



Thermal Stress Has Minimal Effects on Bacterial Communities of Thermotolerant *Symbiodinium* Cultures

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Algae in the dinoflagellate family Symbiodiniaceae are endocellular photosymbionts of corals and other cnidarians. This close relationship is disrupted when seawater temperature increases, causing coral bleaching eventually affecting entire coral reefs. Although the relationship between animal host and photosymbiont has been well-studied, little is known about the bacterial community associated with Symbiodiniaceae in culture. We compared the microbial communities of three isolates from different species of the genus *Symbiodinium* (formerly known as *Symbiodinium* clade A) with different ecophysiology, levels of interaction with the animal host, and thermal adaptations. Two species, *Symbiodinium microadriaticum* and *Symbiodinium necroappettens*, exhibit intermediate thermotolerance, with a decrease of both growth rate and photochemical efficiency with increased temperature. The third species, *Symbiodinium pilosum*, has high thermotolerance with no difference in growth rate or photochemical efficiency at 32°C. Microbial communities were characterized after 27 days of growth under control (26°C) and high temperature (32°C). Data shows stronger grouping of bacterial assemblages based on *Symbiodinium* species than temperature. Microbial communities did not group phylogenetically. We found a shared set of fifteen ASVs belonging to four genera and three families that remained in all three Symbiodiniaceae species. These included *Labrenzia*, Phycisphaeraceae (SM1A02), *Roseovarius*, and *Muricauda*, which are all commonly associated with corals and Symbiodiniaceae cultures. Few ASVs differed significantly by temperature within species. *S. pilosum* displayed significantly lower levels of microbial diversity and greater individual variability in community composition at 32°C compared to 26°C. These results suggest that bacteria associated or co-cultured with thermotolerant *Symbiodinium* might play an important role in thermotolerance. Further research on the functional metabolic pathways of these bacteria might hold the key to understanding *Symbiodinium*'s ability to tolerate thermal stress.

Keywords: microbiome, holobiont, thermotolerance, microbial communities, photosymbionts

INTRODUCTION

The holobiont, a host and its associated microbial community, is a concept that we are beginning to understand is of crucial importance for host health, and in some cases for ecosystem health as well. Not only does the holobiont serve as a unit for natural selection, but each constitutive member can increase the plasticity of the whole by their physiological responses to the environment (Ye et al., 2019). For example, lichen microbiota may shift their metabolism to a fasting state under dry conditions, thereby increasing holobiont tolerance to drought stress (Cernava et al., 2019). Furthermore, the resilience of symbiotic microbial communities can directly affect host fitness. Certain bacteria found growing in the roots of desert plants were found to promote drought resistance in wheat (Zhang et al., 2020). While animal-microbe and plant-microbe holobionts have been intensely studied (Bronstein, 2015; Sitaraman, 2015; Hacquard, 2016; Hassani et al., 2018; Mayoral-Peña et al., 2020), microbe-microbe interactions such as phytoplankton-bacteria, mycorrhiza-helper bacteria, and symbiotic bacteriophages (Rodríguez et al., 2008; Seymour et al., 2017) are only recently being recognized as important components of the holobiont. The coral holobiont encompasses the animal (cnidarian host), its endosymbiotic photosymbionts, bacteria, archaea, fungi, and viruses (Thurber et al., 2009). Coral-associated microorganisms are critical to host fitness and survival (Glasl et al., 2016; Sweet and Bulling, 2017). Coral holobionts are highly sensitive to increases in temperature, with only a +1°C increase above long-term summer maximum temperature resulting in bleaching (i.e., disruption of symbiosis between the coral and endosymbiotic dinoflagellate), and increasingly, in coral mortality (Hoegh-Guldberg, 1999; Webster et al., 2016; McDevitt-Irwin et al., 2017; Peixoto et al., 2017).

