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ORIGINAL ARTICLE

Bacterial diversity associated with a newly described bioeroding sponge, *Cliona thomasi*, from the coral reefs on the West Coast of India

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Abstract

The bacterial diversity associated with eroding sponges belonging to the *Cliona viridis* species complex is scarcely known. *Cliona thomasi* described from the West Coast of India is a new introduction to the *viridis* species complex. In this study, we determined the bacterial diversity associated with *C. thomasi* using next-generation sequencing. The results revealed the dominance of Proteobacteria followed by Cyanobacteria, Actinobacteria and Firmicutes. Among Proteobacteria, the Alphaproteobacteria were found to be the most dominant class. Furthermore, at the genus level, *Rhodothalassium* were highly abundant followed by *Endozoicomonas* in sponge samples. The beta-diversity and species richness measures showed remarkably lower diversity in *Cliona thomasi* than the ambient environment. The determined lower bacterial diversity in *C. thomasi* than the environmental samples, thus, categorized it as a low microbial abundance (LMA). Functional annotation of the *C. thomasi*–associated bacterial community indicates their possible role in photo-autotrophy, aerobic nitrification, coupling of sulphate reduction and sulphide oxidization. The present study unveils the bacterial diversity in bioeroding *C. thomasi*, which is a crucial step to determine the functions of the sponge holobiont in coral reef ecosystem.

Introduction

Marine sponges (phylum Porifera), one of the oldest multicellular animals (Metazoa), are an essential component of aquatic benthic communities (Wulff 2001; de Goeij et al. 2013). Sponges are also important members of coastal food webs and biogeochemical cycles and play a vital role in marine habitats by providing a significant number of important ecosystem services such as the establishment of a 3-dimensional structure that generates habitat for other organisms, water purification and nutrient cycling (Bell 2008). Interestingly, sponges are known to harbour the highest number of prokaryotic symbionts along with other invertebrates (Webster and Thomas 2016). Furthermore, the prokaryotic sponge symbionts were found to be stable, to a certain extent, at different physico-chemical environmental conditions such as variability in nutrient composition (Luter et al. 2014), temperature (Simister et al. 2012; Pita et al. 2013), pH (Ribes et al. 2012), light (Cárdenas et al. 2014) and at geographical (Steinert et al. 2016; Thomas et al. 2016) and temporal scales (Erwin et al. 2015). Such sponge-prokaryotic symbiotic association has recently been suggested to play roles in maintaining the fitness of sponges over other competitors in the same niche (Kiran et al. 2018; Pita et al. 2018). Sponges have also been considered as a reservoir of pharmacologically and biotechnologically important microbes (Villegas-Plazas et al. 2019). Growing evidence suggests that sponge microbial symbionts are the main producers of several documented sponge-derived bioactive compounds rather than the host itself (Pita et al. 2018). A number of sponge-holobiont publications reported microbial metabolic contributions to the host sponge, e.g. heterotrophy and autotrophy, and elemental recycling, along with the production of bioactive compounds

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(Webster and Thomas 2016; Bourne et al. 2016; Slaby et al. 2017).

Coral bioeroding sponges are the least studied for their microbiome association and functions. However, the role of sponge-holobiont in space competition and coral destruction is not clear. A few studies have shown that the sponges of *Cliona viridis* complex are associated with Symbiodiniaceae, which exert thermotolerance to them over their space competitor neighbouring corals (Ramsby et al. 2017; Achlatis et al. 2019). As a result, the invasion of sponge increased over the coral affected by the sudden increase in temperature, for instance, bleaching events (Carballo et al. 2013). The only reports available for microbial diversity analyses in *Cliona viridis* complex included *C. viridis* (Schmidt, 1862) from the Mediterranean (Blanquer et al. 2013; Soares 2016), *C. varians* (Duchassaing & Michelotti, 1864) from the Florida keys (Sacristán-Soriano et al. 2020) and *C. orientalis* Thiele, 1900 from the Great Barrier Reef (Pineda et al. 2016, 2017; Ramsby et al. 2018b). However, the prokaryotic associates of these bioeroding sponges were neglected for such sponge-holobiont interactions. Therefore, it is crucial to define the sponges and their associates as a holobiont unit rather than only the sponge as an autonomous entity (Roughgarden et al. 2017; Pita et al. 2018). Before gaining insights into the physiological plasticity induced in the host by a change in associated microbiome, it is necessary to investigate the communitity structure of this microbiome. Thus, the aim of the present study is to determine the bacterial community associated with coral excavating sponge *Cliona thomasi* Mote, Schönberg, Samaai, Gupta & Ingole, 2019. The same study also described *C. thomasi* as an aggressive benthic competitor in coral reef, which overgrows at a faster rate over the live coral colonies and outcompete live corals (Mote et al. 2019). This species belongs to the *C. viridis* species complex, which is considered to be among the most dominant and destructive macroborers on coral reefs (Schönberg et al. 2017). *C. thomasi* has been observed as a fast-growing, coral-infesting species with high ecological significance.

