Distinct Bacterial Communities Associated with the Coral Model Aiptasia in Aposymbiotic and Symbiotic States with Symbiodinium

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Coral reefs are in decline. The basic functional unit of coral reefs is the coral metaorganism or holobiont consisting of the cnidarian host animal, symbiotic algae of the genus Symbiodinium, and a specific consortium of bacteria (among others), but research is slow due to the difficulty of working with corals. Aiptasia has proven to be a tractable model system to elucidate the intricacies of cnidarian-dinoflagellate symbioses, but characterization of the associated bacterial microbiome is required to provide a complete and integrated understanding of holobiont function. In this work, we characterize and analyze the microbiome of aposymbiotic and symbiotic Aiptasia and show that bacterial associates are distinct in both conditions. We further show that key microbial associates can be cultured without their cnidarian host. Our results suggest that bacteria play an important role in the symbiosis of Aiptasia with Symbiodinium, a finding that underlines the power of the Aiptasia model system where cnidarian hosts can be analyzed in aposymbiotic and symbiotic states. The characterization of the native microbiome and the ability to retrieve culturable isolates contributes to the resources available for the Aiptasia model system. This provides an opportunity to comparatively analyze cnidarian metaorganisms as collective functional holobionts and as separated member species. We hope that this will accelerate research into understanding the intricacies of coral biology, which is urgently needed to develop strategies to mitigate the effects of environmental change.

Keywords: coral reef, cnidarian-dinoflagellate symbiosis, microbial community profiling, 16S rRNA gene, functional profiling

INTRODUCTION

Coral reefs are biodiversity hotspots of enormous ecological and economic importance. In these ecosystems, corals are the foundation species that build the calcium carbonate skeletons that give rise to the massive three-dimensional reef structures providing a habitat for millions of species (Reaka-Kudla et al., 1996) and economic activity worth around US$ 5.7 billion each year for Australia’s Great Barrier Reef alone (Hoegh-Guldberg, 2015). However, reef ecosystems are under threat due to a combination of local (e.g., overfishing, pollution) and global (e.g., ocean warming and acidification) factors (Hughes et al., 2003). While unusually high sea surface temperatures cause...
coral bleaching (i.e., the disruption of the coral- algal symbiosis resulting in algal expulsion and tissue whitening), pollution may cause coral disease and facilitate bleaching susceptibility from high nutrient loads or other toxic substances (Negri et al., 2011; Vega Thurber et al., 2014). In the Caribbean, 80% of coral cover has been lost over the last decades (Gardner et al., 2003). Despite a reasonably good understanding of the environmental conditions that are harmful to corals, we are still missing knowledge on the cellular and molecular basis of coral bleaching and disease, and the contributions of microbes to stress resilience (Mouchka et al., 2010; Bourne et al., 2016), information that is critical to conceive strategies for mitigating future reef loss.

The basic functional unit of stony corals is the coral holobiont, consisting of the cnidarian-animal host, its intracellular dinoflagellate algae of the genus Symbiodinium, and a specific consortium of associated microbes, including bacteria, archaea, fungi, and viruses (among other organisms) (Rohwer et al., 2002). While the dependency on a functional symbiosis between the animal host and its photosynthetic algae has long been acknowledged (Trench, 1993), the importance of bacterial microbes has only recently been elucidated in more detail (Rosenberg et al., 2007; Raina et al., 2009; Ritchie, 2011; Jessen et al., 2013; Radecker et al., 2015; Röthig et al., 2016; Ziegler et al., 2016). Sparked by the development of new genomic tools (e.g., next-generation sequencing), recent years have brought a changing understanding in life sciences (Mcfall-Ngai et al., 2013). The common notion is that all animals and plants are metaorganisms that critically depend on living together with a highly diverse and specific group of microbes that provide functions related to metabolism, immunity, and environmental adaptation, among others (Mcfall-Ngai et al., 2013). These metaorganisms or holobionts cannot be understood in isolation, but must be studied as a consortium of organisms, i.e., as hosts and associated microbes. Consequently, interactions and communication mechanisms among holobiont members presumably play a major role in maintaining host health and microbiome stability.

One of the reasons why progress is slow on gaining a better insight into the molecular mechanisms governing holobiont function is due to the difficulties of working with corals. For instance, corals are difficult to grow in culture, have long generation times, and are difficult to be kept without their associated algal symbionts, prohibiting the study of a non-symbiotic “control” or “reference” state (Voolstra, 2013). To this end, the sea anemone Aiptasia has emerged as a tractable laboratory model to study coral symbiosis (Weis et al., 2008). A key aspect is Aiptasia’s ease of culturing and flexibility in its symbioses (e.g., Aiptasia can host the same algal symbionts as corals), allowing the comparative analysis of symbiotic and non-symbiotic states side-by-side in a laboratory context (Voolstra, 2013). In this regard, the recent assembly and analysis of the Aiptasia genome provides a foundation for its role as a model for coral biology (Baumgarten et al., 2015), but characterization of the associated bacterial microbial community is missing.