A growing body of research has started to reveal the role of bacteria in the coral holobiont (Bourne et al., 2016; van Oppen and Blackall, 2019). They can protect the host against pathogenic microbes by preventing colonization through the physical occupation of their potential niches (Ritchie and Smith, 2004). Coral-associated bacteria are known to contribute to carbon cycling (Kimes et al., 2010), sulfur cycling (Wegley et al., 2007; Raina et al., 2009; Tandon et al., 2020), phosphorus fixation, metal homeostasis, organic remediation, secondary metabolism (Zhang et al., 2015), and production of antibiotics (Ritchie, 2006). Changes in environmental stress can alter the fitness advantages of having such functional traits represented in the microbiome. The Coral Probiotic Hypothesis proposed by Reshef et al. (2006) posits that when environmental conditions are altered, a coral's populations of associated bacteria will undergo rapid change that effectively establishes a new, context-dependent coral holobiont. The modified bacterial community is thought to provide an advantage to cope with the new environment (i.e., warmer water). Recently, experiments involving transplantation of bacterial consortia showed improvements to coral host physiology and temperature tolerance (Doering et al., 2021). Elucidating microbial community dynamics during thermal stress and holobiont acclimatization is increasingly important in

understanding the long-term persistence of coral reefs in the face of global climate change (Bellantuono et al., 2012).

For decades, dinoflagellate algae of the family Symbiodiniaceae have been recognized as the driving force behind the high productivity of coral reefs (Freudenthal, 1962; Hoegh-Guldberg et al., 2007; Roth, 2014). Symbiodiniaceae can supply up to 96% of its host's energy budget (Muscatine, 1990) and may help with other important functions such as irradiation and thermal tolerance, with different assemblages being associated with different light and temperature conditions (Rowan et al., 1997; Rowan, 2004; Goulet et al., 2005; Oliver and Palumbi, 2011). Symbiodiniaceae therefore play a key role in coral microbiomes, and likely in their resilience to environmental disturbance as well (Trench and Blank, 1987; Warner and Suggett, 2016). Although the roles of Symbiodiniaceae in coral reefs have been fairly well-documented, these dinoflagellates harbor microbial communities of their own that remain poorly understood (Matthews et al., 2020). Taking advantage of the ability to grow these algae in culture, only three studies have aimed to identify the core microbiome of Symbiodiniaceae (Lawson et al., 2018; Camp et al., 2020; Maire et al., 2021). These studies used highly evolutionarily divergent lineages with diverse physiology, finding little overlap across isolates as well as closely related, conspecific strains. While such studies offer great insight into the similarities and differences between microbial communities of Symbiodiniaceae species with differing physiologies, comparing divergent taxa (what now includes multiple genera) of Symbiodiniaceae overgeneralized the ecology and evolution of diverse lineages (LaJeunesse et al., 2018). Comparing closely-related species with similar physiology can allow for a more detailed understanding of the ecological and evolutionary mechanisms of host-symbiont interactions.

Understanding symbiotic microbial communities holistically is critical to conservation and restoration efforts, as microbial communities may influence holobiont fitness in the face of highly stressful disturbances such as climate change (Putnam et al., 2017). In order to predict future shifts and mitigate coral reef decline, discerning shifts in host- and photosymbiont-associated microbial species during and after thermal stress is crucial. This study aims to further characterize the microbes associated with three thermotolerant photosymbionts (two intermediately thermotolerant and one highly thermotolerant) within the genus *Symbiodinium* (formerly known as Clade A) (Díaz-Almeyda et al., 2017). Herein, we compare three closely related species of *Symbiodinium* with known thermotolerance, grown under normal and high temperature conditions. We hypothesize that phylogenetically related species of *Symbiodinium* (e.g., *Symbiodinium pilosum*, *Symbiodinium microadriaticum*, and *Symbiodinium necroappettens*) with relatively similar thermotolerance will have overlapping microbial communities. We observe bacterial assemblages strongly grouping according to *Symbiodinium* species, with these communities remaining stable under high temperature conditions. We are yet to determine if the bacteria present in each group have redundant functions.

