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SYMPOSIUM

Microbiome Composition and Diversity of the Ice-Dwelling Sea Anemone, *Edwardsiella andrillae*

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Synopsis *Edwardsiella andrillae* is a sea anemone (Cnidaria: Anthozoa: Actiniaria) only known to live embedded in the ice at the seawater interface on the underside of the Ross Ice Shelf, Antarctica. Although the anatomy and morphological characteristics of *E. andrillae* have been described, the adaptations of this species to the under-ice ecosystem have yet to be examined. One feature that may be important to the physiology and ecology of *E. andrillae* is its microbiome, which may play a role in health and survival, as has been deduced in other metazoans, including anthozoans. Here we describe the microbiome of five specimens of *E. andrillae*, compare the diversity we recovered to that known for temperate anemones and another Antarctic cnidarian, and consider the phylogenetic and functional implications of microbial diversity for these animals. The *E. andrillae* microbiome was relatively low in diversity, with seven phyla detected, yet included substantial phylogenetic novelty. Among the five anemones investigated, the distribution of microbial taxa varied; this trait appears to be shared by many anthozoans. Most importantly, specimens either appeared to be dominated by Proteobacteria-affiliated members or by deeply branching Tenericute sequences. There were few closely related sequence types that were common to temperate and Antarctic sea anemone microbiomes, the exception being an *Acinetobacter*-related representative. Similar observations were made between microbes associated with *E. andrillae* and an Antarctic soft coral; however, there were several closely-related, low abundance Gammaproteobacteria in both Antarctic microbiomes, particularly from the soft coral, that are also commonly detected in Southern Ocean seawater. Although this preliminary study leaves open many questions concerning microbiome diversity and its role in host ecology, we identify major lineages of microbes (e.g., diverse deep-branching Alphaproteobacteria, Epsilonproteobacteria, and divergent Tenericutes affiliates) that may play critical roles, and we highlight the current understanding and the need for future studies of sea anemone–microbiome relationships.

Introduction

The importance of microbes to the health and ecology of marine animals and the ecosystems in which they live is well-appreciated at a conceptual level, yet understanding microbial diversity, interactions, and functions remains elusive in many systems. Although global plankton surveys are shedding light on the diversity and functional capacities of free-living microbes (e.g., Rusch et al. 2007; Sunagawa et al. 2015), the extent of diversity and interactions between marine animals and host-associated microbes (termed the microbiome) is still poorly known across organisms and ecosystems (Bourne et al. 2009; Ainsworth et al. 2010). In addition to playing a foundational role in shaping evolution (McFall-Ngai et al. 2013) and development (e.g., Fraune and Bosch

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2010), microbes can play significant roles in animal health (e.g., Ritchie 2006; Rosenberg et al. 2007; Krediet et al. 2013; Lokmer and Wegner 2015) and nutrient acquisition (e.g., Hoffmann et al. 2009; Roeselers and Newton 2012). Marine microbes that are symbiotic can also play a critical supportive role in the physiological acclimatization or adaptation of their hosts to challenging habitats (e.g., Grzymski et al. 2008; Schönknecht et al. 2013; O'Connor et al. 2014).

The ecosystems of the Southern Ocean and its associated coastal and ice habitats represent a particular deficit in our understanding of microbial symbioses. What is known from this large, diverse set of ecosystems highlights the potential importance of microbes to their host. Microbial diversity in Antarctic sponges (Webster et al. 2004; Rodriguez-Marconi et al. 2015) and soft corals (Webster and Bourne 2007) is host-specific and distinct from that of lower latitude marine ecosystems. The bacterial diversity of the Antarctic ascidian, *Synoicum adareanum*, is limited, but bacterial symbionts are potentially responsible for biosynthesis of the bioactive compound, palmerolide, in the holobiont (Riesenfeld et al. 2008). Further, microbial partners have been implicated in cryoadaptation of the Antarctic ciliate, *Euplotes focardii*, in which two bacterial genome-encoded ice binding proteins were identified (Pucciarelli et al. 2014). These examples are just the "tip of the iceberg" with respect to uncovering the expansive diversity of marine microbial symbioses in the Southern Ocean.

Actiniarian sea anemones are among the most conspicuous inhabitants of marine ecosystems, where they play key roles in benthic-pelagic coupling and primary production through their symbioses with photosynthetic unicellular organisms (e.g., Sebens 1981; Shick 1991). Actiniarians are unique among anthozoans in showing broad physiological tolerance with respect to salinity, temperature, and oxygen levels (reviewed in Shick 1991). Excluding the extensive study of the diversity and interactions between actiniarians and photosynthetic eukaryotes (e.g., LaJeunesse and Trench 2000; Müller-Parker and Davy 2001; Lewis and Müller-Parker 2004), the role of the microbiome in the ecology and physiology of actiniarians, particularly those in habitats devoid of photosynthetically active radiation, is virtually unknown. Reports of actiniarian host-microbe associations were limited to a few microscopic observations and studies of microbial diversity via rRNA gene sequences (Schuett et al. 2007; Williams et al. 2007; Du et al. 2010; Schuett and Doepke 2010; Meron et al. 2013) until Har et al. (2015) conducted

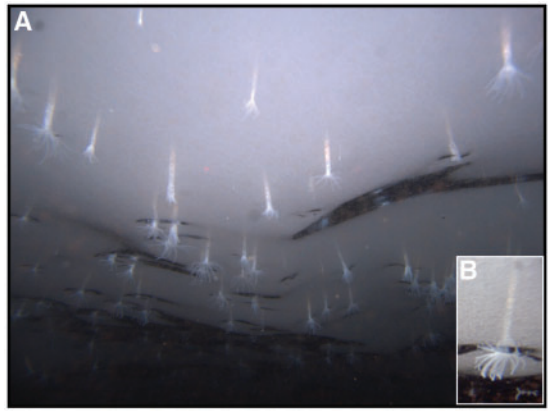

Fig. 1. Underside of the Ross Ice Shelf with *E. andrillae* imbedded in the ice. Image captured using the ROV SCINI at Coulman High.

an extensive investigation into the microbiome of the model anemone, *Nematostella vectensis*. Har et al. (2015) found a pattern of microbial association that suggests that, like their relatives the scleractinian corals, actiniarians may have selective and specific microbial associations and that these may be critical to supporting host survival. Indeed, metatranscriptome and bacterial cultivar genome sequencing efforts in *Nematostella* indicated potential microbial roles in energy metabolism, nutrient acquisition and storage, and environmental acclimation (Har et al. 2015).