In order to further contribute to the establishment of Aiptasia as a model system for coral symbiosis and to contribute to the characterization of the entire Aiptasia holobiont, we set out to analyze the bacterial community associated with Aiptasia. To do this, we compared bacterial communities from Aiptasia strain CC7 that are aposymbiotic and symbiotic with the Symbiodinium strain SSB01 (species S. minutum) (Xiang et al., 2013b; Baumgarten et al., 2015) to investigate how microbial assemblages may change with symbiotic state. Last, we report on the generation of culturable isolates from bacterial taxon of the microbial community providing the opportunity to study host-microbe interactions in detail.

**MATERIALS AND METHODS**

**Animal Rearing**

Aposymbiotic and symbiotic Aiptasia of the clonal strain CC7 were generated and reared as described previously (Baumgarten et al., 2015). Briefly, aposymbiotic animals were obtained through repetitive cold-shock by addition of 4°C cold autoclaved freshly collected seawater (AFSW) from the Red Sea and subsequent incubation at 4°C for 4 h. Anemones were then treated for 1–2 days with 50 μM of the photosynthesis inhibitor diuron (Sigma-Aldrich, St. Louis, MO, USA) at 25°C in AFSW. Aposymbiotic Aiptasia were raised in 1 liter AFSW-tanks at 25°C in the dark for more than 1 year, fed Artemia twice weekly, and supplied with AFSW the day after feeding. Symbiotic Aiptasia were generated by infecting aposymbiotic animals with the clade B Symbiodinium strain SSB01 (Xiang et al., 2013a) at a final concentration of 10⁴ algal cells mL⁻¹. Following infection, symbiotic animals were transferred to a 12 h light: 12 h dark incubator (20–40 μmol photons m⁻² s⁻¹ of photosynthetically active radiation) at 25°C and fed Artemia twice weekly. Two weeks prior to the start of the experiment, aposymbiotic and symbiotic Aiptasia were cultured in 6 multiwell cell culture plates (3–5 organisms per well in 6 mL AFSW), kept on a 12 h light: 12 h dark cycle at 25°C, and repeatedly tested for Symbiodinium re-infection by fluorescent microscopy (Leica DMI3000 B). Additionally, aposymbiotic Aiptasia were regularly tested for the presence of Symbiodinium via PCRs with Symbiodinium-specific primers. Five days prior to experiments food supply was ceased to avoid Artemia contamination.

**Bacterial Microbiome - DNA Isolation and 16S rRNA Gene Sequencing**

For bacterial DNA isolation from anemones, five aposymbiotic and five symbiotic Aiptasia polyps of ~0.8 cm length were collected from the respective multiwell plates with a Pasteur pipette and transferred into 1.5 mL microtubes, washed thrice with AFSW, and remaining water was carefully removed. All 10 microtubes holding the polyps were transferred to −20°C. Aiptasia samples were crushed while thawing using a 10 μL pipette tip, and subsequently 400 μL API buffer (DNeasy Plant Mini Kit, Qiagen) were added. DNA extraction was performed according to the manufacturer’s instructions. For bacterial DNA isolation from water, 300 mL water were collected from each AFSW-container in which symbiotic and aposymbiotic anemones were reared. The collected water was firstly filtered through a 40 μm cell strainer (BD, Franklin Lakes, NJ, USA) to remove debris, and then through a 0.22
µm Durapore PVDF filter (Millipore, Billerica, MA, USA). Filters were frozen at −20°C, thawed, cut in strips using a sterile razorblade, and transferred into 2 mL microtubes. 400 µL API buffer were added (DNeasy Plant Mini Kit, Qiagen) and the microtubes were incubated on a rotating wheel for 20 min. Further procedure followed the manufacturer’s instructions (DNeasy Plant Mini Kit, Qiagen). DNA concentrations of samples were quantified on a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To generate 16S rRNA gene amplicons for sequencing, we targeted the variable regions 5 and 6 of the 16S rRNA gene using the primer pair 784F [5′ TCGTCGCGACCGTCAAGATGTTGATAAAGACAG-AGGATTAGATACCCGTGTAG 3′] and 1061R [5′ GTCTCGGCGCAGATGTATGTTGATAAAGACAG-CRCRCACGAGCTGACGAC 3′] (Andersson et al., 2008) with Illumina (San Diego, CA, USA) adaptor overhangs (underlined above). For each sample, PCRs were performed in triplicate using the Qiagen Multiplex PCR kit, between 10 and 80 ng template DNA, a primer concentration of 0.5 µM, and a final reaction volume of 25 µL. PCRs were performed as follows: One cycle at 95°C for 15 min, 27 cycles each at 95°C for 30 s, 55°C for 90 s, and 72°C for 30 s, followed by a final extension step at 72°C for 10 min. Triplicate PCRs for each sample were pooled and cleaned with the Agencourt AMPure XP magnetic bead system (Beckman Coulter, Brea, CA, USA), and subsequently underwent an indexing PCR to add Nextera XT barcoded sequencing adapters (Illumina) according to the manufacturer’s instructions. Indexed PCR products were cleaned using the Invitrogen SequlPrep normalization plate kit (Thermo Fisher Scientific, Carlsbad, CA, USA) following the manufacturer’s instructions and eluted at normalized concentrations (~4 nM) in 20 µl elution buffer and pooled in equimolar ratios. Pooled samples were quality checked on the BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) for presence of primer dimers. The library was sequenced at 8 pM with 10% phiX on the Illumina MiSeq. 2*300 bp paired-end version 3 chemistry according to the manufacturer’s specifications.