MATERIALS AND METHODS

Sample Culture, Collection, and DNA Extraction

Algal isolates of three species of *Symbiodinium*, formerly known as clade A, were used for this study (Table 1). Cultures were grown in ASP-8A media [pH 8.5, 35ppt, Blank (1987)] at control (26°C) and high (32°C) temperature conditions. Cultures were grown on Percival incubators and placed on a glass shelf on top of full-spectrum fluorescent lights at 100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ facing up, with a 12:12 light/dark cycle photoperiod. Light intensities were measured inside a 300 ml culture flask with media using a 4 π sensor (Biospherical, United States). Cultures were maintained by adjusting cell density to 1×10^5 cells ml^{-1} every 10 days by adding fresh media, acclimated to the control temperature (26°C) for at least 2 months (Supplementary Figure 1A). To measure cell density, a sterile rubber policeman was used to scrape all cells from the flask walls to ensure collection of all cells. The culture was homogenized manually and 1 ml aliquot was collected into a 2 ml screwcap tube. To detach cell aggregations, lugol was added (200 μl) and tubes were vortexed for 5 min. Cells were then counted manually with a hemocytometer. To begin the experiment, replicate 15 ml clear glass tubes containing 8 ml of culture with 1×10^5 cells ml^{-1} were placed vertically on top of a glass shelf with full spectrum lights facing up, in an incubator set at 26 or 32°C ($n = 5$ per isolate per temperature) (Supplementary Figure 1B). Tubes were undisturbed until after 27 days of incubation, when F_v/F_m was measured for all replicates ($n = 5$ per isolate per temperature) and cells were collected for DNA extraction ($n = 3$ per isolate per temperature). Cells were collected by scraping all cells attached to the glass tube with a disposable inoculating loop and centrifuged at approximately 2,000 g for 1 min, removing the supernatant and flash freezing with liquid nitrogen immediately. DNA was isolated using the MO BIO PowerSoil DNA isolation Kit following manufacturer's instructions. A previous study established that *S. microadriaticum*

(strain CassKB8) and *S. necroappetens* (strain RT80) have intermediate thermotolerance, since they were able to grow at high temperature but not as well as at normal temperature. *S. pilosum* (strain RT130) grew at the same rate in normal and high temperature conditions, suggesting that it has higher thermotolerance (Díaz-Almeyda et al., 2017). *S. pilosum* and *S. necroappetens* were collected in the Caribbean and are part of the same isolate collection (formerly known as Trench collection) while *S. microadriaticum* was isolated in the North Pacific (formerly known as BURR collection) (Table 1).

16S rRNA Gene Amplification and Sequencing

Samples were amplified using specific primers pairs (Caporaso et al., 2011) for the V4 region of the 16S rRNA gene. A 25 μL PCR reaction was prepared with 10 μL 5Prime HotMaster Mix (QuantaBio), 0.5 μL forward primer 515F (GTGCCAGCMGCCGCGCGTAA) (10 μM), 0.5 μL reverse primer 806R (GGACTACHVGGGTWTCTAAT) (10 μM), 1 μL of BSA (10 mg/ml), 1 μL template DNA (10 ng), and 12 μL PCR-grade water. PCR amplifications consisted of a 3 min denaturation at 94°C; 30 cycles of 45 s at 94°C, 60 s at 50°C and 90 s at 72°C; and 10 min at 72°C. Samples were submitted for sequencing to the Department of Energy's Joint Genome Institute (JGI) and were processed according to their iTag sequencing protocol. Individual libraries were prepared and barcoded before being multiplexed and sequenced on an Illumina MiSeq sequencer machine in a 2×300 run mode.