Here we describe the bacterial microbiome of *Edwardsiella andrillae*, an ice-inhabiting actiniarian that lives with its body column in the ice at underside of the Ross Ice Shelf, with the tentacle crown and mouth at the ice–water interface (Fig. 1; Rack et al. 2012). *E. andrillae* is the first actiniarian known to have an ice-associated lifestyle. Although broad salinity tolerance is fairly rare across sea anemones (Shick 1991), it is most common in Edwardsiidae, the family that includes *E. andrillae* (Shick 1991; Hand and Uhlinger 1994; Daly et al. 2012). In its burrows in the dynamic basal ice shelf frontal zone, *E. andrillae* may encounter conditions that are challenging and variable. Their food sources are unknown, as is their role as prey for, e.g., fishes, crustaceans, or other organisms. Temperatures in this aphotic habitat are close to $-2\,^{\circ}C$, although in summer, waters up to $0.4\,^{\circ}C$ above freezing can be advected under the ice shelf from the Ross Sea Polynya (Arzeno et al. 2014). Salinity can range from near zero in meltwater in the ice-boundary layer to 34.6–34.9 ppt in ambient sea water (Jacobs et al. 2002).

Microbes are key to the ecosystems of the "cryosphere" (reviewed in Boetius et al. 2015) and may

play a role in the adaptive capability of *E. andrillae* to life in the ice shelf. Our preliminary observations of the *E. andrillae* microbiome include its diversity, community structure, and phylogenetic and functional context. Our study of the microbiome of *E. andrillae* benefits from its close phylogenetic relationship to *N. vectensis*, facilitating comparisons with the *N. vectensis* microbiome (Har et al. 2015). We also compared the *E. andrillae* microbiome to the only other Antarctic anthozoan microbiome data set known to us (*Alcyonia antarctica*; Webster and Bourne 2007).

Materials and Methods

Sample collection

The Remotely Operated Vehicle Submersible Capable of Under-Ice Navigation and Imaging (ROV SCINI; Cazenave et al. 2011) was deployed through a ~ 30 cm borehole in the Ross Ice Shelf. Specimens of *E. andrillae* were recovered using a makeshift suction sampler (see Daly et al. 2013 for details) at dive site 3 (−77.5267 S, 171.3350 E), 10 km south of the ice shelf edge in late December 2010 (Rack et al. 2012). At that location, the ice shelf was ~ 260 m thick, with ~ 220 m below mean sea level, and the sea floor ~ 570 m below the bottom of the ice (Rack et al. 2012). Once recovered to the surface, anemone specimens were initially stored in sterile tubes with denatured alcohol (the only preservative available on site) then transferred to 96% ethanol after ~ 2 weeks and stored at 4°C. The sample numbers in the manuscript indicate the numbers given to each anemone at the time of collection. Seawater physical and chemical parameters were measured at the ice shelf–seawater interface using a Seabird Electronics SBE19+ CTD and Niskin bottle to collect water for chemical analysis. Results from three replicate CTD casts are presented here.

Microbiome sampling

Five specimens of *E. andrillae* were each rinsed in sterile seawater and then homogenized by hand with a sterile pellet pestle (Sigma-Aldrich, St. Louis, MO) in 1 mL of sterile seawater in a 1.5 mL microcentrifuge tube. Homogenates were centrifuged for 2 min at 3000 g to pellet the larger cellular debris, and then the supernatant was removed and centrifuged at maximum speed for 10 min to pellet the microbial cells. The cells were resuspended in 200 μL sucrose lysis buffer, and DNA was extracted using an enzymatic digestion [lysozyme (Roche, Branchburg, NJ), proteinase K (Thermo Fisher Scientific, Waltham, MA), and RNAse A (Ambion, Foster City, CA)] followed by phenol:chloroform:isoamyl alcohol purification following Massana et al. (1997). Extracts resuspended in 10 mM Tris-Cl were quantified by fluorescence with Picogreen (Life Technologies, Carlsbad, CA) on a Spectramax Gemini (Molecular Devices, Sunnyvale, CA). The DNA extracts were screened by polymerase chain reaction (PCR) using primers targeting the three domains of life. The bacterial rRNA gene was targeted using primers 27F and 1931R (Lane 1991); the archaeal rRNA gene was targeted using primers 20F (Massana et al. 1997) and 958R (DeLong 1992); and the eukaryal rRNA gene was amplified using primers 960F and 1200R (Gast et al. 2004). Following successful amplification of the 16S rRNA gene to verify amplifiability of the extracts, DNA was prepared for pyrosequencing (MRDNA, Lubbock, TX). Briefly, barcoded amplicon sequencing used bacteria-targeted primers (27Fmod GRGTTTGATCMTGGCTCAG and 519R GWATTACCGCGGCKGCTG) in a single-step 30 cycle PCR with a HotStarTaq Plus Master Mix Kit (Qiagen, Valencia). Amplification conditions were: denaturation at 94°C for 3 min; followed by 28 cycles at 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min; followed by a final elongation step at 72°C for 5 min. The amplicon product libraries were mixed in equal proportions and purified using Agencourt Ampure beads (Agencourt Bioscience Corp., Beverly, MA).

Libraries were sequenced using Roche 454 FLX titanium reagents and instrument following the manufacturer's guidelines at a scale targeting ~3K sequences per library. Combined SFF files received from MRDNA were analyzed using Mothur (version 1.36.1) following the 454SOP (Schloss et al. 2011), which incorporates the PyroNoise algorithm to mitigate errors in amplification and sequencing (Quince et al. 2009). The pipeline utilized seed alignment and taxonomy files provided by SILVA rRNA database project (www.arb-silva.de) Silva.seed_v119.align silva.seed_v119.tax and Perseus (Quince et al. 2011) to detect chimeras, which removed 225 sequences. Sequences with ambiguities were removed, as were all sequences with >8 homopolymers, and sequences shorter than 341 bases. The final data set contained 12,052 sequences (1660–3919 per library; 341–386 bases) that fell into 771 unique Operational Taxonomic Units (OTUs), and 163 OTUs defined at a distance of 0.03 (97 OTUs when singletons were removed). The sff data sets and associated MIMARKS file have been submitted to the National Center for Biotechnology Information (NCBI) under BioProject PRJNA315709, and the

sample metadata are also available at the Microbial Antarctic Resource System (mARS.biodiversity.aq).