Cliona thomasi has also been listed as one of the WORMS top ten new marine species of 2019 (http://lifewatch.be/en/worms-top10-2018), thereby highlighting its high ecological importance. Interestingly, *C. thomasi* has shown an increase in its prevalence over coral after a mass bleaching event in the year 2015–2016. Our subsequent continuous monitoring of the site showed an increased abundance of *C. thomasi*. This observed increase in *C. thomasi*, at first instance, may be attributed to an increased SST induced coral bleaching providing favourable ground for the sponge to proliferate. The sea surface temperature (SST) data from the NOAA Coral Reef Watch (NOAA-CRW) platform revealed that the SST of the sponge habitat had exceeded the thermal bleaching threshold in the summer months (April–June) in the years 2015, 2016, 2017, 2018 and 2019 (Supplementary Fig. 1). During the monitoring of this site, an anomalous increase in SST was observed during summer months. In order to gain insights into the host associate–derived physiological benefits, this study defines the bacterial diversity associated with *C. thomasi* as a first step investigation.

Materials and methods

Sample collection

Samples of the bioeroding sponge, *C. thomasi* growing on colonies of the hard coral *Turbinaria mesenterina* (Lamarck, 1816), were collected from the Malvan Marine Sanctuary (16° 3′ 46.76″ N, 73° 27′ 17.18″ E) located on the central West Coast of India. Corals in the Malvan Marine Sanctuary are under stress due to elevated temperature-induced bleaching events (De et al. 2015), coral diseases (Hussain et al. 2016), sedimentation and physical damages from unregulated tourism (De et al. 2020). The samples were collected from a depth of 5–7 m, and care was taken while collecting the samples that each sample was distinct and separated from others by at least 5 m. The growth of *C. thomasi* over the coral species *T. mesenterina* is shown in Fig. 1. The sponge samples were collected in replicates of four. To compare the community with the ambient environment, surrounding seawater samples ($n = 3$) were collected from the same depth 1 m away from the sponge. Samples were immediately brought on board, fixed in liquid nitrogen and transported to the laboratory for further processing.

DNA extraction and library preparation

The collected samples were homogenized in liquid nitrogen and processed for DNA extraction using a tissue DNA

Fig. 1 In-situ observation of sponge *Cliona thomasi* growing over *Turbinaria mesenterina* in Malvan Marine Sanctuary

extraction kit according to the manufacturer's protocol (Invitrogen, CA, USA). About 2 L of seawater was filtered using a 0.22-μm polycarbonate membrane filter (Whatman ®), and residue that remained on filter paper was used for DNA isolation. For bacterial community analysis, amplification of the 16S rRNA V3-V4 region was performed (Muyzer et al. 1993; Li et al. 2009). Illumina MiSeq 16S rRNA amplicon libraries were generated following a standard protocol (New England Biolabs, Frankfurt, Germany). The libraries were validated using 2100 Bioanalyzer (Agilent Technologies) for quality, and samples were sequenced using the 2 × 300 paired-end chemistry (MiSeq Reagent Kit).

Sequence assembly and prokaryotic community analysis

Illumina Miseq platform generated raw 16S rRNA amplicon reads for all the samples were processed for quality filtering and adaptor removal using FastQC V0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). After trimming, V3–V4 region sequences were assembled using the FLASH program 1.2.11 with a minimum overlap cutoff of 10 bp and maximum overlap cutoff of 240 bp. By default mismatches were allowed (default is 0) and as a result produced an average contig length 350–450 bp. The chimera sequences were then removed using the de novo based UCHIME method implemented in the USEARCH tool version 11.0.667. Assembled reads were subjected to QIIME version 2.0 pipeline for downstream bioinformatics analysis (Bolyen et al. 2019). First, assembled reads were clustered using UCLUST (Edgar, 2010) and operational taxonomic units (OTUs) were picked at a 97% similarity. The singleton OTUs (read abundance < 2) were discarded from the analysis (Caporaso et al. 2010). The most abundant reads for the remaining OTUs were selected as representatives and then mapped against rRNA database SILVA release (132 version using PyNAST) (Caporaso et al. 2010). Taxonomy for each mapped OTUs was assigned using the RDP classifier using a threshold value of 0.8 (Wang et al. 2007). The OTUs that did not match to any reference taxonomy were considered as unknown. We used METAGENassist (Arndt et al. 2012) for assessing putative functional analysis of sponge bacterial community.

