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SHORT COMMUNICATION

Coral reef invertebrate microbiomes correlate with the presence of photosymbionts

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Coral reefs provide habitat for an array of marine invertebrates that host symbiotic microbiomes. Photosynthetic symbionts including *Symbiodinium* dinoflagellates and diatoms potentially influence the diversity of their host-associated microbiomes by releasing carbon-containing photosynthates and other organic compounds that fuel microbial metabolism. Here we used 16S ribosomal RNA (rRNA) gene amplicon pyrosequencing to characterise the microbiomes of 11 common Great Barrier Reef marine invertebrate species that host photosynthetic symbionts and five taxa in which they are absent. The presence of photosynthetic symbionts influenced the composition but not the species richness, evenness and phylogenetic diversity of invertebrate-associated microbiomes. Invertebrates without photosynthetic symbionts were dominated by *Alphaproteobacteria,* whereas those hosting photosynthetic symbionts were dominated by *Gammaproteobacteria*. Interestingly, many microbial species from photosymbiont-bearing invertebrates, including *Oceanospirillales* spp., *Alteromonas* spp., *Pseudomonas* spp., *Halomonas* spp., are implicated in the metabolism of dimethylsulfoniopropionate (DMSP). DMSP is produced in high concentrations by photosynthetic dinoflagellates and is involved in climate regulation by facilitating cloud formation. Microbiomes correlated with host taxa and replicate individuals from most sampled species grouped in distance-based redundancy analysis of retrieved 16S rRNA gene sequences. This study highlights the complex nature of invertebrate holobionts and confirms the importance of photosynthetic symbionts in structuring marine invertebrate bacterial communities.
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Coral reefs harbour abundant and diverse marine invertebrates that perform important ecosystem functions such as: calcification, bioerosion, consolidation and benthic-pelagic coupling (Glynn and Enochs, 2011). Animal–plant/microbe symbioses are vital to these ecosystems as they facilitate photosynthetic productivity, mineral recycling, nutrient provision to the host and secondary metabolite production (Smith and Douglas, 1987). Although patterns of microbial diversity and putative symbiotic functions have been well explored in corals and sponges (Sunagawa *et al.*, 2009; Mouchka *et al.*, 2010; Webster and Taylor, 2012; Bourne and Webster, 2013), there is a lack of data on microbial associations in other reef taxa including Bivalves, Foraminifera and Ascidians.

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The diversity of microbial communities associated with corals and sponges is known to be influenced by host interactions (Wegley *et al.*, 2007; Kimes *et al.*, 2010; Raina *et al.*, 2010; Fan *et al.*, 2012), the production of antimicrobial compounds (Ritchie, 2006; Shnit-Orland and Kushmaro, 2009) and environmental conditions (Hong *et al.*, 2009; Ceh *et al.*, 2011). Recent studies, however, indicate that other members of the coral holobiont (in particular *Symbiodinium* dinoflagellates) also influence microbial community structure through release of complex carbon-containing exudates including dimethylsulfoniopropionate (DMSP; Ikeda and Miyachi, 1995; Raina *et al.*, 2009, 2010). DMSP can be degraded to dimethylysulphide, a central molecule in the global sulphur cycle, which diffuses from the ocean into the atmosphere where it influences cloud formation, with consequences for atmospheric chemistry, local climate and water temperature (Ayers and Gras, 1991; Andreae and Crutzen, 1997). A complex array of other organic exudates including amino acids and polysaccharides can also

influence invertebrate-associated microbiomes, which may affect holobiont fitness. For example, *Symbiodinium* spp. have been shown to influence the response of bacterial communities to thermal stress, which affects the susceptibility of the holobiont to bleaching (van Oppen *et al.*, 2009; Stat *et al.*, 2012), disease (Stat *et al.*, 2008) and colonisation by opportunistic potential pathogens (Littman *et al.*, 2010).