Data analysis

OTUs were taxonomically classified in Mothur. Comparative analyses between the microbiomes of anemones Ea3–7 were conducted with data that had been subsampled to the lowest number of sequences for all five anemone microbiomes using Mothur for α (Chao1) and β-diversity (Bray–Curtis) statistics and Daisy Chopper (Gilbert et al. 2009) for presence/absence and relative abundance of taxonomic classes. The subsampled microbiome data sets were clustered based on Spearman rank correlation (we used a nonparametric approach, given the non-linear nature of the occurrence data) using Cluster 3.0 (adapted from Eisen et al. 1998), and the heatmap representation and clustering were visualized using Java Treeview 3.0 (https://sourceforge.net/projects/jtreeview/). Rarefaction and rank abundance curves derived from Mothur were based on observed samples of uneven size. Phylogenetic diversity comparisons of microbiomes from *E. andrillae* and *N. vectensis* (Har et al. 2015) were a focus of this study, although we also included the clone library and culture-based sequence data from *Alcyonium antarcticum* sampled in McMurdo Sound ($\sim$ 118 km from Coulman High; Webster and Bourne 2007). All sequence data sets were aligned using the SILVA Incremental Aligner (SINA) aligner and bacterial variability profile (Pruesse et al. 2012). The alignment was inspected and trimmed in MEGA (version 6; Tamura et al. 2013), and analyses included generating a distance matrix, calculating evolutionary divergence of sequence pairs between groups, and neighbor-joining phylogenetic analysis using 1000 bootstrap resampling. In all pairwise comparisons, ambiguous positions were removed from each pair. Subtrees were generated from the same analysis.

Results and Discussion

Other than the taxonomic characterization of *E. andrillae*, virtually nothing is known of the life history of this anemone, nor the ecosystem characteristics at the ice shelf–seawater interface. One potentially critical aspect for understanding *E. andrillae* and its ecology is understanding the associations between the anemone and natural microbiota in the under-ice shelf ecosystem. We attempt to view the *E. andrillae* holobiont in a similar framework as is currently done with corals and other species for which species-specific associations, metagenome-encoded metabolic functions, and even metatranscriptome-expressed responses provide a much more complete picture of host life, in an ecosystem bathed in microbial food sources, allies, and adversaries (Kelly et al. 2014; Ainsworth et al. 2015; Daniels et al. 2015).

The waters at the ice shelf–seawater interface (mean depth of 256 m) had a mean ($\pm$ standard deviation) temperature of $-1.949 \pm 0.053^{\circ}$C ($n = 3$) and salinity of 34.573 ± 0.017 psu, suggesting little fresh water at the interface, which was close to the pressure-dependent freezing temperature. The inorganic nutrients were replete with NO_3-N (419 mg m^{-3}) and dissolved reactive phosphorus (DRP; 65 mg m^{-3}), giving a N:P molar ratio of 14.25 that is lower than the Redfield ratio of 16, and not indicative of waters depleted either in P by *Phaeocystis* or in N by diatoms in Ross Sea waters (Arrigo et al. 2002).

Despite unconventional sample preservation methods for molecular analysis of microorganisms, DNA yields from microbial cell-enriched preparations of *E. andrillae* retrieved by SCINI in late 2010 were adequate for downstream analysis (0.8–3.3 μg), and 16S rRNA gene PCR products were amplified using bacterial and eukaryal primer sets. Although Archaea have been found in association with other Antarctic marine invertebrates (e.g., Webster et al. 2004; Rodriguez-Marconi et al. 2015), we did not recover any archaeal sequences in the *E. andrillae* microbiome. Thus far, we have not investigated the nature of the eukaryal rRNA gene signal to determine whether there are parasites, fungi, or other commensal eukaryotes associated with the *E. andrillae* host.

Taxonomic composition of the microbiome

Low coverage pyrosequencing of the V1-3 regions of the bacterial rRNA gene was performed to obtain an initial picture of the *E. andrillae*-associated bacterial diversity. Sequences were clustered into OTUs at a distance of 0.03. The complete (non-subsampled) data set had 163 OTUs. The subsampled data set had 126 OTUs, of which 47 were represented by a single sequence and not included in the analysis here, given the risk of errors in sequencing (Kunin et al. 2010; Flynn et al. 2015). The 79 OTUs with two or more sequences remaining in the data set indicated a low–moderate level of diversity, with 10–36 OTUs per anemone microbiome. The OTUs could be classified into six phyla, with Bacteroidetes and Proteobacteria represented by multiple lineages (Fig. 2A). We found two OTUs that were related to each other but could not be placed in a known phylum and thus may represent an undescribed phylum. One phylum, Firmicutes, represented in