Statistical analysis

The diversity indices such as species richness and Shannon index were determined for all the investigated samples in R, package vegan v.2.5-6 (Oksanen 2017). The rarefaction curves for the samples were derived from the genus summary using the same package. The significant differences among the diversity indices values were determined by the Kruskal-Wallis test (with Dunn's post hoc test) in Past version 3. The differences in the beta-diversity of the bacterial community composition between the sponge and environmental samples were tested using PERMANOVA with the Bray Curtis test followed by 999 permutations in PRIMER v7 (Clarke and Gorley 2015). Similarity percentage (SIMPER) analysis was performed to calculate the contribution of each bacterial community to the dissimilarity within and between samples in the PRIMER v7 (Clarke and Gorley 2015). Bacterial community composition across samples was ordinated using non-metric multidimensional scaling (nMDS) and ANOSIM performed in R, package vegan v.2.5-6 (Oksanen 2017).

Results

Prokaryotic diversity associated with *C. thomasi*

In this study, high-throughput 16S rRNA gene (V3 and V4 regions) sequencing analysis was performed for the newly defined coral-eroding sponge *C. thomasi* to assess its associated bacterial diversity. The 16SrRNA analysis determined 3,17,333–5,51,000 quality reads assigned to 2531–9581 sponge OTUs while 3,64,284–4,79,774 reads were assigned to 18,672–34,773 environmental OTUs (Supplementary Table 1). The rarefaction curve showed high sequencing coverage and higher taxonomic assignments to the environmental samples than sponge samples (Supplementary Fig. 2). Alpha diversity indices obtained from the analysis showed higher diversity and richness in environment samples than the sponge samples. Shannon's diversity index determined for the sponge and environment samples were 1.79 ± 0.12 and 3.16 ± 0.99, respectively (Supplementary Table 1). The determined diversity indices were found to be significantly different for sponge and environment samples based on the Kruskal-Wallis test, followed by Dunn's posthoc test ($p < 0.05$).

The beta-diversity composition of sponge samples was found to be significantly different from the environment samples (PERMANOVA; $p = 0.02$, at permutation N: 999). The significant difference in the bacterial composition between sponge and environment samples were further confirmed by ANOSIM (Global $R = 0.66$, $p < 0.023$). The multivariate clustering from nMDS also confirmed the variation in the bacterial community composition among sponge and the surrounding environment. The clustering of samples based on bacterial taxonomic abundance variation is reflected in nMDS as well as in heatmap (Supplementary Figs. 3 and 4). SIMPER analysis determined the key bacterial genera that contributed to the differences in the sponge and environmental samples. The bacterial genera which contributed to 75.65% dissimilarity between the sponge and environmental samples were *Rhodothalassium*, *Thalassomonas*, *Neptuniibacter*, *Vibrio*, *Endozoicomonas*, *Lutibacter* and *Synechococcus* (Supplementary Table 2).

A total of 23 phyla were found to be associated with the sponge species *C. thomasi* while 33 phyla in environmental samples (ES). At the phylum level, all the samples included in this study were found to be dominated by Proteobacteria (sponge: 64.7.9 ± 4.90%, ES: 66.30 ± 20.0%), followed by Cyanobacteria (sponge: 12.69 ± 10.69%, ES: 1.20 ± 1.16%), Actinobacteria (sponge: 4.85 ± 3.94, ES: 3.07 ± 2.09%) and Bacteroidetes (sponge: 1.79 ± 3.94, ES: 14.53 ± 9.05%) (Fig. 2). Cyanobacteria was found significantly higher in sponge while Bacteroidetes in environmental samples (p value < 0.05).

