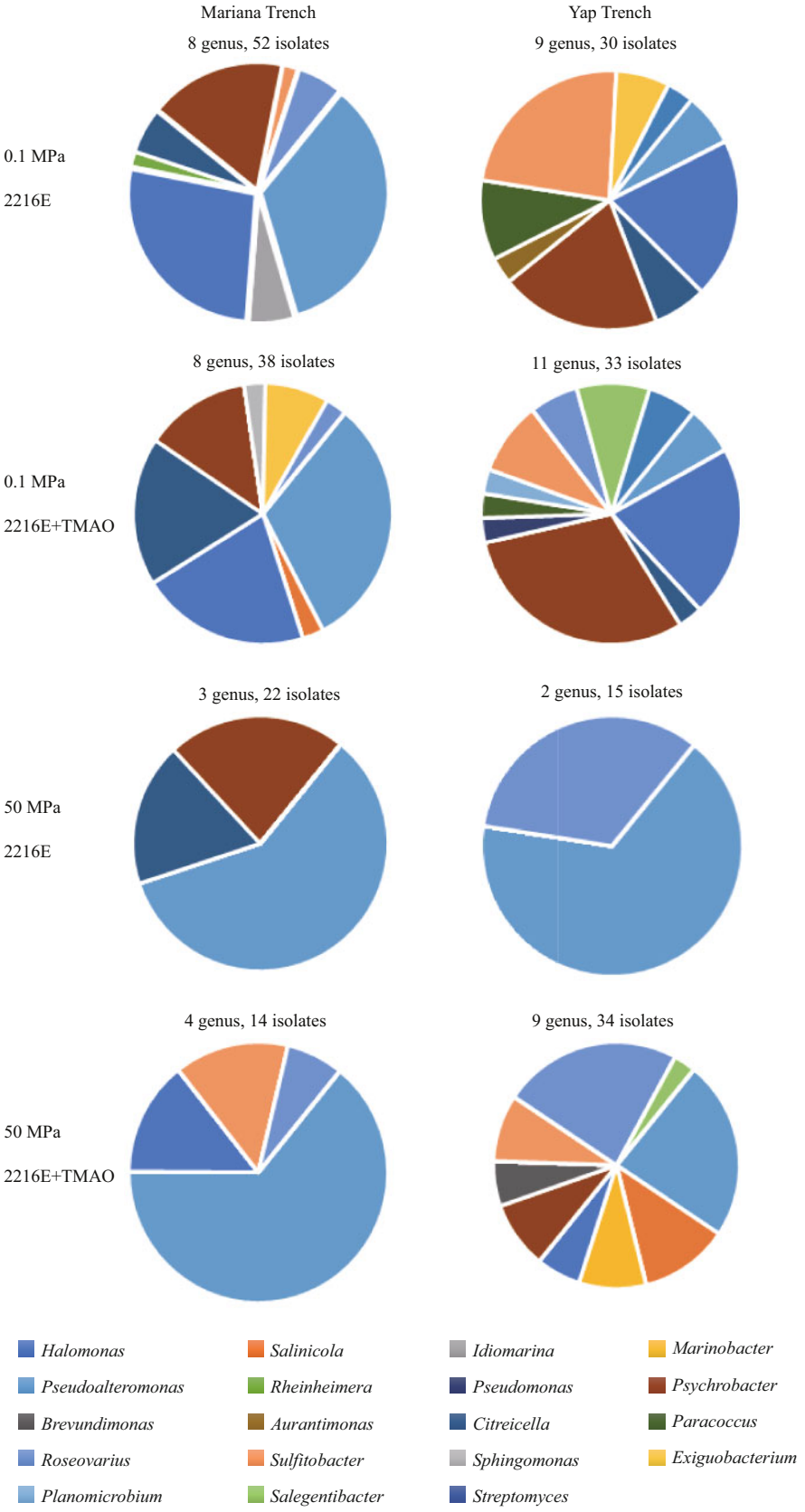

Fig.5 The composition of strains isolated after pre-incubation under different conditions

The origin of samples utilized for bacterial isolation is listed on top of each column and the conditions of pre-incubation were stated on the left. The number of genus and species of strains isolated under each condition is listed above the pie chart.

Table 3 Number of strains isolated from samples after different treatments

Genus	Condition of pre-incubation			
	50 MPa		0.1 MPa	
	2216E	2216E+TMAO	2216E	2216E+TMAO
Halomonas	23	17	20	14
Planomicrobium	5	9	3	3
Psychrobacter	5	3	15	15
Pseudomonas	4	-	5	8
Pseudoalteromonas	-	4	20	15
Sulfitobacter	-	5	8	3
Salegentibacter	-	1	-	3
Salinicola	-	4	-	1
Paracoccus	-	-	3	1
Exiguobacterium	-	-	2	3
Streptomyces	-	-	1	2
Marinobacter	-	3	-	-
Brevundimonas	-	2	-	-
Aurantimonas	-	-	1	-
Idiomarina	-	-	3	-
Rheinheimera	-	-	1	-
Citreicella	-	-	-	1
Roseovarius	-	-	-	1
Sphingomonas	-	-	-	1
Total	37	48	82	71

at 50 MPa (*Colwellia*, *Moritella*, *Shewanella* and *Psychrobium*) has been isolated. Strains from three genera (*Halomonas*, *Planomicrobium* and *Psychrobacter*) have been obtained after treatment under all the four conditions. Among them, *Planomicrobium* is the only genus of which more strains have been isolated from samples incubated at 50 MPa, suggesting strains from this genus were more tolerant to high-pressure condition comparing to others.

It appears that addition of TMAO had little influence on the abundance of isolates (71 vs 82 in samples pre-incubated under 0.1 MPa and 48 vs 37 in samples pre-incubated under 50 MPa). However, the presence of TMAO obviously affected the diversity of isolates obtained. Strains of certain genera were isolated only when TMAO was supplemented, such as *Marinobacter* and *Brevundimonas* (isolated only in samples incubated under 50 MPa), *Citreicella*, *Roseovarius*, and *Sphingomonas* (only in samples incubated under 0.1 MPa), and *Salinicola* and *Salegentibacter* that were isolated under both pressure conditions. However, caution should be taken when drawing any conclusions as only limited number of isolates has been obtained and the result may not be statistically significant. Among the genera enriched by TMAO, *Marinobacter*, *Citreicella*, *Roseovarius*, and *Sphingomonas* were possibly capable of metabolizing TMAO as genes encoding Tdm and/or TorA have been identified in their genomes (Fig.2), whereas no clues indicated that *Brevundimonas*, *Salinicola*, and *Salegentibacter* could utilize TMAO directly. Cross feeding among different species might be one possible explanation for their presence only after treatment with supplementation of TMAO.

4 CONCLUSION

Having analyzed bacterial community structure of enrichments under different conditions in order to understand the influence of TMAO on deep-sea microorganisms, we found that pressure was the primary factor in shaping bacterial community, whereas the impact of nutrient such as TMAO varied under different pressure conditions. Presence of TMAO imposed greater influence on bacterial community structure under atmospheric pressure condition while the sampling site had a greater effect under high-pressure condition. In addition, bacterial isolates were shown pressure-dependent effect of TMAO. Collectively, we for the first time described the distinct influence of TMAO on deep-sea bacteria under different pressure conditions, which enriched the knowledge of physiological characteristic of deep-sea bacteria.

5 DATA AVAILABILITY STATEMENT

The 16S rRNA genes of bacterial isolates obtained and the raw seuqncing data in the study were submitted to NCBI sequence Research Archive under accession number MH725320 to MH725557and SRP157419.

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