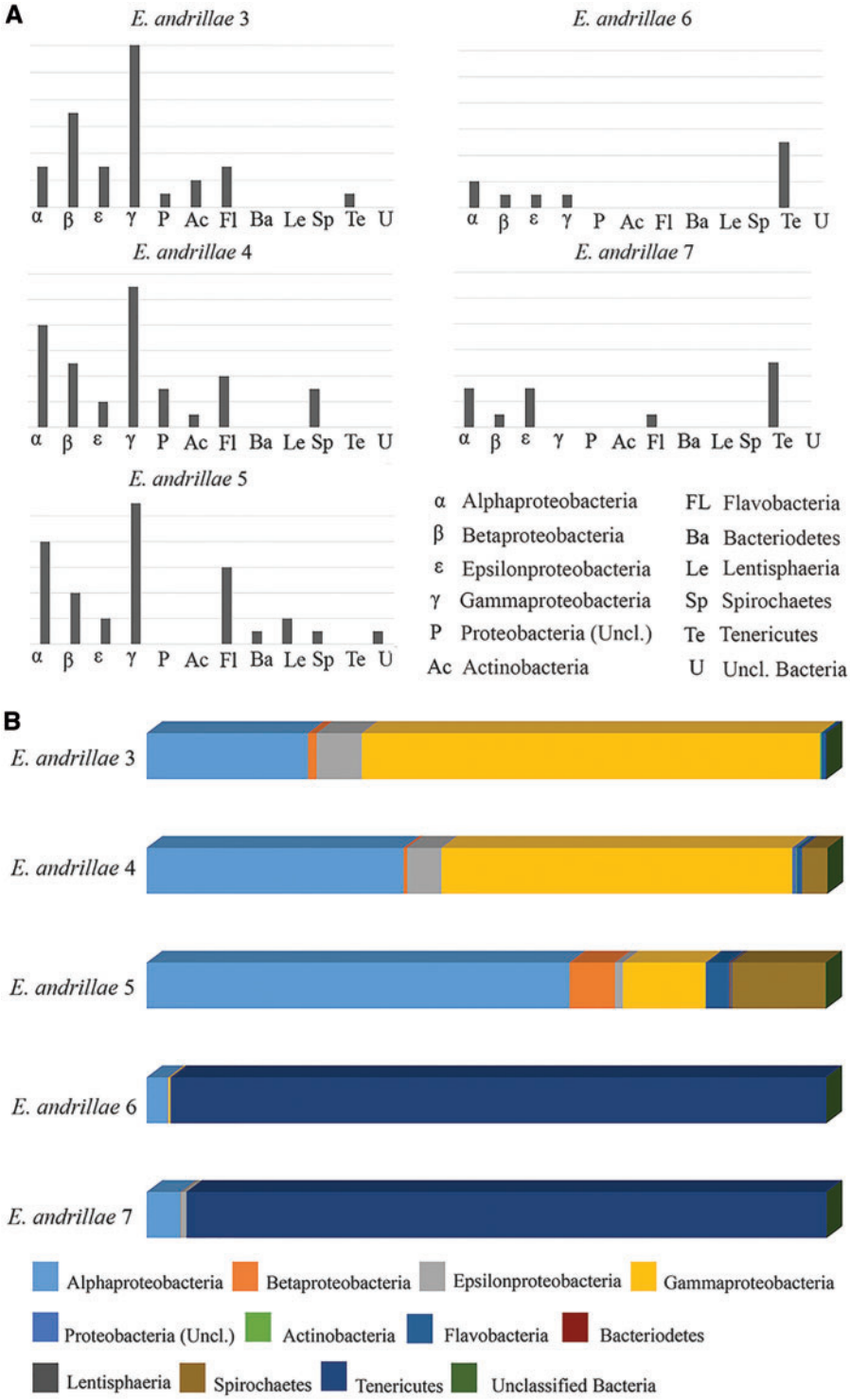

Fig. 2. Class-level taxonomic diversity of microbial OTUs from each specimen of *E. andrillae*. (A) OTU presence/absence across all *E. andrillae* microbiomes sampled and (B) the relative abundance of sequences assigned to taxonomic class. Singleton OTUs are not included. Anemone samples are abbreviated with respect to their collection ID.

the initial data set by one OTU, was eliminated through the subsampling process.

The lineage with the greatest number of OTUs was Gammaproteobacteria, for which there were 12 representatives in one microbiome (Ea5), 11 in two others (Ea3 and Ea4), and interestingly only 1 in Ea6, and none in Ea7 (Fig. 2A). OTUs associated with Flavobacteria, Alphaproteobacteria,

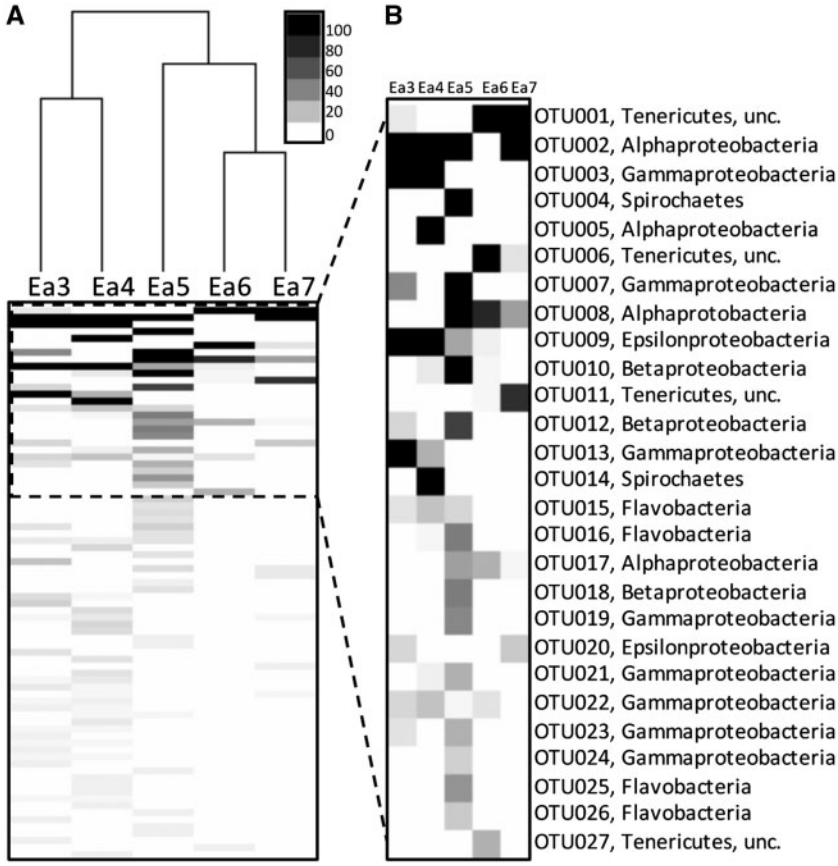

Fig. 3 Heatmap representation of OTU sequence depth across the five specimens of *E. andrillae*. (A) All OTUs with 2 or more sequences detected are shown. (B) Heatmap representation of OTUs >0.5% relative abundance across the five anemone microbiomes. More highly represented OTUs are shown in darker colors (max 92%); white represents a value of 0%.

Epsilonproteobacteria, and Gammaproteobacteria were found in each of the five microbiomes, although there was no single OTU common to all anemones analyzed (Fig. 3). Spearman rank correlation-based clustering indicated that the microbiomes grouped into two pairs of two members each (Ea3 and 4; Ea6 and 7) and one (Ea5) that was more distantly related (Fig. 3). The lack of a common or "core" set of microbial OTUs could be due to undersampling, as the rarefaction curves (Fig. 4A) suggest that the microbiomes were not sampled to saturation; likewise, sample preservation or extraction procedures may not have fully retained the original microbiome diversity.