Within the Proteobacteria at class level, sponge samples were found to be dominated by Alphaproteobacteria (43.73 ± 11.11%) followed by Deltaproteobacteria (09.64 ± 6.89%), Gammaproteobacteria (2.20 ± 1.10%) and Betaproteobacteria (1.10 ± 0.01%) (Fig. 3a). The environmental samples, however, were found to be dominated with Gammaproteobacteria (50.33 ± 26.81%) followed by Alphaproteobacteria (8.05 ± 3.83%), Deltaproteobacteria (4.48 ± 3.21%) and Betaproteobacteria (0.06 ± 0.02%) (Fig. 3b). The significantly higher abundance of bacterial genera (p value < 0.05) found in sponge samples were *Rhodothalassium* (sponge: 24.17 ± 8.96%, ES: 0.007 ± 0.003%), *Synechococcus* (sponge: 5.82 ± 4.11%, ES: 0.24 ± 0.34%), *Endozoicomonas* (2.08 ± 1.47%, ES: 0.02 ± 0.01%), *Prochlorococcus* (sponge: 0.07 ± 0.11%, ES: 0.07 ± 0.01%), *Shewanella* (sponge: 0.55 ± 0.30%, ES: 0.02 ± 0.01%) and *Corynebacterium* (sponge: 0.10 ± 0.05%, ES: 0.01 ± 0.01%) (Fig. 4). The sponge-specific bacterial genera were dominated by *Methylobacterium*, *Brevibacterium*, *Cupriavidus* and *Massilia*, while the environmental samples were dominated by *Vibrio*, *Neptuniibacter*, *Alteromonas*, *Thalassomonas*, *Photobacterium* and *Lutibacter* (Fig. 4). Both the sponge and environmental samples showed significantly higher unidentified bacterial communities-58% and 62%, respectively. The unknown bacterial diversity was excluded from the study while analysing the data.

Functional annotation

This study further determined the taxonomic based predictive functions of sponge-specific prokaryotic communities using the METAGENassist web portal (Supplementary Fig. 5). The sponge-associated bacterial population–based predicted metabolic processes mainly include the breakdown of complex organic molecules such as xylan, chitin and chlorophenol degraders, and sugar fermenters. Metabolism functions revealed that ammonia oxidizers, nitrite reducers, nitrogen fixers, sulphate reducers and sulphate oxidizers were enriched in the sponge. Other processes identified in sponge were Chlorophenol degradation, Dehalogenation, Lignin degradation and Naphthalene degradation.

Discussion

The present study describes the bacterial diversity associated with bioeroding sponge *C. thomasi*, belonging to the family Clionaidae. This species was known to harbour photosymbiont dinoflagellate Zooxanthallae (Mote et al. 2019), which may provide essential nutrients to the host as it does to other *C. viridis* complex species (Hill et al. 2011).

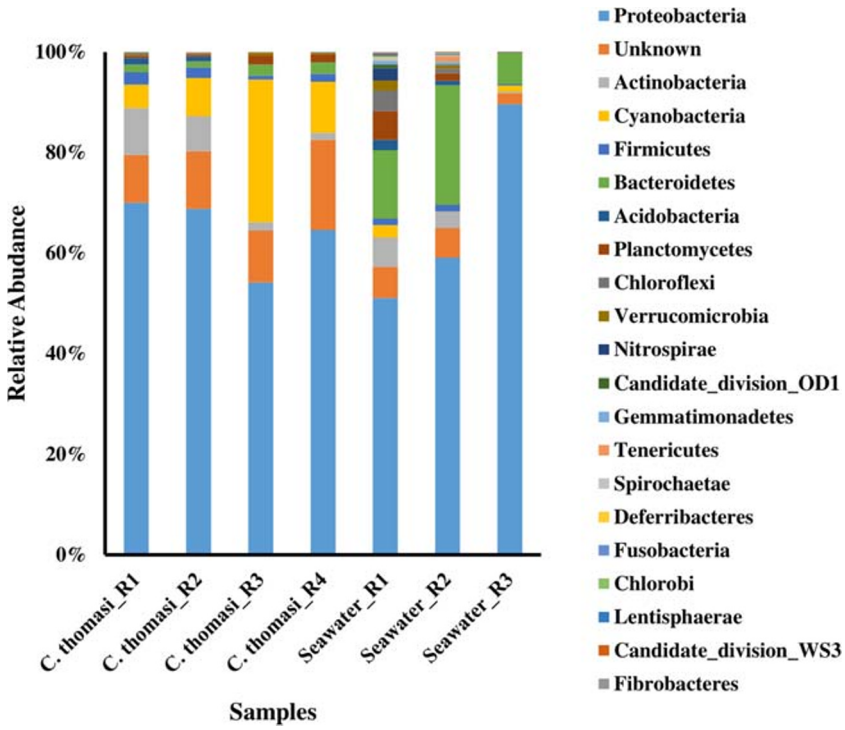

Fig. 2 Relative abundance of prokaryotic communities associated with the sponge *Cliona thomasi* and environmental samples at the phylum level