**Bacterial Microbiome—Analysis**

The sequence data set comprised 2.48 million sequence reads. Reads were demultiplexed and adapters and barcodes were removed in MiSeq Reporter (v. 2.4.60.8). Data were imported into mothur version 1.36.1 (Schloss et al., 2009) and 1,239,574 contigs were assembled using the “make.contigs” command. Contigs were quality trimmed, i.e., sequences with ambiguous nucleotides, sequences with excessively long homopolymers (>5), and sequences of insufficient length were removed. Additionally 432,543 singletons were removed. Remaining sequences were aligned against SILVA release 119 (Pruesse et al., 2007), preclustered (2 bp difference) (Huse et al., 2010), and chimeric sequences were removed using UCHIME (Edgar et al., 2011). Sequences were classified against the Greengenes database (release gg_13_8_99) with a minimum bootstrap of 60 (McDonald et al., 2012), and unwanted sequences (i.e., unknown, eukaryota, archaea, mitochondria, and chloroplasts) were removed. From the remaining 575,354 sequences alpha diversity indices for bacterial communities were calculated in mothur, and the composition of samples was compared on the family level by creating stack column plots in R (R Core Team, 2014). For taxon-based analysis, samples were subsampled to 11,000 sequences and clustered into Operational Taxonomic Units (OTUs) using a 97% similarity cutoff. Rarefaction curves, non-metric multidimensional scaling (nMDS), and analysis of molecular variance (AMOVA) (Excoffier et al., 1992) were conducted as implemented in the software mothur. Differences between alpha diversity indices of samples were assessed after testing for normality and homoscedasticity (Shapiro-Wilk and Levene’s test performed in R) using one-way ANOVAs (STATISTICA 10, StatSoft Inc.). nMDS results were plotted in SigmaPlot 11 (Systat Software, Point Richmond, CA, USA). The commands make.shared, classify.OTU, and getOTUrep were used to create a list of all OTUs and their distribution across samples. Based on these data, we obtained a putative “core microbiome” (i.e., all OTUs present in 100% of all Aiptasia polyps), an aposymbiotic microbiome or “apobiome” (i.e., all OTUs present in 100% of all aposymbiotic polyps), and a symbiotic microbiome or “symbiome” (i.e., all OTUs present in 100% of all symbiotic polyps). Of note, the respective OTUs may be members of multiple “biomes” and can be present in the water samples. To identify previous occurrences of identical or highly similar bacteria, the representative sequence of each OTU occurring in at least one “biome” was BLASTed against NCBI’s GenBank nr and the three best matches were considered (e-value cutoff e−20). Putative functions encoded in the microbial communities of anemones were based on phylogenetic inference and assessed using METAGENassist for automated taxonomic-to-phenotypic mapping (Arndt et al., 2012). We created input files in mothur using the make.shared and classify.OTU commands. During data processing, OTUs present in anemones were assigned, mapped, and condensed into 236 functional taxa in METAGENassist. Data were further filtered based on interquartile range (Hackstadt and Hess, 2009), and the remaining 225 functional taxa were normalized across samples by sum and over taxa by Pareto scaling. We analyzed the dataset for “metabolism by phenotype” using the Spearman distance measure to cluster the 15 most differentially abundant metabolic processes.

**Generation of Bacterial Cultivates**

Reared and starved aposymbiotic and symbiotic anemones (see above) were collected in 1.5 mL microtubes with 500 µL of sterile seawater, crushed using a pestle, and subsequently spread out on either M1 (MO) Agar (10 g Starch, 4 g yeast extract, 2 g peptone, 18 g agar, 1 L sterile seawater) or Marine (MA) Agar (55.1 g Difco™ Marine Agar 2216 in 1 L sterile seawater) plates and incubated at 28°C for up to 24 h. To determine the identity of cultured isolates, bacterial colonies were picked from the agar plates into 96 well plates using sterile 10 µL pipette tips. Each well contained 10 µl PCR mix (5 µl Qiagen Multiplex PCR kit, 1 µM of 27F and 1492R primers, adjusted to the final volume with dH2O). The PCR conditions were set as follows: 95°C for 15 min, followed by 35 cycles of each: 30 s at 95°C, 90 s at 55°C, and 90 s at 72°C. A final extension step was set at 72°C for 15 min.