In comparison, 16S rRNA gene sequence composition of the *N. vectensis* microbiomes from anemones (pooled) at four sites were characterized by bacteria associated with seven phyla (Har et al. 2015). Four of these (Proteobacteria, Bacteroidetes, Spirochaetae, and Tenericutes) also were found in *E. andrillae*. Lentispherae and sequences affiliated with the Betaproteobacteria class of Proteobacteria were unique to *E. andrillae*, and OTUs associated with Chloroflexi, Cyanobacteria, Deferribacteres, and Verrucomicrobia were only detected in the *N. vectensis* microbiome. The microbiome of *Anemonia viridis*, which resides in a different superfamily than *E. andrillae* and *N. vectensis* and which hosts photosymbionts, had three bacterial phyla in common with *E. andrillae* (Proteobacteria, Bacteroidetes, and Actinobacteria) and shows broad representation of Proteobacteria classes, similar to what we detected in *E. andrillae* (Meron et al. 2013). Proteobacteria, Bacteroidetes, and Actinobacteria were also detected in the soft coral, *A. antarcticum* (Webster and Bourne 2007), with Alpha-, Beta-, and Gammaproteobacteria classes in common with *E. andrillae*. Thus at a very coarse level of taxonomic resolution, the groups common to all four of these Anthozoa microbiome studies include Alphaproteobacteria, Gammaproteobacteria, and Bacteroidetes-related bacteria.

Microbiome community structure

The rank abundance distributions varied across the anemone microbiomes (Fig. 4B), with the most distinct difference found in microbiome Ea5, which had

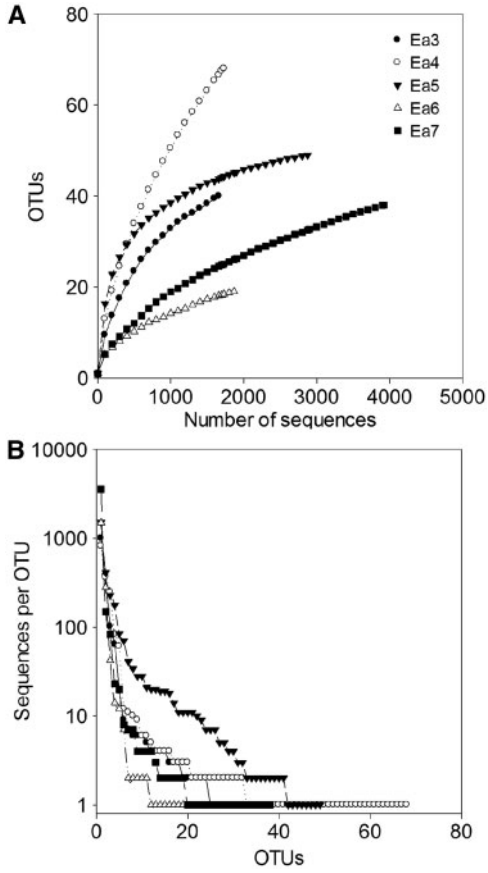

Fig. 4 Microbiome sequence distribution across OTUs are represented in terms of (A) rarefaction plot of observed OTUs for anemone (Ea) microbiomes and (B) rank abundance. The legend applies to both panels.

higher numbers of sequences distributed across more OTUs compared to the other four anemones. The others (Ea3, 4, 6, and 7) had a fewer OTUs with high numbers of sequences and many rare members. Differences in structure are also reflected in the Bray–Curtis pairwise similarity values among the five *E. andrillae* microbiomes: Ea3 and 4, and Ea6 and 7 have values greater than 0.69, whereas all other comparisons have values below 0.26 (Supplementary Fig. S1) These differences are driven by the strong differences in OTUs across the microbiomes (Figs. 2B and 3), most strikingly between samples dominated by a single Tenericutes-related OTU (Otu001; Ea6 and 7; 80.3 and 91.5% relative abundance), those with substantial representation of an Alphaproteobacteria OTU (Otu002; 14–53% relative abundance in Ea3, 4, and 5), and those with abundant Gammaproteobacteria-affiliated *Acinetobacter* sequences (Otu003; 48–60.5% relative abundance in Ea3 and 4). The difference in membership between these two clusters could potentially reflect different microbiome-produced antimicrobial compounds that influence host selection and specificity, as has been observed in corals (e.g., Mao-Jones et al. 2010). Another cnidarian, *Hydra*, has been shown to resist infection through antifungal production by cooperating bacteria associated with the epithelium (Fraune et al. 2015).

The differences among microbiomes from individual *E. andrillae* hosts should be interpreted with caution, as they could reflect limitations of the sequencing effort that would be reduced upon deeper sequencing that provides access to less-well represented sequences. However, the use of short sequences is likely to inflate the perception of similarity rather than of difference, as differences may not be detected in the short fragment used (but may be present in longer sequences). The unconventional sample preservation (storage in ethanol at 4°C for several years) might influence the outcome in ways that would be difficult to predict. Although this is not standard practice for microbial samples, ethanol is widely used for biological sample preservation (e.g., Gaither et al. 2011). Although Anthozoan microbiome preservation approaches have not been rigorously evaluated, host-associated bacterial and mitochondrial DNA can persist for extremely long periods in ethanol-preserved human tissues (Spigelman et al. 2001). Alternatively, the observed differences could reflect real differences in the microbiomes between anemones, an interpretation bolstered by the high degree of similarity within the two pairs of *E. andrillae* microbiomes described here (Ea3 and 4 and Ea6 and 7).

The variable patterns we observed resemble trends in other anemone, gorgonian, and coral microbiomes. Direct comparisons of structure are made difficult by different sampling approaches (e.g., pooling individuals for DNA extraction and sequencing; Har et al. 2015). Nevertheless, between-site and inter-seasonal variation clearly occur in *N. vectensis* (Har et al. 2015), and some samples were highly skewed in representation of single phylotypes (e.g., Epsilonproteobacteria) compared to others, which were much more even in terms of community structure. The microbial community structure of cold-water gorgonians was also similar to *E. andrillae* in that some colonies were dominated by a single Tenericutes phylotype (Gray et al. 2011). The Antarctic soft coral *A. antarcticum*, has a diverse yet perhaps stable and specific microbial community dominated by Gammaproteobacteria (Webster and Bourne 2007). Initial studies that address global trends of coral microbiome structure suggest that habitat and environmental conditions drive composition (Pantos et al. 2015; Roder et al. 2015; Zhang

et al. 2015). Although coral microbiomes vary with species and environment, meta-analysis has also indicated a small, broadly distributed ''core'' of coral-associated bacteria (Ainsworth et al. 2015).