In this study, we used 16S ribosomal RNA (rRNA) gene amplicon pyrosequencing (Supplementary Methods) to characterise the microbiomes of 16 common Great Barrier Reef marine invertebrate species representing five invertebrate families (Table 1). These families included 11 species that host photosynthetic symbionts (*Symbiodinium* and diatoms) and five species that do not host these symbionts. The microbiomes for three replicate samples from each invertebrate species and seawater controls were characterised. Briefly, 16S rRNA gene amplicons generated using primers 63F and 533R (Engelbrektson *et al.*, 2010) were subjected to 454 pyrosequencing. Sequences were checked for chimeras using UCHIME ver. 3.0.617 (Edgar *et al.*, 2011), denoised using Acacia (Bragg *et al.*, 2012) and then parsed using the QIIME pipeline with default settings (Caporaso *et al.*, 2010). We tested the hypotheses that: (1) the presence of photosynthetic symbionts influences the diversity of marine invertebrate-associated microbiomes, and (2) that the diversity of marine invertebrate-associated microbiomes differs between host species.

Table 1 List of samples, phylogentic classification, associated pyrosequence reads and symbiont type

Sample/species name	*Taxa/group*	*No. of raw reads*	*No. of cleaned reads*	*% Removed*	*Symbiont*	*Symbiont type*
Acropora millipora #1	Scleractinea	9016	8043	10.8	Yes	*Symbiodinium*
Acropora millipora #2	Scleractinea	6618	6221	6.0	Yes	*Symbiodinium*
Acropora millipora #3	Scleractinea	15 986	13 909	13.0	Yes	*Symbiodinium*
Pocillopora damicornis #1	Scleractinea	10 168	9254	9.0	Yes	*Symbiodinium*
Pocillopora damicornis #2	Scleractinea	14 733	13 636	7.4	Yes	*Symbiodinium*
Pocillopora damicornis #3	Scleractinea	12 701	11 380	10.4	Yes	*Symbiodinium*
Seriatopora hystrix #1	Scleractinea	12 302	11 758	4.4	Yes	*Symbiodinium*
Seriatopora hystrix #2	Scleractinea	12 839	11 857	7.6	Yes	*Symbiodinium*
Seriatopora hystrix #3	Scleractinea	11 151	10 500	5.8	Yes	*Symbiodinium*
Nephtea sp. #3	Octocorallia	10 596	9505	10.3	Yes	*Symbiodinium*
Sarcophyton sp. #1	Octocorallia	18 070	16 505	8.7	Yes	*Symbiodinium*
Sarcophyton sp. #2	Octocorallia	10 750	9680	10.0	Yes	*Symbiodinium*
Sarcophyton sp. #3	Octocorallia	14 179	11 761	17.1	Yes	*Symbiodinium*
Sinularia flexibilis #1	Octocorallia	12 068	11 595	3.9	Yes	*Symbiodinium*
Sinularia flexibilis #2	Octocorallia	12 231	10 947	10.5	Yes	*Symbiodinium*
Sinularia flexibilis #3	Octocorallia	11 358	10 522	7.4	Yes	*Symbiodinium*
Tridacna cf. crocea #1	Bivalvia	12 910	11 523	10.7	Yes	*Symbiodinium*
Tridacna cf. crocea #2	Bivalvia	4516	4015	11.1	Yes	*Symbiodinium*
Tridacna cf. crocea #3	Bivalvia	3931	3495	11.1	Yes	*Symbiodinium*
Tridacna cf. maxima #1	Bivalvia	13 246	10 453	21.1	Yes	*Symbiodinium*
Tridacna cf. maxima #2	Bivalvia	12 597	10 579	16.0	Yes	*Symbiodinium*
Tridacna cf. maxima #3	Bivalvia	8403	6945	17.4	Yes	*Symbiodinium*
Heterostegina depressa #1	Foraminifera	13 868	12 056	13.1	Yes	Diatom
Heterostegina depressa #2	Foraminifera	14 328	12 860	10.2	Yes	Diatom
Heterostegina depressa #3	Foraminifera	15 993	14 126	11.7	Yes	Diatom
Marginopora vertebralis #1	Foraminifera	10 917	9626	11.8	Yes	*Symbiodinium*
Marginopora vertebralis #2	Foraminifera	16 713	15 239	8.8	Yes	*Symbiodinium*
Marginopora vertebralis #3	Foraminifera	28 568	25 942	9.2	Yes	*Symbiodinium*
Sorites sp. #1	Foraminifera	5467	4855	11.2	Yes	*Symbiodinium*
Sorites sp. #2	Foraminifera	7361	6439	12.5	Yes	*Symbiodinium*
Sorites sp. #3	Foraminifera	15 979	14 292	10.6	Yes	*Symbiodinium*
Bryozoan sp. #1	Bryozoa	14 520	12 493	14.0	No	NA
Bryozoan sp. #2	Bryozoa	11 004	9488	13.8	No	NA
Bryozoan sp. #3	Bryozoa	16 807	13 562	19.3	No	NA
Diademnum molle #1	Ascidiacaea	24 826	23 150	6.8	No	NA
Diademnum molle #2	Ascidiacaea	12 348	8590	30.4	No	NA
Diademnum molle #3	Ascidiacaea	14 772	11 266	23.7	No	NA
Lissoclinum patella #1	Ascidiacaea	13 130	11 493	12.5	No	NA
Lissoclinum patella #2	Ascidiacaea	15 511	13 994	9.8	No	NA
Lissoclinum patella #3	Ascidiacaea	13 700	12 621	7.9	No	NA
Polycarpa aurata #1	Ascidiacaea	15 176	14 203	6.4	No	NA
Polycarpa aurata #2	Ascidiacaea	13 526	12 576	7.0	No	NA
Polycarpa aurata #3	Ascidiacaea	10 968	9925	9.5	No	NA
Rhopaloiedes odorabile	Porifera	16 382	11 999	26.8	No	NA
Seawater #1	Seawater	56 633	45 068	20.4	No	NA
Seawater #2	Seawater	19 560	15 614	20.2	No	NA