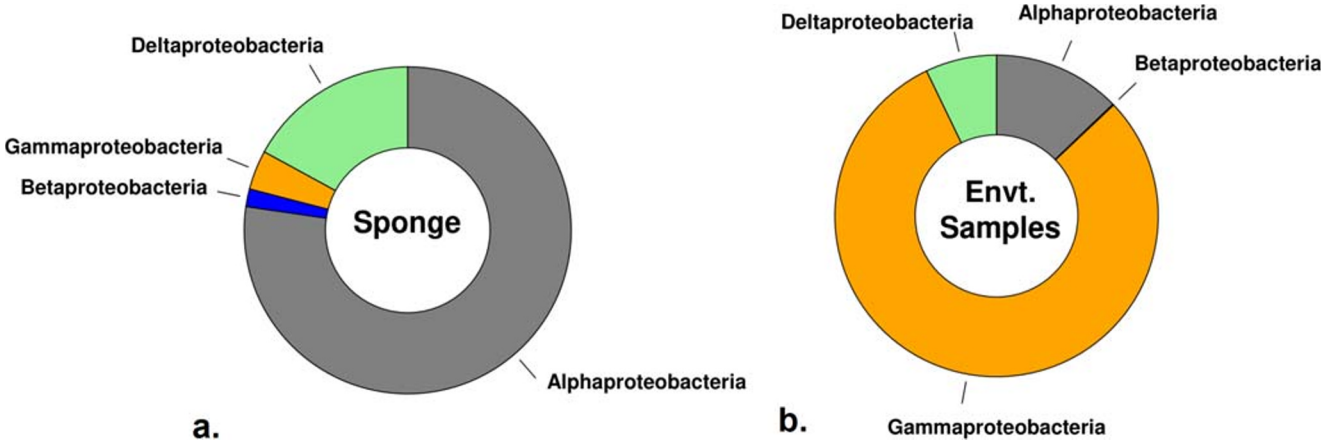

Fig. 3 **a**, **b** Relative abundance variations within Proteobacteria associated with the sponge and environmental samples

Determining the bacterial diversity associated with *C. thomasi* in this study is the first step towards developing a baseline microbial diversity structure of *C. thomasi*.

Similar to earlier reports, *C. thomasi* investigated in this study was found to be dominated by Proteobacteria, together with the significant contributions by Cyanobacteria and Actinobacteria and frequent occurrence of Firmicutes, Bacteroidetes and Planctomycetes (Thomas et al. 2016). In the present study, *C. thomasi* showed the dominance of Alphaproteobacteria similar to the previous reports of sponge species from *C. viridis* complex and other Symbiodiniaceae-bearing sponges (Supplementary Table 3) (Blanquer et al. 2013; Soares 2016; Pineda et al. 2016, 2017; Ramsby et al. 2018b; Sacristán-Soriano et al. 2020). However, azooxanthellate sponges (without Symbiodiniaceae association) such as *C. deltrix* Pang, 1973 and *C. celata* Grant, 1826, were reported for the predominance of Gammaprotoeobacterial communities (Jeong et al. 2015; Thomas et al. 2016; Sacristán-Soriano et al. 2020).

The identification of cyanobacteria as sponge-specific photosynthetic associate of *C. thomasi* corroborates with the earlier reports of finding the Cyanobacteria (*Synechococcus*) as sponge-specific cluster and absent in seawater (Simister et al. 2012). The association of cyanobacteria in sponges benefits the host in various ways. For instance, compounds isolated from the cyanobacterial symbionts of the sponge *Lamellodysidea herbacea* deterred fish feeding (Sawhney and Mishra 2019; Schorn et al. 2019). Also, increased cyanobacteria abundance was shown to be an opportunistic proliferation in bleached *C. orientalis*, fulfilling the energy requirement of the host (Ramsby et al. 2018a). Morrow et al. (2015) showed the higher prevalence of *Synechococcus* in two different sponge species colonized in the acidified environment of a CO_2 seep. Photosynthetic symbionts not only act