Phylogenetic context for microbial associates of *E. andrillae*

We used SSU rRNA gene sequences of OTUs from the microbiomes of *E. andrillae, N. vectensis*, the Antarctic octocoral *A. antarcticum*, and cloned sequences and closely related neighbors from GenBank to contextualize the microbial diversity associated with *E. andrillae* (Fig. 5A and Supplementary Fig. S2). Our primary objective was to determine the relative relationship between OTUs we recovered from *E. andrillae* to OTUs from other Antarctic (or cold-water) species *versus* OTUs from other sea anemones The anemone microbiomes show a high degree of evolutionary novelty across several lineages. For example, six of the 10 most abundant OUT's in *E. andrillae* are at most 94% identical to previously known sequences, based on Blastn searches of Genbank's nr database. An initial evolutionary comparison among the three microbiome groups indicated considerable distance between all pairs: 0.402 between *N. vectensis* and *E. andrillae*, 0.349 between *N. vectensis* and *A. antarcticum* and 0.278 between *E. andrillae* and *A. antarcticum*. These coarse comparisons suggest that at the level of the microbial community, habitat may be more important than phylogenetic relatedness among hosts.

Most of the sequences that are closely related to the OTUs from *E. andrillae* are environmentally derived and from cold-water, host-associated studies. The phylogenetic diversity of Alphaproteobacteria from *E. andrillae* microbiome sequences is more extensive than in *N. vectensis* and *A. antarcticum* and includes many of the recognized subclasses of this group (Rickettsiales, Pelagibacterales, Holosporales, Rhodospirillales, Sphingomonadales, Rhizobales, and Rhodobacterales; Feria et al. 2013). Given this diversity and the limited length of the sequences ($\sim$ 350 bases), the Alphaproteobacteria were not monophyletic in the complete data set neighbor-joining tree (Supplementary Fig. S2); thus, all sequences in this group were run through a separate neighbor-joining analysis (Fig. 5B). Alphaproteobacteria OTU002, common to four of five *E. andrillae* microbiomes, did not have any related sequences in the *N. vectensis* microbiome but is distantly related to a microbial sequence from a cold-water scleractinian coral (92% sequence identity; FJ041553; Kellogg et al. 2009). This Alphaproteobacterium is likely to be novel at least at the family level, as it is not closely related to any cultivated strains. There were other divergent *E. andrillae*-associated Alphap roteobacteria sequences that also did not cluster with known taxonomic groups; for example, the *N. vectensis* and *A. antarcticum* microbiome data sets had very limited representation in this class. The perception of novelty is not an artifact of the short sequence length, which would tend to obscure novelty: because there are fewer positions at which a sequence can vary, there is a lower chance of these being unique.

There was only one OTU (OTU003; well-represented in Ea3 and 4) affiliated with Gammaproteobacteria related to *Acinetobacter sp.* that was nearly identical (99.7% sequence identity) between the two anemone microbiomes (Fig. 5C). There were closely related sequences associated with *Pseudomonas* present in *E. andrillae* and *N. vectensis* (distance: 0.032) and two cultures from *A. antarcticum* that were closely related to a different *E. andrillae* OTU (Fig. 5C). Two other genera of Gammaproteobacteria also have closely related sequences in some of these microbiomes: there were several highly related *Psychrobacter* sp.-related sequences in both *E. andrillae* and *A. antarcticum* (distance: 0.015–0.026), and sequences affiliated with *Endozoicomonas* were common to *N. vectensis* and *A. antarcticum* (distance: 0.044; Fig. 5D). Although *Endozoicomonas* appear frequently in anthozoan microbiomes (Ainsworth et al. 2015) sequences closely related to *Endozoicomonas* were not detected in our survey of the *E. andrillae* microbiome.

Phylogenetic novelty was also found in spirochaete-related sequences, which were recovered in both *E. andrillae* and *N. vectensis*. These were relatively abundant, as in the case of OTU004, yet only distantly related to cultivated spirochaetes (85% identical to Spirochaeta sp. SR, FJ80060; Supplementary Fig. S2). Likewise, although both anemone species harbor Epsilonproteobacteria-affiliated Campylobacteriales related to known sulfur-oxidizers, the most closely related sequences between the two anemone microbiomes were quite distantly related to each other (distance: 0.172). The most common OTU, OTU009, detected in four of the five *E. andrillae* microbiomes (Fig. 3) is affiliated with a commensal Helicobacteraceae-affiliated sequence reported from a marine gastropod (Supplementary Fig. S2).

Tenericutes were common to both anemone microbiomes, though the dominance of this group in the microbiome of *E. andrillae* was not observed