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RESULTS

Bacterial Community of Aiptasia and Rearing Water

We produced 12 16S rRNA gene libraries containing a total of 1,239,574 sequences from 5 aposymbiotic and 5 symbiotic Aiptasia animals and 2 water samples (from both rearing conditions, i.e., 1 aposymbiotic and 1 symbiotic). After quality trimming and removal of singletons and unwanted sequences, 575,354 sequences with an average length of 292 bp were available for subsequent analyses. Classification of sequences on the family level revealed noticeable differences between the microbial community associated with aposymbiotic and symbiotic anemones (Figure 1). On average, aposymbiotic Aiptasia were overall dominated by Alteromonadaceae (between 29% and 52%, mean 47%), Rhodobacteraceae (between 6% and 15%, mean 11%), and Oceanospirillaceae (between 1% and 22%, mean 12%). In contrast, microbial communities from symbiotic anemones showed an increased amount of Pseudomonadaceae (between 17% and 24%, mean 20%) and Dermabacteraceae (between 10% and 15%, mean 12%), but contained noticeably less Alteromonadaceae (between 16% and 23%, mean 19%). By comparison, water samples were markedly different from all Aiptasia samples and also different from each other. On average, water samples were more diverse, i.e., more bacterial families with a more even abundance were present (e.g., Alteromonadaceae, Rhodobacteraceae, and unclassified families of the order Flavobacteriales and the class Gammaproteobacteria made up >50% of sequences).

To assess differences between bacterial communities of aposymbiotic and symbiotic Aiptasia in more detail, we clustered sequences into operational taxonomic units (OTUs) at a 97% similarity cutoff after subsampling to 11,000 reads and calculated alpha diversity indices (Table 1, Supplementary File S1). We identified a total of 486 OTUs, 379 associated with Aiptasia (251 OTUs were exclusively found in Aiptasia) and 235 found in water (of these 107 exclusively in water) (Supplementary File S2). Average Chao1 estimator of species richness was significantly higher for aposymbiotic samples than for symbiotic samples (average 166 vs. 131, respectively) (t-test < 0.05). Simpson's evenness and the inverse Simpson index, however, were significantly higher (t-test < 0.05) in symbiotic samples (average 0.095 and 11.8, respectively) than in aposymbiotic samples (average of 0.053 and 8.0, respectively). Water samples showed a higher Chao1 (average 257) and inverse Simpson index (average 14.7), but a similar evenness (average 0.059) in comparison to Aiptasia samples. Differences in bacterial communities from aposymbiotic and symbiotic Aiptasia and water samples were visualized in a non-metric multidimensional scaling (nMDS) plot based on the Yue & Clayton theta similarity coefficient (Supplementary File S3). As expected, we found a clear separation between the water samples and all Aiptasia samples (P<sub>AMOVA</sub> = 0.014) demonstrating the presence of a specific and selected microbiome associated with Aiptasia. To focus on differences between aposymbiotic and symbiotic Aiptasia, we excluded water samples from subsequent analyses.

Distinct Bacterial Communities of Aposymbiotic and Symbiotic Aiptasia

Bacterial communities associated with aposymbiotic and symbiotic Aiptasia were significantly different in an OTU framework (P<sub>AMOVA</sub> = 0.008). To further identify OTUs associated with different symbiotic states, we determined the “core microbiome” (i.e., all OTUs present in 100% of all Aiptasia samples), the aposymbiotic microbiome or “aposymbiotic microbiome” (i.e., all OTUs present in 100% of aposymbiotic Aiptasia), and the symbiotic microbiome or “symbiotic microbiome” (i.e., all OTUs present in 100% of symbiotic Aiptasia) (Figure 2).

We identified 24 OTUs in the core microbiome (Table 2, Supplementary File S2), which included the 10 most abundant OTUs, comprising >60% of all OTU sequence counts. We next looked for patterns of differential abundance among core microbiome members in aposymbiotic and symbiotic Aiptasia, since their relative abundance may indicate functional differences (Figure 2, Table 2). Interestingly, only three OTUs showed a comparatively modest fold-change between 1.2- and 1.7-fold (OTU004, OTU010, OTU024), while the remaining 21 OTUs, i.e., the vast majority of all core microbiome taxa, showed marked differences in abundance (between 2.4- to 18-fold) between aposymbiotic and symbiotic anemones. For the “aposymbiotic microbiome”, we identified 50 distinct OTUs, including 11 OTUs that were exclusively found in aposymbiotic animals (Supplementary File S2). The 50 bacterial taxa of the “aposymbiotic microbiome” represented abundant and rare members of the microbiome (mean abundance of 1–3318 sequence counts in aposymbiotic conditions). Similarly, the “symbiotic microbiome” consisted of 37 OTUs, including only 1 OTU that was exclusively found in symbiotic anemones (Supplementary File S2). The average abundance of OTUs from the “symbiotic microbiome” ranged between 6 and 2173 sequence counts in symbiotic Aiptasia.

Taxonomy-Based Functional Profiling of Bacterial Communities in Aiptasia

To assess putative functional changes underlying the different bacterial communities in aposymbiotic and symbiotic Aiptasia, we used METAGENassist (Figure 3, Supplementary File S4). Symbiotic Aiptasia clustered together tightly indicating homogeneity in enrichment and depletion of functions. By comparison, aposymbiotic samples seemed more diverse and did not cluster together. In particular, one of the samples (Apo5, Figure 3) exhibited higher similarity to the symbiotic
samples as indicated by the clustering of this sample with symbiotic Aiptasia. In general, we found processes to be enriched in aposymbiotic and depleted in symbiotic samples (e.g., “Sulfate reducer”, “Sulfide oxidizer”, “Selenate reducer”, “Denitrifying”) or vice versa enriched in symbiotic and depleted in aposymbiotic samples (e.g., “Sulfur oxidizer”, “Chlorophenol degrading”, “Degrades aromatic hydrocarbons”, “Sulfur metabolizing”, “Naphthalene degrading”), besides some processes that were more inconsistent (e.g., “Xylan degrader”, “Atrazine metabolism”, “Iron oxidizer”) (Figure 3, Supplementary File S4).