Abbreviation: NA, not applicable.

The presence of photosynthetic symbionts influenced the composition (Figure 1), but not the species richness, evenness and phylogenetic diversity ($P>0.05$, linear regression; Supplementary Table S1) of invertebrate-associated microbiomes. At the class level, the presence of photosynthetic symbionts explained 21% of variation in the composition of microbial communities between samples (PERMANOVA, $F_{1,44}=11.37$, $P<0.001$). At the level of operational taxonomic units (OTUs), defined as groups of sequences that shared 97% nucleotide sequence similarity ('species' level), the presence of photosynthetic symbionts explained a significant, albeit smaller (6%) proportion of variation in the composition of microbial communities between samples (PERMANOVA, $F_{1,44}=2.66$, $P<0.001$). Unifrac analysis based on OTUs also confirmed that the presence of photosynthetic symbionts influenced microbial community composition (unweighted $P=0.002$, weighted $P<0.001$).

Alphaproteobacteria and *Gammaproteobacteria* were the dominant classes of bacteria associated with reef invertebrates. Invertebrates without photosynthetic symbionts (with the exception of one replicate *Bryozoan* sp.) were associated with a larger abundance of *Alphaproteobacteria*, whereas those with photosynthetic symbionts generally hosted a higher relative abundance of *Gammaproteobacteria* (Supplementary Figure S1). The only exceptions were *Seriatopora hysterix* and two of the *Sinularia* sp. samples in which *Flavobacteria* were particularly abundant. Other bacterial classes including the *Deltaproteobacteria, Sphingobacteria* and *Cyanobacteria* differed between invertebrate species but were not influenced by the presence of photosymbionts (Supplementary Figure S1). Although present in all samples, the *Cyanobacteria* were particularly abundant (8–24% relative abundance) in *Heterostegina depressa* and in one *Marginopora vertebralis* sample (17% relative abundance). The composition of microbial communities associated with the sponge *Rhopaloeides odorabile* was different to those associated with other invertebrates, although this community pattern is consistent with a previous investigation (Webster *et al*., 2010).