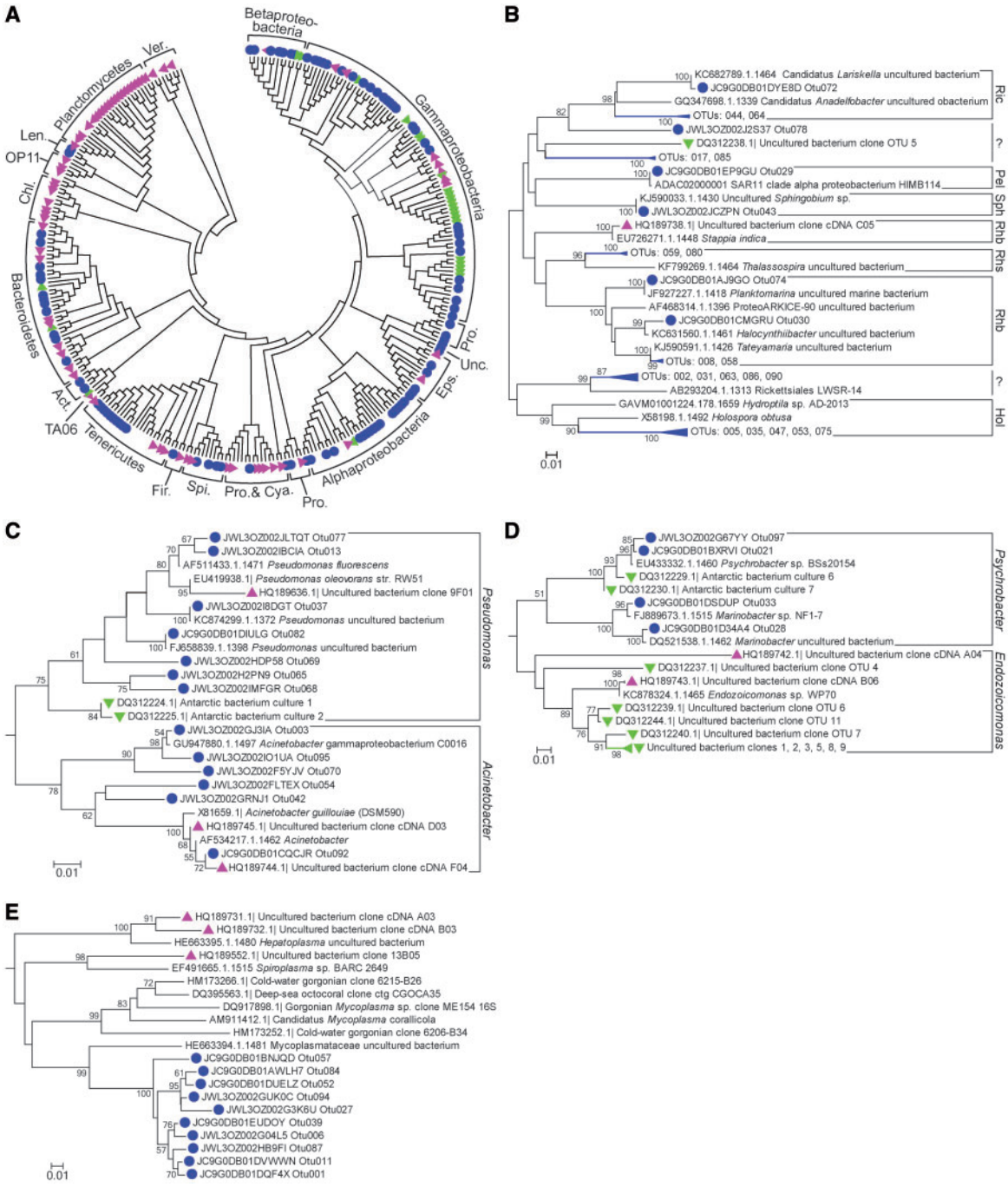

Fig. 5. Neighbor-joining phylogenetic tree and subtrees. (A) Sequences include the *E. andrillae* pyrosequenced data set (blue circles; representatives from each OTU, not including singletons), the *N. vectensis* clone library (pink triangles; Har et al. 2015), the *A. antarcticum* clone library and cultivated bacteria (green inverted triangles; Webster and Bourne 2007) and near sequence neighbors. Abbreviations: Pro, Proteobacteria; Unc, unclassified; Eps, Epsilonproteobacteria; Cya, Cyanobacteria; Spi, Spirochaetes; Fir, Firmicutes; Act, Actinobacteria; Chl, Chloroflexi; Len, Lentisphaera; Ver, Verrucomicrobia. Subtrees highlighting particular aspects of the full tree demonstrate deep diversity of *E. andrillae*-associated Alphaproteobacteria (B), close relationships between Anthozoan microbiome sequences affiliated with Gammaproteobacteria genera *Acinetobacter* and *Pseudomonas* (C) and *Psychrobacter* and *Endozoicomonas* (D), and diverse Tenerricutes-related sequences in microbiomes of Anthozoans including *E. andrillae* and *N. vectensis*, and octocorals from cold water habitats. Subtree abbreviations: Ric, Rickettsiales; Pel, Pelagibacterales; Sph, Sphingomonadales; Rhb, Rhodobacterales; Rhs, Rhodospiralalles; Hol, Holobacterales,? Unclassified sequences. A total of 303 16S rRNA gene sequences were included in the tree; all ambiguous positions were removed for each sequence pair resulting in a total of 504 positions in the final data set. Numbers at the nodes indicated percent of 1000 bootstrap resamplings for values >49. The neighbor-joining subtree in (B) was recalculated with all Alphaproteobacteria sequences using the same parameters for the complete data set, while the subtrees in (C-E) were clipped from the large tree.

in *N. vectensis*, and the Tenericutes sequences in each microbiome were only distantly related (75.4% sequence identity). The nearest neighbor of the Tenericutes OTU (OTU001) that dominated the Ea6 and 7 microbiomes is distantly related to a sequence from the microbiome of a cold-water chiton (88% sequence identity; HE663394.1) and to sequences from other marine invertebrates, including an isolate from the scleractinian coral *Lophelia pertusa* (Fig. 5E; 81% identity, Neulinger et al. 2008).

The microbiome of *E. andrillae*: phylogenetic and functional diversification

Seawater samples were not collected at the same time as our samples of *E. andrillae*. However, based on the biology and patterns of association of the microbial lineages we found in our samples, we expect that some members of the *E. andrillae* microbiome may be free-living or particle-associated bacterioplankton. Of the likely free-living bacterioplankton, the most noteworthy is OTU007 (detected in Ea3 and 5), which is 100% identical to the 16S rRNA gene of Ant4D3, a marine Gammaproteobacterium that was initially reported from a metagenomic study in Antarctic Peninsula waters (Grzymski et al. 2006) and that has been shown to be active in the uptake of amino acids and proteins from Dissolved Organic Matter (DOM) (Straza et al. 2010, Nikrad et al. 2014). Other Gammaproteobacteria associated with *E. andrillae* that are likely free-living and in most cases are highly related to well-characterized "true" psychrophiles from polar marine ecosystems (e.g., sequences were affiliated with Gammaproteobacteria-related *Colwellia psychrerythraea*, *Psychrobacter*, *Marinobacter*, and Flavobacteria-related *Polaribacter, Crocinitomix*, and *Flavobacterium frigidarium*) were not detected in the microbiome of *N. vectensis*. Some of these, e.g., those related to *Psychrobacter* sp. (Fig. 5D), were detected in *A. antarcticum* (Webster and Bourne 2007). Likewise, several of these microbes are frequently particle-associated and could have been ingested with marine snow from the water column.

Several lineages represented in the *E. andrillae* microbiome appear to be diversified, having five or more closely related OTUs across the specimens we sampled. This level of microheterogeneity at the 16S rRNA gene level is common in environmental molecular surveys of both free-living (Thompson et al. 2005) and host-associated (Grzymski et al. 2008) systems. The lineages diversified in *E. andr illae* include several Proteobacteria: (i) Alphaproteobacteria-affiliated Rickettsiales and *Holospora obtusa*-related OTUs (Fig. 5B), (ii) Gammaproteobacteria-related *Acinetobacter* (Fig. 5C), and (iii) Betaproteobacteria-affiliated Comamonadaceae (one sequence was highly related to *Acidovorax* sp. and a denitrifying strain *Comomonas* sp. R-25060). The Tenericutes OTUs in *E. andrillae* also clustered into two groups, with a total of 10 OTUs (Fig. 5E). In all but *Holospora*, the microdiverse sequences were spread across 2–4 specimens; the *Holospora* sequences were all from Ea4. Further interrogation of the data will be necessary to tease out the nature of these microdiverse lineages, and to understand how environmental selection is acting on these genomes within the same population (Shapiro and Polz 2014) and whether they play important roles in host ecology.