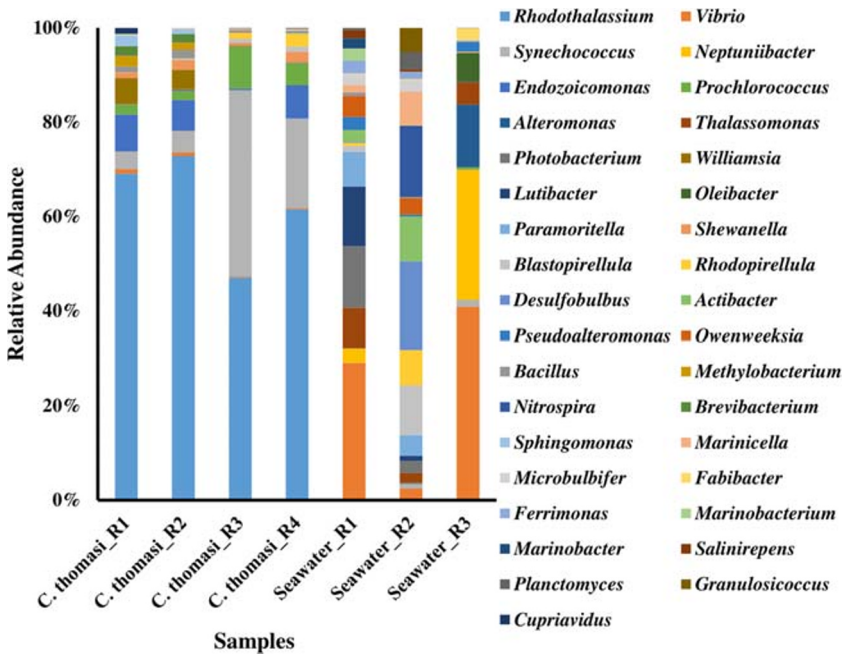

Fig. 4 Relative abundance of prokaryotic communities associated with the sponge *Cliona thomasi* and environmental samples at the genus level

as the energy source to the sponge but also provide photoprotective effects against the intermittent high light exposure to sponge (Steindler et al. 2002) and also known to produce cytotoxic secondary metabolites (Teruya et al. 2004; Matthew et al. 2010). In addition to photosynthetic symbionts, the other sponge-enriched microbes include *Endozoicomonas*, *Williamsia*, *Shewanella* and *Vibrio*. *Endozoicomonas* is a common bacterial genus widely reported from different sponges (Nishijima et al. 2013), a wide range of coral species and several other marine organisms (Jensen et al. 2010; Esteves et al. 2013). *Endozoicomonas* are known for multiple functions in sponges such as nutrient acquisition, structuring the sponge microbiome via signalling molecules or in host health stability (Nishijima et al. 2013; Gardères et al. 2015). The actinomycetes that belonged to the genera *Williamsia* are known for bioactive natural product synthesis in sponges (Audia et al. 2017). *Shewanella* and *Vibrio* are frequently encountered as sponge-associated microbes (Hentschel et al. 2012; Esteves et al. 2013).

The beta-diversity measures in this study showed a significant difference among bacterial diversity associated with the investigated sponge and environment samples (Supplementary Fig. 4). Recently, Steinert et al. (2019) also showed that the sponge's prokaryotic community was significantly different from the surrounding water. Higher species richness and Shannon diversity indices for the environment samples attribute to their higher diversity than the sponge. The results highlighted less complexity of bacterial community in *C. thomasi* than its surrounding environment suggesting it as a low microbial abundance (LMA) sponge (Gloeckner et al. 2014). These results corroborate well with previous reports on other closely related *Cliona* species such as *C. orientalis*, *C. varians* and *C. viridis* (Blanquer et al. 2013; Poppell et al. 2014; Soares 2016; Pineda et al. 2016; Thomas et al. 2016; Sacristán-Soriano et al. 2020). The LMA characteristic of *C. thomasi* indicates a unique metabolic feature of higher availability of carbon in sponge choanosome than N and P through higher photosynthetic activity (Thacker and Freeman 2012). This fact is further supported by the identification of higher autotrophy through specifically determined *Synechococcus* together with sulphur metabolism–related bacteria in *C. thomasi*.