Most dominant OTUs (that is, >5% relative abundance) were affiliated with bacterial populations previously retrieved from marine environments including corals and sponges (Figure 2). Invertebrates that host photosynthetic symbionts were positively correlated with OTUs related to *Oceanospirillales* spp., a *Roseivirga* sp., an *Alteromonas* sp., *Pseudoalteromonas* spp., *Halomonas* spp., *Pseudomonas* spp. and *Flavobacteriacae* spp. (Figure 1). Indicator species analysis (Dufrene and Legendre, 1997) confirmed these OTUs were significantly correlated with the presence of photosynthetic symbionts by having high relative abundance and frequency of occurrence (Figure 2). These OTUs are all affiliated with species implicated in the metabolism of complex organic molecules such as DMSP and dimethylsulphide. For example, previous studies have identified abundant bacteria within the *Oceanospirillales* that are able to metabolise DMSP in the coral *Acropora millepora* (Raina *et al*., 2009). *Halomonas* spp. have been shown to be capable of metabolism of DMSP and its breakdown product acrylic acid (Todd *et al*., 2010),

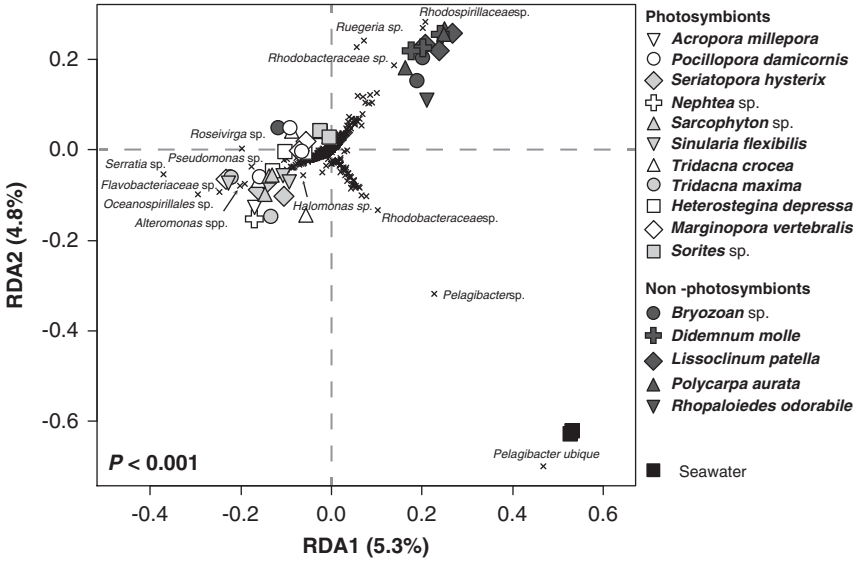

Figure 1 Redundancy analysis (RDA) summarising variation in the composition of marine invertebrate-associated microbial communities that was attributable to the presence–absence of photosymbionts. The filled shapes represent individual samples collected from each invertebrate species. The black crosses represent bacterial OTUs. For clarity, taxonomic affiliations are shown for the most discriminating OTUs only. The distance of an object (sample or OTU) from the origin is proportional to its variance along an axis and its angle relative to the axes reflects its correlation with those axes. Full sample collection and processing details can be found in the Supplementary Methods and the sequence data set deposited in the NCBI Sequence Read Archive (SRA) database with the accession number SRA4494953.