Cultured Isolates of Aiptasia-Associated Bacteria

In order to enable functional studies on bacteria-host interactions in Aiptasia, it is of great benefit to have culturable isolates of bacterial associates, as previously demonstrated for Hydra (Fraune et al., 2015). To obtain cultured isolates, we used lysates of aposymbiotic and symbiotic animals and compared the isolated bacteria to the native microbial community. We retrieved approximately 700 bacterial colonies. Subsequent 16S marker gene sequencing and comparison to the native microbiome revealed about 200 distinct cultivates with a similarity of ≥97% (data not shown). Importantly, 14 cultivates displayed a similarity of 100% to the 16S rRNA gene amplicon, which were further considered (Table 3). These 14 OTUs included 3 of the 10 most abundant bacteria (i.e., OTU001, OTU004, and OTU006) and were members of the most abundant family Alteromonadaceae in aposymbiotic and symbiotic anemones (Table 3, Supplementary File S2). Importantly, we could culture the most abundant member (OTU001) from the core microbiome and identified it to the genus Glaciecola, which was possible based on the longer Sanger sequence (~900 bp) in comparison to the MiSeq amplicon. The 14 OTUs contained 6 OTUs (25%) of the core microbiome, 9 OTUs (18%) of the apobiome, and 7 OTUs (19%) of the symbiome. The use of two different growth media retrieved different cultures. For instance, a bacterial cultivate representing OTU001 was obtained from bacterial colonies grown on Marine Agar, but not M1 Agar. Further, while
Marine Agar retrieved a higher taxonomic diversity, M1 Agar showed an increased selectivity for the genera *Alteromonas* and *Pseudoalteromonas*.

**DISCUSSION**

The Microbiome of *Aiptasia*

Despite the importance of bacteria to animal and plant function (Mcfall-Ngai et al., 2013), the microbiome of model systems has only begun to be studied in earnest over the past few years. While studies in *Hydra magnipapillata* show that co-operation between host-selected microbes exist (Fraune et al., 2015), the microbiome of *Nematostella vectensis* has only been characterized very recently and functional studies are not yet available (Har et al., 2015). Here we describe the microbiome of *Aiptasia* associated with aposymbiotic and symbiotic states. We find that the bacterial microbiome, irrespective of the symbiotic state with *Symbiodinium*, is comprised of a fairly consistent number of OTUs (between 96 to 133 OTUs). In *Hydra* a similar number of OTUs (∼100) has been found in 15 week old polyps (Franzenburg et al., 2013). In corals, numbers of associated OTUs vary more pronouncedly between species and prevailing environmental conditions, but are also on the order of tens to hundreds of OTUs (Bayer et al., 2013; Jessen et al., 2013; Roder et al., 2014, 2015; Neave et al., 2016; Röthig et al., 2016; Ziegler et al., 2016).

Although the core microbiome was comparably small, the 24 bacterial taxa made up the majority of sequence counts. The ubiquity and high abundance of these OTUs suggest functional importance to the animal host, regardless of the symbiotic state. Yet, the majority of core microbiome taxa considerably differed in their abundance in aposymbiotic and symbiotic anemones. At present, it is unknown why these bacteria display differential abundance, but these data suggest a link between the bacterial community and the cnidarian-algal symbiosis, strongly arguing to integrate bacterial communities in research of the cnidarian-algal symbiosis.

**TABLE 1** | Summary statistics of 16S rRNA gene bacterial community sequencing of *Aiptasia*.

| Sample name | # of sequences | # of OTUs | Chao1 | Inverse Simpson | Simpson’s evenness |
|-------------|----------------|-----------|-------|----------------|-------------------|
| Apo1        | 81,766         | 102       | 192   | 8.5            | 0.048             |
| Apo2        | 68,978         | 96        | 171   | 7.7            | 0.051             |
| Apo3        | 61,293         | 110       | 168   | 5.5            | 0.035             |
| Apo4        | 33,248         | 110       | 146   | 5.2            | 0.038             |
| Apo5        | 24,223         | 128       | 154   | 12.9           | 0.093             |
| Sym1        | 22,415         | 115       | 138   | 13.2           | 0.102             |
| Sym2        | 25,755         | 133       | 142   | 12.3           | 0.088             |
| Sym3        | 26,144         | 113       | 127   | 10.9           | 0.090             |
| Sym4        | 11,277         | 109       | 112   | 12.4           | 0.113             |
| Sym5        | 23,692         | 121       | 135   | 10.3           | 0.081             |
| WaterApo    | 91,201         | 181       | 280   | 19.1           | 0.071             |
| WaterSym    | 105,362        | 142       | 234   | 10.3           | 0.045             |

Apo, aposymbiotic; Sym, symbiotic.