Sequence Generation and Processing

Sequences were demultiplexed and processed according to the JGI's iTag amplicon sequencing protocol for taxonomic identification. Initial processing of 16S rRNA gene sequence libraries was performed using the Quantitative Insights Into Microbial Ecology (QIIME2; 2019.7.0) package (Bolyen et al., 2019). Read trimming, filtering, denoising, and amplicon sequence variant (ASV) inference was performed with the DADA2 implementation of QIIME2 (Callahan et al., 2016). Taxonomy was assigned to ASVs using the Naive Bayes classifier pre-trained on the Silva database version 138 clustered at 99% sequence similarity and optimized for the 515F/806R primer pairs. Extraction blanks and template PCR controls were not included as part of our sequencing effort. We therefore applied additional filters to the reads to remove possible contaminants. Initially, ASVs were removed if they were not present in a minimum of three of the 18 samples sequenced. Additionally, low abundance ASVs totaling less than 63 reads (0.2 percent of the total reads from the sample with the fewest reads) were removed from each sample. The additional filtering removed rare ASVs, many of which were present in a single sample and highly likely to be contamination of the culture. Filtering reduced the total ASV count from 255 to 124. Phylogenetic diversity metrics were generated with alignments performed with MAFFT and trees generated with FastTree (Supplementary File 1), which was followed by alpha and beta diversity calculations.

TABLE 1 | Cultures used in this study, invertebrate species from which the culture was isolated, and geographic location where sample was collected.

	<i>Symbiodinium microadriaticum</i>	<i>Symbiodinium necroappetens</i>	<i>Symbiodinium pilosum</i>
Culture name	CassKB8	RT80	RT130
Collected from	<i>Cassiopea xamachana</i>	<i>Condylactis gigantea</i>	<i>Meandrina</i> sp.
Origin	North Pacific	Caribbean	Caribbean
Temperature tolerance	intermediate	intermediate	tolerant
Growth rate (K) at 26°C	0.446 \pm 0.163	0.423 \pm 0.005	0.42 \pm 0.008
Growth rate (K) at 32°C	0.354 \pm 0.014	0.331 \pm 0.112	0.407 \pm 0.125
F_v/F_m at 26°C	0.594 \pm 0.047	0.538 \pm 0.045	0.631 \pm 0.014
F_v/F_m at 32°C	0.373 \pm 0.069	0.354 \pm 0.035	0.545 \pm 0.027

For detailed physiological data summarized here go to Díaz-Almeyda et al. (2017). Cultures are kept at The Pennsylvania State University.

Differential Abundance and Distance Contribution

Differential abundance testing was performed with DESeq2 [v1.32.0, Love et al. (2014)], against sample abundances normalized by the geometric mean of positive counts. Fold change values were adjusted for dispersion using the adaptive shrinkage estimator from the *ashr* package [v2.2-47, Stephens et al. (2022)], and ASVs differentially abundant beyond a false discovery rate of 0.01 were collected (**Supplementary Table 1**). Contributions of each ASV to Bray-Curtis dissimilarity were calculated for using the SIMPER function from the *vegan* package (v2.5-7) (**Supplementary Table 2**). Permutational multivariate analysis of variance (PERMANOVA, 999 permutations) was performed as well (**Supplementary Table 3**). Initially, samples were rarefied to 30,000 reads and pairwise comparisons were performed between 26 and 32°C (**Supplementary Table 4**).

Data Visualization

Data visualization, including PCoA plots of unweighted and weighted UniFrac distance results calculated in QIIME2 were generated with qiime2R (v0.99.6)¹ and phyloseq [v1.36.0, McMurdie and Holmes (2013)] R packages (RStudio Team, 2020). Overlapping taxa (grouped at the genus level) between species and temperature were identified using the online tool from VIB/Ugent Bioinformatics and Evolutionary Genomics Website.²

RESULTS

Bacterial Communities Are Specific to *Symbiodinium* Species

The microbial communities of each *Symbiodinium* sp. were characterized via sequencing of the V4 region of the 16S rRNA gene. We recovered an average of 50,623 raw reads per sample. After filtering and processing the sequences, the average number of sequences per sample was 48,507, with a minimum of 31,090 and maximum of 68,716. After removal of low abundance taxa, we identified 124 bacterial ASVs across all samples and we summarized them at the genus level by replicate (**Supplementary Figure 2**) and by treatment and species (**Supplementary Figure 3**). Samples grouped largely by *Symbiodinium* species when clustered by Bray-Curtis dissimilarity (**Figure 1**). Permutational multivariate analysis of variance (PERMANOVA) confirmed diversity composition significantly varied by species (999 permutations, q -value < 0.05), indicating *Symbiodinium* species harbor specific microbial communities (**Supplementary Table 3**).