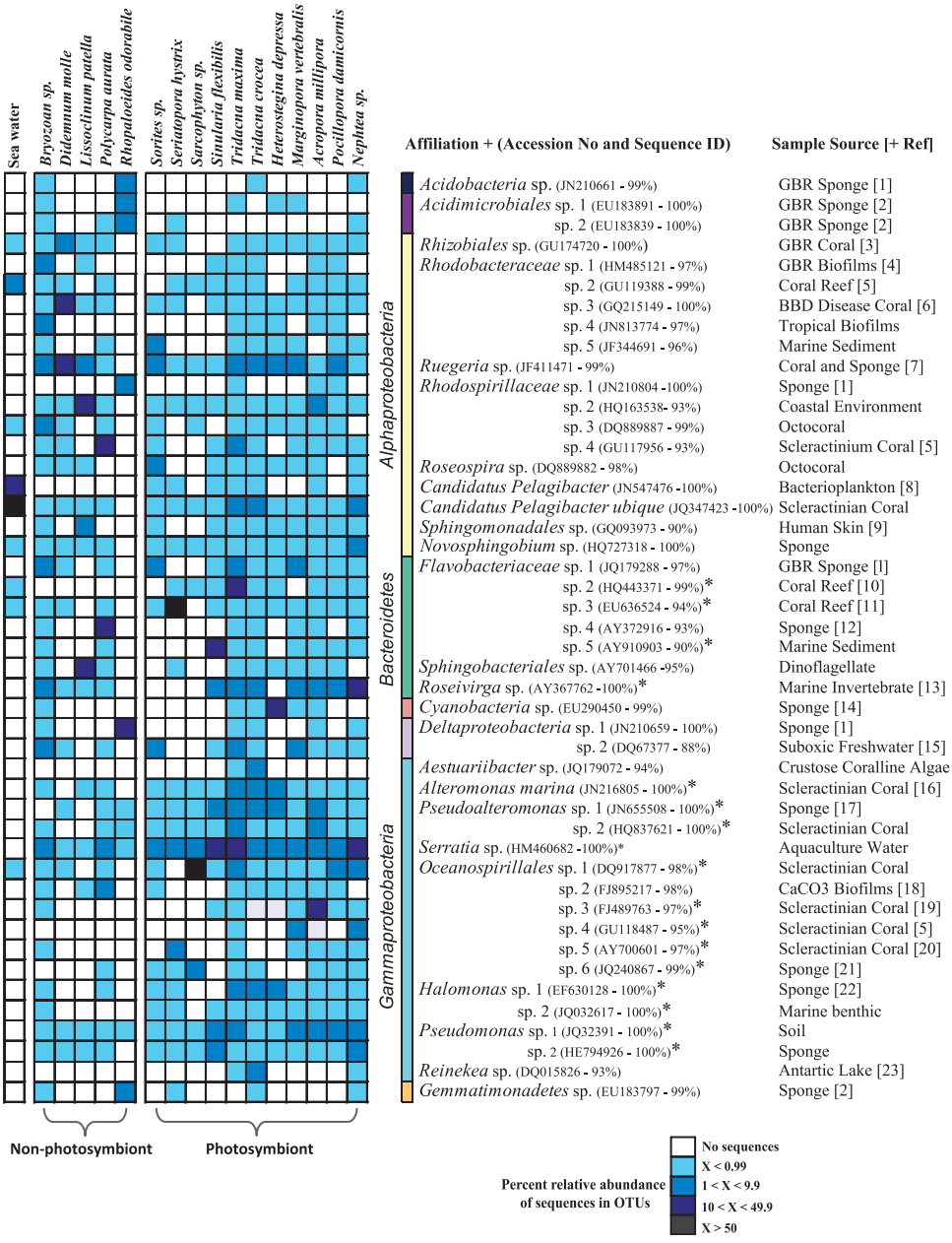

Figure 2 Heatmap of OTUs (97% sequence identity, averaged within each invertebrate species) that represent >5% of sequence tags within a particular sample type or represented an OTU with a significant indicator value (representative of high relative abundance and high relative frequency of occurrence in photosymbiont-bearing invertebrates). OTUs with highest indicator values are represented by an asterisk (*). The closest sequence match determined in a BLAST database query (Altschul *et al.*, 1997) and its corresponding accession number and derived sample source are also represented. If these affiliated sequences are represented in a published study the associated reference can be found in Supplementary References.

while members of the *Flavobacteriacae* respond rapidly to high DMSP concentrations in phytoplankton blooms, although the genetic pathways for metabolism of this compound in this group of bacteria is unknown (Howard *et al.*, 2011). In the marine environment, DMSP has been the focus of considerable attention because of its fundamental role as carbon and sulphur sources for bacteria (Sievert *et al.*, 2007). Coral reefs are one of the largest producers of DMSP with the source thought to be derived from marine invertebrates harbouring symbiotic dinoflagellates (Broadbent *et al.*, 2002; Van Alstyne *et al.*, 2006). In fact, the concentrations of DMSP and its breakdown products dimethylsulphide and acrylate in reef-building corals are the highest recorded in the marine environment (Broadbent and Jones, 2004). These results further support the concept that sulphur-based organic compounds derived from photosymbionts influence the microbial communities of marine invertebrates by providing nutrient sources readily available for metabolism by associated microbiomes. Although

compounds such as DMSP appear to have a role in structuring microbial communities associated with the host organism, there are likely to be many other organic exudates derived from photosymbionts that also influence microbial associations. In addition, host animal factors can have an important role in structuring microbial communities. Results from this study and other recent reports highlight that members of the *Oceanospirillales*, specifically, *Endozoicomonas* spp. are commonly found in marine invertebrates with and without photosymbionts and potentially have important functional roles within their host species (Yang *et al*., 2010; Nishijima *et al*., 2012; Speck and Donachie, 2012).