**FIGURE 2** | *Aiptasia* “core microbiome”, “apobiome” (aposymbiotic microbiome), and “symbiome” (symbiotic microbiome). Bacterial members were determined by assessing presence of OTUs over samples. Only OTUs present in all anemones, all aposymbiotic anemones, and all symbiotic anemones were included in the “core microbiome”, “apobiome” (aposymbiotic microbiome), and “symbiome” (symbiotic microbiome), respectively. Each color represents a distinct OTU of the 14 most abundant taxa; 49 rare OTUs have been summarized in gray in the category “others.”
### TABLE 2 | Aiptasia “core microbiome”, “apobiome” (aposymbiotic microbiome), and “symbiome” (symbiotic microbiome).

| OTU   | “Core microbiome” | “Apobiome” | “Symbiome” | Taxonomy (bootstrap value) |
|-------|------------------|------------|------------|----------------------------|
|       | # of OTUs        | 24         | 50         | 37                         |
| OTU001| 2072             | 3318       | 826        | unclassified Alteromonadaceae (100) |
| OTU002| 1236             | 299        | 2174       | *Pseudomonas veronii* (88) |
| OTU003| 792              | 252        | 1332       | *Brachybacterium* sp. (100) |
| OTU004| 668              | 849        | 486        | *Alteromonas* sp.(100) |
| OTU005| 512              | 827        | 197        | *Thalassobius mediterraneus* (86) |
| OTU006| 265              | 85         | 445        | *Alteromonas* sp.(100) |
| OTU007| 560              | 1003       | 117        | *Oceanospirillum* sp. (100) |
| OTU008| 535              | 175        | 894        | *Deltzia* sp. (100) |
| OTU009| 443              | 108        | 777        | *Pelomonas puraeae* (100) |
| OTU010| 428              | 515        | 342        | *Alteromonas* sp.(100) |
| OTU011| NaM              | 497        | NaM        | unclassified Cohaesibacteraceae (100) |
| OTU012| NaM              | 239        | 405        | 73                         |
| OTU013| 220              | 414        | 27         | *Bacteriovorax* sp. (100) |
| OTU014| 189              | 74         | 303        | *Francisella* sp. (100) |
| OTU015| 179              | 19         | 339        | *Propionibacterium* acnes (100) |
| OTU016| NaM              | NaM        | 95         | unclassified Oleiphilaceae (100) |
| OTU017| 149              | 270        | 28         | unclassified Rhodobacteraceae (100) |
| OTU018| NaM              | 144        | NaM        | *Thalassomonas* sp. (100) |
| OTU019| NaM              | 240        | NaM        | *Tenacibaculum* sp. (100) |
| OTU020| 77               | 84         | 69         | *Nautilia italica* (100) |
| OTU021| 89               | 145        | 33         | *Photobacterium* angustum (100) |
| OTU022| 97               | 25         | 168        | *Glucanacetobacter liquefaciens* (100) |
| OTU023| 81               | 140        | 21         | *Plesiocystis* sp. (100) |
| OTU024| NaM              | 83         | NaM        | unclassified Rhodobacteraceae(100) |
| OTU025| 87               | 21         | 152        | *Brevibacterium aureum* (100) |
| OTU026| NaM              | NaM        | 71         | *Shimia* sp. (100) |
| OTU027| NaM              | NaM        | 90         | *Candidatus Rhodochlamydia* sp. (100) |

(Continued)
TABLE 2 | Continued

| OTU    | “Core microbiome” | “Apobiome” | “Symbiome” | Taxonomy (bootstrap value) |
|--------|-------------------|-------------|-------------|----------------------------|
|        | # of OTUs | 24 | 50 | 37 |

OTU002  NaM  13  NaM  Okeibacter sp. (100)
OTU004  NaM  10  NaM  unclassified Flavobacteriales (100)
OTU008  NaM  10  NaM  Ferrimonas sp. (100)
OTU102  NaM  NaM  9  Methylobacterium hispanicum (86)
OTU104  NaM  7  NaM  unclassified Alteromonadaceae (80)
OTU105  NaM  7  NaM  unclassified Cohaeisibacteraceae (82)
OTU112  NaM  2  NaM  unclassified Alphaproteobacteria (100)
OTU121  NaM  2  NaM  unclassified Phycisphaeraceae (100)
OTU122  NaM  NaM  5  Brevundimonas diminuta (94)
OTU213  NaM  1  NaM  unclassified Thalassomonas (100)

Members were determined by assessing presence of OTUs over samples. Only OTUs present in all anemones, aposymbiotic anemones, and symbiotic anemones were considered members of the “core microbiome”, “apobiome”, and “symbiome”, respectively. NaM: not a member; numbers denote average abundance in respective “biomes”.