Comparison of community composition with SIMPER identified less than 20 ASVs driving 85% of the observed dissimilarity between species (**Supplementary Table 2**). Additionally, OM182_clade, which accounted for the greatest percentage of dissimilarity between *S. microadriaticum* and

S. necroappetens, was absent from *S. microadriaticum*, while present in both *S. necroappetens* and *S. pilosum* (**Figure 2**). *Labrenzia* accounted for 18.9% of dissimilarity between *S. pilosum* and both other *Symbiodinium* species. These findings were further corroborated by differential abundance analysis with DESeq2. We found the aforementioned taxa to be differentially abundant between *Symbiodinium* species (**Supplementary Table 1**).

A closer inspection of these ASVs revealed that they belong to a small subset of the genera found across the three species, including *Muricauda*, *Balneola*, *Labrenzia*, *Phaeodactylibacter*, *Sphingorhabdus*, and OM182_clade, each with >8% influence in respective species comparisons (**Figure 3** and **Supplementary Table 2**). Notably, four genera (*Muricauda*, *Labrenzia*, *Roseovarius*, and the uncultured SM1A02 group of the family Phycisphaeraceae) were commonly found across the three *Symbiodinium* species at both 26 and 32°C, with each dinoflagellate taxa associating with presumably a unique strain represented by a single ASV.

Symbiodinium microadriaticum harbored the greatest number of unique microbial genera (37 at 26°C and 36 at 32°C), whereas *S. pilosum* harbored the least, with seven species specific genera at both 26 and 32°C (**Figure 4**). No additional genera were shared between *S. pilosum* and *S. necroappetens* beyond the aforementioned four taxa, whereas *S. microadriaticum* and *S. necroappetens* shared 13 to 14 genera at 26 and 32°C, respectively.

While the four shared genera were often found to exhibit high relative abundance within the samples, other taxa were represented in greater abundance within each *Symbiodinium* species. In *S. microadriaticum*, *Phaeodactylibacter* (15.21–16.75%) and usually a member of the OM60(NOR5) clade of Halieaceae (6.08–15.80%) were the dominant genera. In *S. necroappetens*, OM182_clade of Gammaproteobacteria (17.51–21.96%), *Balneola* (13.89–24.78%), *Marinobacter* (5.89–17.18%), and *Labrenzia* (11.14–18.01%) were the dominant genera. In *S. pilosum*, *Muricauda* (15.09–21.61%), *Sphingorhabdus* (8.60–18.06%), and *Labrenzia* (38.32–49.74%) were the dominant genera (**Figure 3**).