The sponge-enriched specific bacterial community is supportive of sponge holobiont with their distinct functions. The functions of sponge holobiont may broadly be classified into two categories (1) metabolism and (2) defence (Pita et al. 2018). The present study predicts the function of heterotrophic metabolism in *C. thomasi* through the degradation of complex polysaccharides such as xylan and chitin by the sponge-specific bacterial community. Heterotrophic carbon metabolism through hydrolysis of complex polysaccharide has widely been reported from the different sponge-associated bacterial community from different regions (Slaby et al. 2017). Heterotrophically metabolizing community association is further supported by the fact that the microbes are also dependent upon the sponge biomass itself or the components of the sponge extracellular matrix (Slaby et al. 2017; Tout et al. 2017). The enrichment of N metabolism–related functions identified in this study highlights the putative role of *C. thomasi*–associated microbes in the Nitrogen cycle, which is one of the limiting nutrients in the marine environment (Kiran et al. 2018; Pita et al. 2018). For instance, *Rhodothalassium* may play a role in nitrogen cycling in sponge (Parfrey et al. 2018; Ramsby et al. 2018b). Sponges are known to produce an excess of ammonia as a waste by-product, which is toxic to their photosymbionts if not being assimilated (Hentschel et al. 2012). The higher prevalence of functions related to ammonia oxidization in this study suggested the ammonia metabolism through either aerobic (nitrification, nitrogen fixation) or anaerobic (denitrification, anammox) manner (Hoffmann et al. 2009; Fiore et al. 2015; Ribes et al. 2015). Since nitrogen is the limiting factor for primary productivity in the marine ecosystem, an adaptation of microbial community capable of enriching N is essential in sponges for maintaining the symbiosis (Fan et al. 2012; Bayer et al. 2014; Moitinho-Silva et al. 2014). The determination of the higher abundance of nitrifying function in this study compared to denitrifying suggest that the sponge excrete nitrogen mainly in the form of ammonia (Southwell et al. 2008; Ribes et al. 2012; Morganti et al. 2017). The higher relative percentage of the function corresponding to nitrification further indicates that the *C. thomasi* holobiont act as a net source of bioavailable nitrogen (Fiore et al. 2013).

The other dominant function determined in *C. thomasi* based specific bacterial community was related to sulphur metabolism, mainly the sulphide oxidizer and sulphate reducer. Similar, endosymbiotic sulphur cycle through the association of sulphate-reducing and sulphate-oxidizing symbionts has been described in a marine oligochaete worm (Dubiller et al. 2001) and the cold water sponge *Geodoa barrette* (Hoffmann et al. 2003). These functions are mainly attributed to the Alphaproteobacteria and Gammaproteobacteria community (Jensen et al. 2017). The present study also determined the higher abundance of *Rhodothalassium* (Alphaproteobacteria) and *Endozoicomonas* (Gammaproteobacteria) community in *C. thomasi*. The coupling of sulphide oxidizer and sulphate reducer supports detoxification of sulphide produced by the metabolic activity of sponge or other associates under oxic or anoxic condition (Hoffmann et al. 2003). In the presence of oxygen, the sulphide is oxidized to sulphate by sulphide oxidizer. Nevertheless, under anoxic conditions in sponge tissue, the detoxification of sulphide was performed by sulphide reducers but necessitated the biologically available ferrous ions (Le Pennec et al. 2003; Orcutt et al. 2011). The presence of higher abundance of *Endozoicomonas* in Symbiodiniaceae-associated sponge *C. thomasi* indicates that it may play role in

the sulphur cycle metabolizing of dimethylsulfoniopropionate (DMSP) to form dimethylsulphate (DMS) and dimethyl sulfoxide (DMSO) (Gardner et al. 2016; Osman et al. 2020). These molecules have potential roles in osmoregulation and antioxidant capacity of the host (Gardner et al. 2016).

In conclusion, the present study determined microbial community structure associated with a newly discovered coral-eroding sponge species. The results revealed that the sponges had microbial signature different from its surrounding environment. The microbial specificity associated with the *C. thomasi* highlights their putative roles benefiting the sponge holobiont. Investigating the sponge-holobiont is essential to realize a holistic regulatory, physiological and functional attributes to understand the space competitive mechanisms of sponge in the reef ecosystem.

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Data availability Accession numbers of nucleotide sequences submitted to NCBI are coded as follows: SRA submission SUB7245986, submission ID PRJNA623169 and Bio project PRJN623169.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed by the authors.

Sampling and field studies All necessary permits for sampling and field observation have been obtained by the authors from the competent authorities.

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