Interestingly, *Pseudomonas veronii* was identified as a highly abundant member of the core microbiome (Table 3). *P. veronii* was strongly enriched in a recent study on fungus corals exposed to hypersalinity in the Red Sea (Röthig et al., 2016). The repeated association with different symbiotic cnidarians might point toward the functional importance of this bacterial taxon and makes it an interesting candidate for cultivation and functional studies. We also identified *Nautella italica*, a bacterial pathogen able to colonize and invade different algae (Fernandes et al., 2011; Gardiner et al., 2015), which at least hypothetically shows how host-associated bacteria can affect the animal host-algal symbiosis. Many of the remaining core microbiome members were found previously associated with corals, sponges, echinoids, algae, and sediments (Supplementary File S2).

In the future, improved resolution of taxonomical classification could be obtained by compiling databases harboring sequences specifically associated with cnidarians, as done for members of the human intestinal microbiota (Ritari et al., 2015). Recent efforts to establish cnidarian-specific databases include the Coral Microbiome Portal (CMP) at https://vamps.mbl.edu/portals/coral_microbe/coral.php and reefgenomics.org (Liew et al., 2016) at http://reefgenomics.org that, besides serving as a data repository for genomics data associated with reef organisms, also anticipates to hold microbial data such as those arising from the ReFuGe 2020 consortium (Voolstra et al., 2015).

**Functional Differences Associated with the Microbial Community**

Interestingly, 4 out of the 15 most pronounced differences in metabolic processes were involved in sulfur cycling (i.e., “Sulfate reducer”, “Sulfide oxidizer”, “Sulfur oxidizer”, “Sulfur metabolizing”). Sulfur utilization is enhanced by the presence of *Symbiodinium* in juvenile corals (Yuyama et al., 2016). Similarly, we argue that symbiotic *Aiptasia* have higher levels of dimethylsulfoniopropionate (DMSP), which accordingly provides a source of sulfur for the bacterial community (Supplementary File S5). In support, aposymbiotic *Aiptasia* seem unable to produce DMSP as it was only found in symbiotic animals (Van Alstyne et al., 2009). Taken together, DMSP is an important substrate of bacterial sulfur cycling (Raina et al., 2010), and its increased synthesis in symbiotic *Aiptasia* likely explains the enrichment of sulfur cycling bacteria, as shown previously for coral holobionts (Frade et al., 2016). Besides differential abundance of functions related to sulfur cycling, we identified differences in nitrogen cycling (Supplementary File S5). Nitrogen is a limiting nutrient in the coral holobiont and algal symbiont densities are controlled, in part, by nitrogen availability (Falkowski et al., 1993; Rädecker et al., 2015). The bacterial processes “nitrite reduction” and “denitrification” were increased in aposymbiotic *Aiptasia*, indicating either increased nitrogen availability and/or increased recycling. Given that *Symbiodinium* is the major sink for nitrogen compounds released by the host in symbiotic coral holobionts (Pernice et al., 2012), nitrogen may no longer be a limiting factor in aposymbiotic animals. Hence, excess nitrogen availability may stimulate growth of denitrifying bacteria, allowing for the efficient removal of these nitrogen compounds from the holobiont. Future studies using metagenomics and metatranscriptomics to study aposymbiotic and symbiotic states have the potential to provide further insight and a more direct assessment of the functional attributes of the microbiome (see e.g., Daniels et al., 2015).

**Cultured Isolates of Aiptasia-Associated Bacteria—Toward Functional Microbiome Studies**

Even though functional studies of corals exist (Lema et al., 2015; Pollock et al., 2015), a laboratory model is needed in order to conduct more elaborate studies, such as experimental replacement of native bacteria in order to assess functional contribution of a specific bacterial species. For this type of experiment, it is essential to obtain bacterial cultivates that represent key microbial symbionts. In this study, we could culture a range of abundant and rare *Aiptasia*-associated bacteria, including isolates that were specific to the aposymbiotic or symbiotic condition. The cultured isolates here present a starting point for functional studies, especially with regard to the notion that abundant and rare bacteria in cnidarians are functionally important (Bosch, 2013; Golberg et al., 2013; Fraune et al., 2015; Glasl et al., 2016). Of note, this is an ongoing effort, and we anticipate that further application of different culture media and conditions will enable a much more complete
cultivation of *Aiptasia*-associated bacteria. These efforts will be complemented by whole genome sequencing of key bacterial associates, as conducted by Har et al. (2015), in order to gain further understanding of the putative functions encoded and provided by the bacterial microbiome. In addition, an important accompanying step to culturing and characterization of bacterial isolates is the generation of axenic *Aiptasia* that may then be used for infection studies with bacterial cultivates in order to unequivocally assign function (Fraune et al., 2015), with the ultimate aim of identifying bacteria that affect holobiont traits of significance to environmental change, such as those that confer increased thermotolerance (Moran and Yun, 2015).
### TABLE 3 | Overview of cultured isolates of *Aiptasia*-associated bacteria.