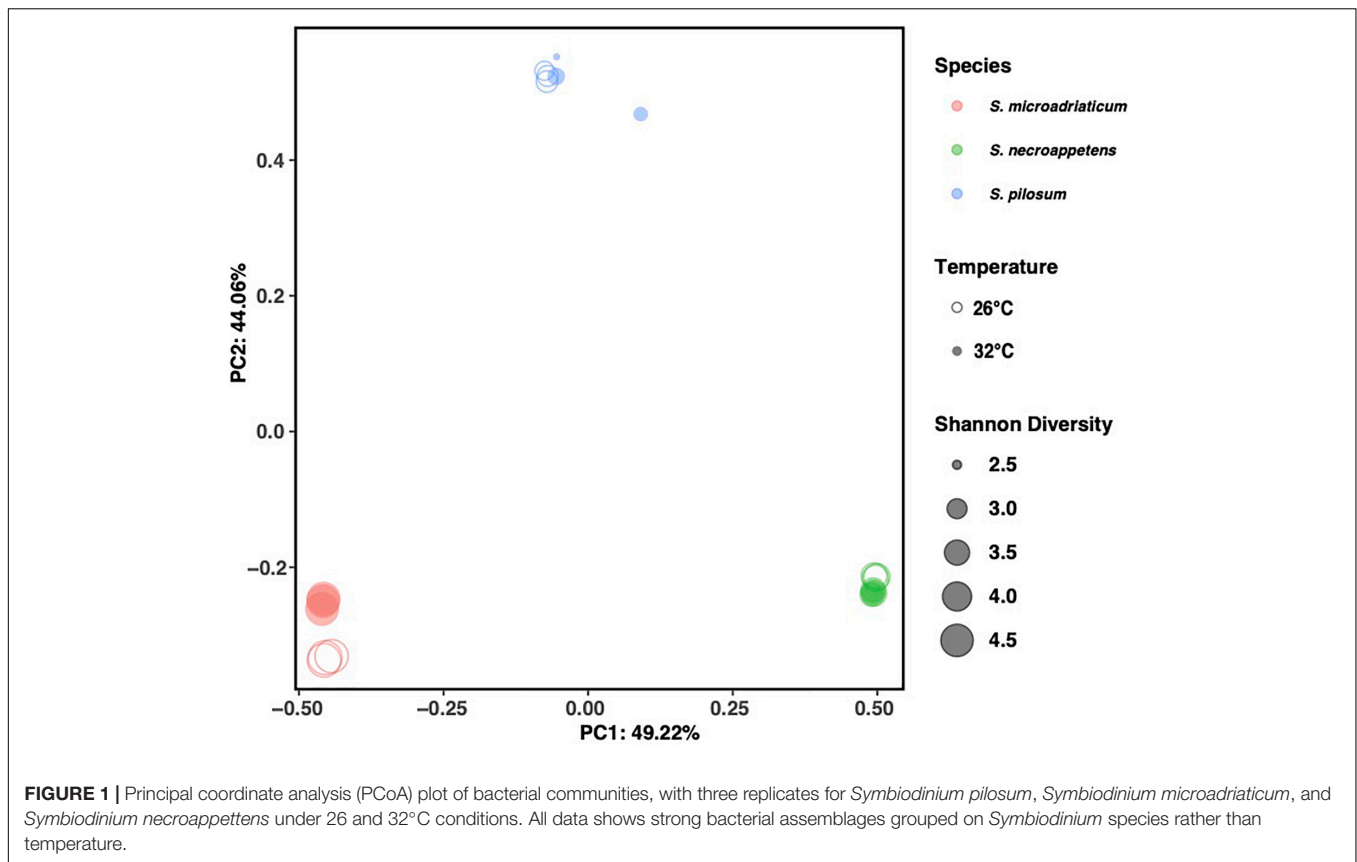
Diversity of Intermediately-Thermotolerant *Symbiodinium* Remains Relatively Stable Under Elevated Temperatures

Shannon Diversity Index (H') values were calculated for each sample (**Figure 5**). *S. microadriaticum* had the highest average H' with no significant differences between temperatures (4.67 at 26°C and 4.78 at 32°C), followed by *S. necroappetens* ($H' = 3.60$ at 26°C and 3.61 at 32°C). *S. pilosum* had the lowest average H' of 3.04 at 26°C and 2.67 at 32°C. One-way ANOVA analysis showed significant differences between species [$F_{(5,12)} = 218.3432$, $p < 0.0001$] but only *S. pilosum* had a significant change in H' between the two temperatures (Tukey HSD, $p = 0.0053$).

Our samples did not cluster well by temperature (q -value > 0.05). We did, however, find ASVs that were significantly

¹<https://github.com/jbisanz/qiime2R>

²<http://bioinformatics.psb.ugent.be/webtools/Venn/>



differentially abundant between 26 and 32°C. Seventeen ASVs were differentially abundant in cultures of *S. microadriaticum*. Two of the differentially abundant taxa belonged to the shared genera *Labrenzia* and *Muricauda*, and increased by 8- and 2-fold (\log_2), respectively. In *S. necroappetens*, both bacterial genera exhibited differential abundance, with *Labrenzia* increasing 5-fold (\log_2) and *Muricauda* decreasing in relative abundance by 5-fold (\log_2). Nine ASVs were differentially abundant in *S. pilosum*, although none belonged to the four genera commonly found between the three dinoflagellate species.