Given the large phylogenetic distances between many of the members of the *E. andrillae* microbiome and organisms with well-characterized physiological attributes, as well as the lack of information about the biology and physiology of *E. andrillae*, our understanding of the functional roles played by the members of the *E. andrillae* microbiome is very limited. Nevertheless, there are a few members whose phylogenetic affiliation with lineages having known functions (across a range of coarse to fine distances) are provocative in light of the ecology and biology of *E. andrillae*. The cell-wall free Tenericutes, including the genera *Mycoplasma*, *Spiroplasma*, and *Ureaplasma*, are frequently endosymbiotic, parasitic and/or pathogenic, and are often associated with metazoan guts. Interestingly, this group is relatively commonly detected in cnidarian microbiomes, including those of gorgonians (Gray et al. 2011), stony corals (Neulinger et al. 2008), and jellyfish (Weiland-Bräuer et al. 2015). Although there is shared representation at the phylum level for Tenericutes between the two anemone microbiomes (and with other cnidarians), the phylogenetic distance between the *Mycoplasma*-related *N. vectensis* sequences, those associated with *E. andrillae* (distance = 0.244–0.301), and other cultivated microorganisms is too large to infer robust functional roles.

Another group of potentially parasitic or pathogenic microorganisms abundant in the mircrobiomes of three of five of the anemones were the Alphaproteobacteria-affiliated order Holosporales. These microorganisms are frequently found to be associated with single celled eukaryotes, and the most closely related microbe (*H. obtusa*) to the OUT's in *E. andrillae* (distance of 0.145) is an endosymbiont of *Paramecium caudatum* that has been shown to aid in host thermotolerance (Hori and Fujishima 2003). Though the phylogenetic distance

prohibits detailed functional interpretation, these relationships points to three questions for future study: (i) does the *E. andrillae* holobiont harbor persistent relationships with unicellular eukaryotes; (ii) do members of the microbiome confer assistance in thermotolerance (in this environment-freezing resistance), and (iii) what is the ecology of the host-pathogen relationship in the under-ice-shelf ecosystem, and what means do the anemones use to select for synergistic versus pathogenic microbial invasions? The dynamic habitat in which *E. andrillae* occurs is affected by ice melt and a temporally variable regime of currents and food supply that could influence *E. andrillae* physiology, stress levels, or susceptibility to microbial invasions. However, the relationship between these factors, the anemones and their microbiomes is presently unknown.

The microbiomes of sessile marine invertebrates in general and anthozoans in particular are believed to play a role in antifouling and antimicrobial defense (reviewed by Satheesh et al. 2016). One of the interesting features of marine *Acinetobacter* is that some strains produce compounds that assist the host in through antifouling (Olguin-Uribe et al. 1997) and antimicrobial (Graça et al. 2013) activities. The *Pseudomonas*-related OTUs, too, may be involved in production of antimicrobial compounds.

Understanding the functional roles between microbiome constituents and the host will be best accomplished using complimentary 'omics approaches, if this ecosystem is accessed in the future. However, there are links between elemental cycling and organisms detected in the microbiomes of *E. andrillae, N. vectensis*, and even *A. viridis*. For example, Campylobacterales-related Epsilonproteobacteria in all three anemone microbiomes and Gammaproteobacteria-related Thiotrichales OTUs in *E. andrillae* may be involved with sulfur oxidation either through chemolithoautotrophic or mixotrophic pathways. Har et al. (2015) cultivated strains (*Limnobacter thiooxidans* and *Stappia stellulata*) from *N. vectensis* that are capable of mixotrophic sulfur oxidation, further suggesting the potential for sulfur metabolism and alternative energy metabolisms in the holobiont. The exchange of carbon also may be important between host and microbiome, or even between the host and the free-living plankton. Based on phylogenetic coherence, the majority of the OTUs in the microbiome have relatives with heterotrophic metabolisms. Corals in temperate regions have recently been recognized to function as suppliers of both organic carbon and nitrogen to the microbial loop (Fonvielle et al. 2015). In the under-ice-shelf ecosystem, it is possible that *E. andrillae* might create hot spots of productivity that have a positive feedback in terms of microbial production that then can provide food resources for the community. Understanding this and other potential roles of *E. andrillae* in the under-ice-shelf ecosystem, too, will be an important focus of future studies.

Conclusions and Future Directions

Edwardsiella andrillae lives in a newly recognized and difficult to access ice shelf habitat ~260 m under the ice surface (Rack et al. 2012). The limited number of specimens provides only a glimpse into the biology of the animal and the ecosystem in which it lives (Daly et al. 2013). Although necessarily limited by the material at hand, the present study of the microbial community associated with these anemones broadens our perspective on the ecosystem and hints at a dynamic biological system with high variability among individuals and high novelty in the microbial members.

Our preliminary characterization of the microbiome of *E. andrillae* is unusual, not only because of the host ecology, but also because we have examined the microbiome at the level of the individual host. Although pooling samples may provide a more representative snapshot of diversity at the level of the host population, it obscures potentially important differences among individual hosts. Understanding the ecology of the holobiont requires deeper sampling across individuals than is found in most studies. For example, a minimum of six replicates has been recommended to infer microbiome ecology (Kvennefors et al. 2010), and the variation among our five *E. andrillae* individuals attests to the importance of replicate sampling for recovering the breadth of microbial diversity associated with a host species. Inter-sample variation, at either the individual or population level, and differences among sequencing approaches and depths, make comparative approaches challenging but important.

Although this initial characterization of the *E. andrillae* microbiome provides some additional perspective on both the anemone and its habitat, we find no microbial "smoking gun" capable of explaining how this sea anemone survives at the ice shelf–water interface as has been done, e.g., for ciliates (Pucciarelli et al. 2014). Instead, we have identified a suite of microbes that could contribute to the success of the animal: some bacteria that are affiliated with broad, often endosymbiotic lineages that may span the range of commensal, parasitic, or pathogenic relationships, and others that may be involved

in elemental cycling or chemical defense. Variation across anemones, the lack of comparative samples from anemones in other habitats or from other members of the ice shelf community, and the high degree of novelty of many of the microbial OTUs limit our ability to make inferences about the function and diversity of the microbiome of *E. andrillae*. Future 'omics-enabled studies of host and microbiome, in addition to a deeper survey of microbiome community structure, will be necessary to provide valuable insights into the ecology of *E. andrillae* and other metazoan species in this newly-recognized ice-associated habitat.

Supplementary data

Supplementary Data available at *ICB* online.

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