| Bacterial isolate | OTU    | Average abundance | Average abundance | Annotation MiSeq | Annotation Sanger |
|-------------------|--------|-------------------|-------------------|------------------|-------------------|
| MA2A18, MA2S3     | OTU001 | 3318              | 826               | Altermomonadaceae | Altermomonadaceae |
| MOA1, MOS1        | OTU004 | 849               | 436               | Altermomonadaceae | Altermomonadaceae |
| MOA2, MOS2        | OTU006 | 85                | 445               | Altermomonadaceae | Altermomonadaceae |
| MA6S5             | OTU019 | 28                | 270               | Rhodobacteraceae  | Rhodobacteraceae  |
| MA4S4             | OTU024 | 69                | 84                | Rhodobacteraceae; Nautella italica | Rhodobacteraceae; Nautella italica |
| MOA3, MA6S1       | OTU026 | 145               | 33                | Vibroniaceae; Photobacterium angustum | Vibroniaceae; Vibrio sp. |

MA2S4, OTU032      | 71     | 4                 |                   | Rhodobacteraceae; Shimia sp. | Rhodobacteraceae |
| MA2A12, MA2S1     | OTU048 | 55                | 0                 | Altermomonadaceae; Marinobacter sp. | Altermomonadaceae; Marinobacter sp. |
| MA2S24            | OTU057 | 17                | 6                 | Altermomonadaceae; Marinobacter sp. | Altermomonadaceae; Marinobacter sp. |
| MA4A2             | OTU075 | 10                | 10                | Pseudoalteromonadaceae; Pseudoalteromonas porphyrae | Pseudoalteromonadaceae; Pseudoalteromonas porphyrae |
| MA4A5, MOS3       | OTU076 | 18                | 5                 | Pseudoalteromonadaceae; Pseudoalteromonas ruthenica | Pseudoalteromonas ruthenica |
| MA2A13            | OTU106 | 1                 | 1                 | Rhizobiaceae | Hyphomicrobiaceae |
| MQS4              | OTU362 | 0                 | 0                 | Altermomonadaceae; Altermomonas sp. | Altermomonas sp. |
| MA4S9             | OTU392 | 0                 | 0                 | Rhodobacteraceae | Rhodobacteraceae; Ruegeria sp. |

Only cultured bacteria with 100% sequence similarity to determined OTUs were considered. Annotation of family; genus; species (all bootstrap 100). Culture Names: MA, Marine Agar; MO, M1 Agar; S/A, extracted from symbiotic/aposymbiotic anemones; number denotes respective culture replicate.

### CONCLUSIONS

The unprecedented decline of coral reef cover in the last decades and in particular in recent years has heightened the need to better understand the mechanistic and molecular underpinnings of coral holobiont function. The growing popularity of the *Aiptasia* coral model promises to yield new insights and allows for the design of novel experiments, such as the comparison of apsymbiotic and symbiotic states. Our data show that apsymbiotic and symbiotic *Aiptasia* harbor distinct bacterial microbiomes with strong implications for the coral holobiont, namely that bacteria putatively play an important role in the coral-algal symbiosis and that the entire holobiont adjusts to the symbiotic condition. This is further corroborated by taxonomy-based functional profiling indicating that the bacterial microbiome of symbiotic *Aiptasia* is highly structured, less variant, and enriched for functions of putative relevance to the algal symbiosis. We hope that cultivation of members of the bacterial community of *Aiptasia* provides a foundation to conduct functional studies with the aim of better understanding the contributions of bacteria to holobiont function and identifying the members that are critical for environmental resilience of *Aiptasia*, and by extension of stony corals.

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### DATA ACCESSIBILITY

Sequence data determined in this study have been deposited on NCBI under BioProject accession no. PRJNA325476 (http://www.ncbi.nlm.nih.gov/bioproject/325476).

### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmars.2016.00234/full#supplementary-material

Supplementary Data Sheet 1 | Rarefaction curves illustrating OTU richness as a function of sequencing depth for subsampled dataset (n = 11,000 sequences per sample).

Supplementary Data Sheet 2 | OTU abundance counts over samples with annotation, reference OTU sequence, and affiliation to “core microbiome”, “apobiome” (apsymbiotic microbiome), and “symbiome” (symbiotic microbiome). For OTUs constituting a member of any “biome,” the
closest BLASTn match for the reference OTU sequence including source environment and available literature is denoted.

**Supplementary Data Sheet 3** | Non-metric multidimensional scaling (nMDS) plot of bacterial communities of apostymotic and symbiotic *Aiptasia* and water samples. Clustering of samples based on Yue & Clayton theta similarity coefficient of microbial community abundances ($R^2 = 0.95$, lowest stress = 0.108).

**Supplementary Data Sheet 4** | Taxonomy-based functional profiling of bacterial communities on average bacterial community composition of apostymotic and symbiotic *Aiptasia*. Heatmap displaying putative functional differences based on the bacterial community composition of apostymotic and symbiotic *Aiptasia*. Changes are displayed on a relative scale with enrichment in red and depletion in blue. Syn, symbiotic *Aiptasia*, Apo, apostymotic *Aiptasia*.

**Supplementary Data Sheet 5** | Conceptual model of cnidarian holobiont functioning and differences between apostymotic and symbiotic states (model extended from Rohwer et al., 2002). Functions proposed in the original conceptual holobiont model are in black, putative functions related to the presence of Symbiodinium in green, functions enriched in apostymotic *Aiptasia* in red, and functions present in the apostymotic and symbiotic state in gray.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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