Indicator species analysis demonstrated that no OTUs were significantly correlated with invertebrates that do not host photosymbionts, although *Rugeria*-, *Rhodobacteraceae*- and *Rhodospirillaceae*-related sequences were more commonly retrieved in these samples as observed in the redundancy analysis (Figure 1). Microbial communities associated with the seawater samples were distinct from those associated with both photosymbiont and non-photosymbiont-bearing invertebrates ($P=0.002$, PERMANOVA). This difference was related to a larger abundance of the ubiquitous bacterioplankton *Candidatus pelagibacter* (SAR11) comprising ~66% of sequence reads from this control group (Figure 1).

The composition of microbial communities also differed between invertebrate groups (Foramaninfera, Scleractinia, Octocorallia, Bivalvia, Bryozoa and Ascidiacaea; $P=0.001$, see Supplementary Figure S2) and this was further supported by both unweighted and weighted unifrac distances ($P<0.001$, redundancy analysis). Many replicate microbiomes from the same invertebrate species grouped well at both class and OTU taxonomic assignment levels and was reflected by the composition of microbial communities being different between invertebrate species ($P<0.001$, PERMANOVA). Most within-species variability existed for the samples derived from *A. millepora, Sinularia flexibilis, Tridacna* spp*. and Bryozoan* sp. (Supplementary Figure S2). Although the redundancy analysis and Heatmap/Cluster analysis generally group individual specimens from one species closely together and showed significant relationships at the taxa level, there is no apparent higher phylogenetic grouping. However, both analyses clearly separate samples by presence or absence of photosymbionts. Further studies comparing microbiomes among taxa with and without photosymbionts will be useful in further clarifying the strength of these relationships and the role photosymbionts have in driving microbial associations.

Rarefaction analysis demonstrated that all three Foraminifera species hosted the largest bacterial diversity among the invertebrate samples (Supplementary Figure S3), which may reflect their lifestyle closely associated with reef rubble, filamentous algae and reef sediment. *Heterostegina depressa* was the only diatom-bearing invertebrate species and richness of these samples (1123 OTUs) exceeded that of all other invertebrate taxa. The two Octocoral species, *Sarcophyton* sp. and *Sinularia flexibilis* hosted the lowest microbial richness (80–180 OTUs, respectively), which may relate to the high antimicrobial activity previously identified in these species (Kim, 1994; Jensen *et al*., 1996; Harder *et al*., 2003). Many invertebrate species including all Foraminifera, Bivalvia, Bryozoa and the Scleractinian corals *A. millepora* and *Pocillopora damicornis* hosted higher bacterial richness than the surrounding seawater (Supplementary Figure S3). Rarefaction analysis of the Scleractinian corals in this study is consistent with earlier estimates of coral microbial diversity (Sunagawa *et al*., 2010).

The documented decline in coral reef ecosystems worldwide (Wilkinson, 2008) has prompted research into understanding how changing environmental conditions affect the close symbiotic associations of marine invertebrates (Webster *et al*., 2001; Bourne *et al*., 2008; Vega Thurber *et al*., 2008; Webster *et al*., 2008; Littman *et al*., 2010, 2011; Webster *et al*., 2011). Only by studying marine invertebrates as holobionts (the host and all associated microbial communities) and better characterising the forces that structure their microbial associations will we be able to fully assess their capacity to adapt or acclimatise to environmental stress. From this study, 16S rRNA gene amplicon pyrosequencing revealed high diversity of bacterial symbionts within 16 common Great Barrier Reef species. Importantly, although microbial composition was related to host species, a significant amount of the variation in community composition was attributed to the presence or absence of photosymbionts. These results highlight the importance of photosymbionts in structuring reef bacterial symbioses.

Conflict of Interest

The authors declare no conflict of interest.

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