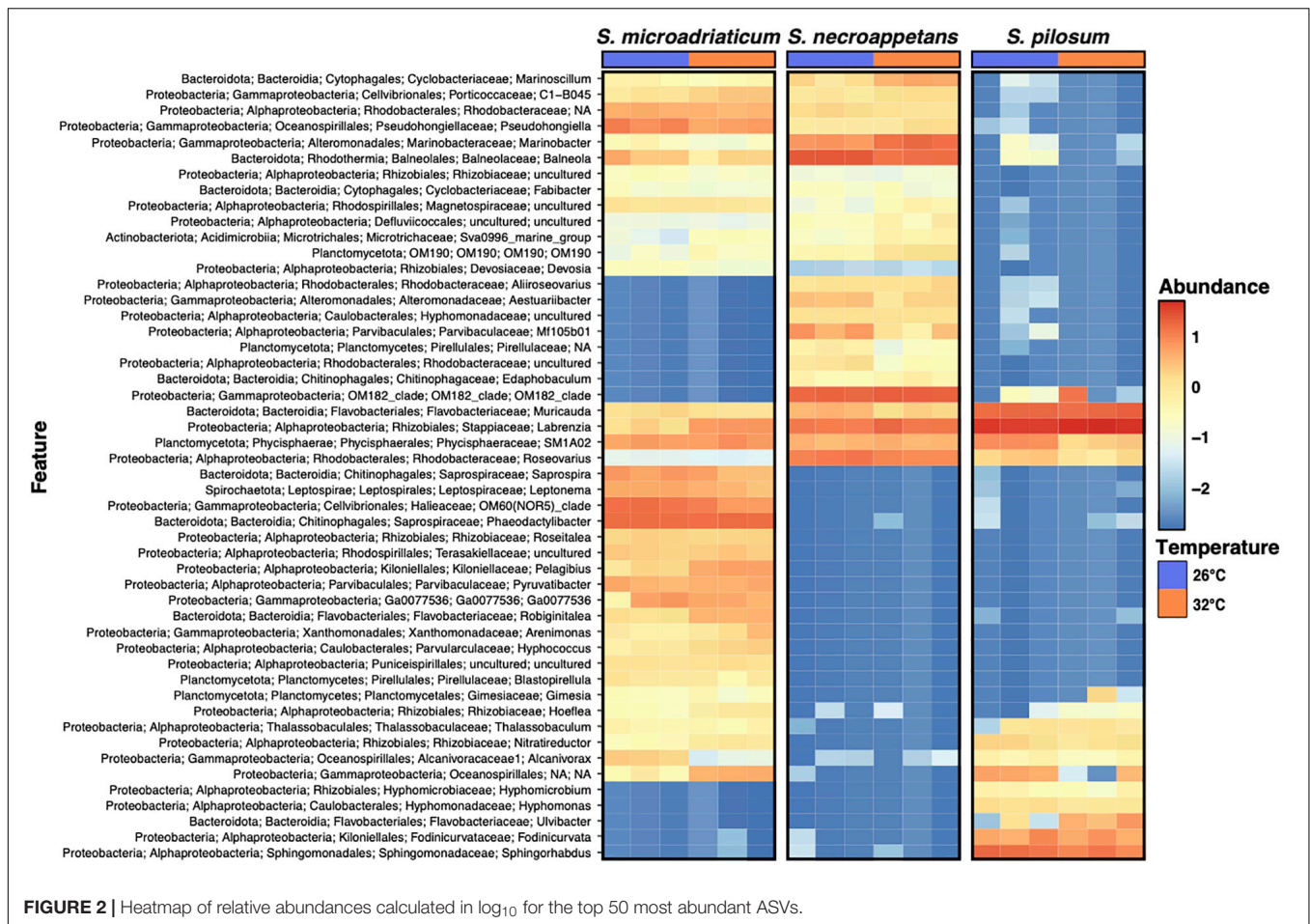
DISCUSSION

We compared the bacterial communities of closely related *Symbiodinium* isolates that have been previously characterized with similar thermotolerance in culture (Díaz-Almeyda et al., 2017). Similar to previous studies, we found microbial communities of *Symbiodinium* clustered primarily by species (Lawson et al., 2018; Camp et al., 2020; Maire et al., 2021). Of the three *Symbiodinium* species included in our study, two intermediately thermotolerant species (*S. necroappetens* and *S. microadriaticum*) had greater microbial diversity than the highly thermotolerant species (*S. pilosum*). The community composition of *S. necroappetens* remained relatively stable at 32°C, while that of *S. microadriaticum* clustered separately by temperature, and the community composition of *S. pilosum*

became more dispersed at 32°C (Figure 1). Relatively few ASVs differed by temperature, and there were no ASVs that consistently increased or decreased in abundance across all species, although we detail why some of them may be notable. More evidence is needed to determine whether differences in the relative abundance of any thermally differing taxa influence *Symbiodinium* thermotolerance (or vice-versa). Our results demonstrate that even closely related *Symbiodinium* species with similar physiology are host to unique assemblages of microbes which may differ in their responses to increased temperature.

Symbiodinium Species Share Few Associated Bacterial Genera

All three *Symbiodinium* species shared members of *Muricauda* (Flavobacteriaceae), *Labrenzia* (Rhodobacteraceae), *Roseovarius* (Rhodobacteraceae), and a member of Phycisphaeraceae (SM1A02). These findings are consistent with previous studies of *Symbiodiniaceae*-associated microbial communities (Lawson et al., 2018; Camp et al., 2020). Importantly, although microbial communities grouped by *Symbiodinium* species, they did not group phylogenetically by *Symbiodinium* species. Rather, they grouped by location and collection of origin (Supplementary Figure 4). *S. necroappetens* and *S. microadriaticum* are more closely related phylogenetically than *S. pilosum* (LaJeunesse et al., 2015). However, *S. necroappetens* had a microbial community more similar to *S. pilosum* than to *S. microadriaticum*. *S. necroappetens* was isolated by Robert

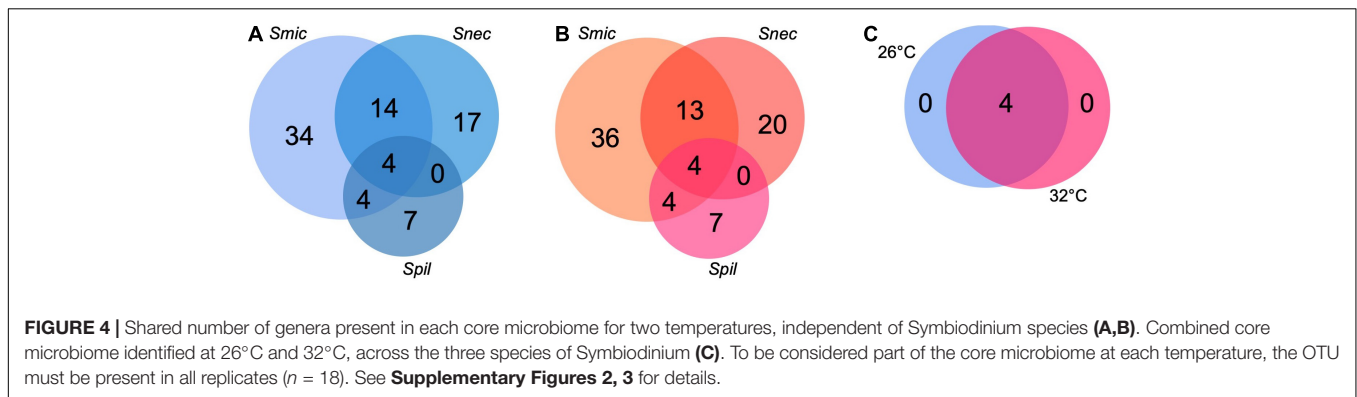
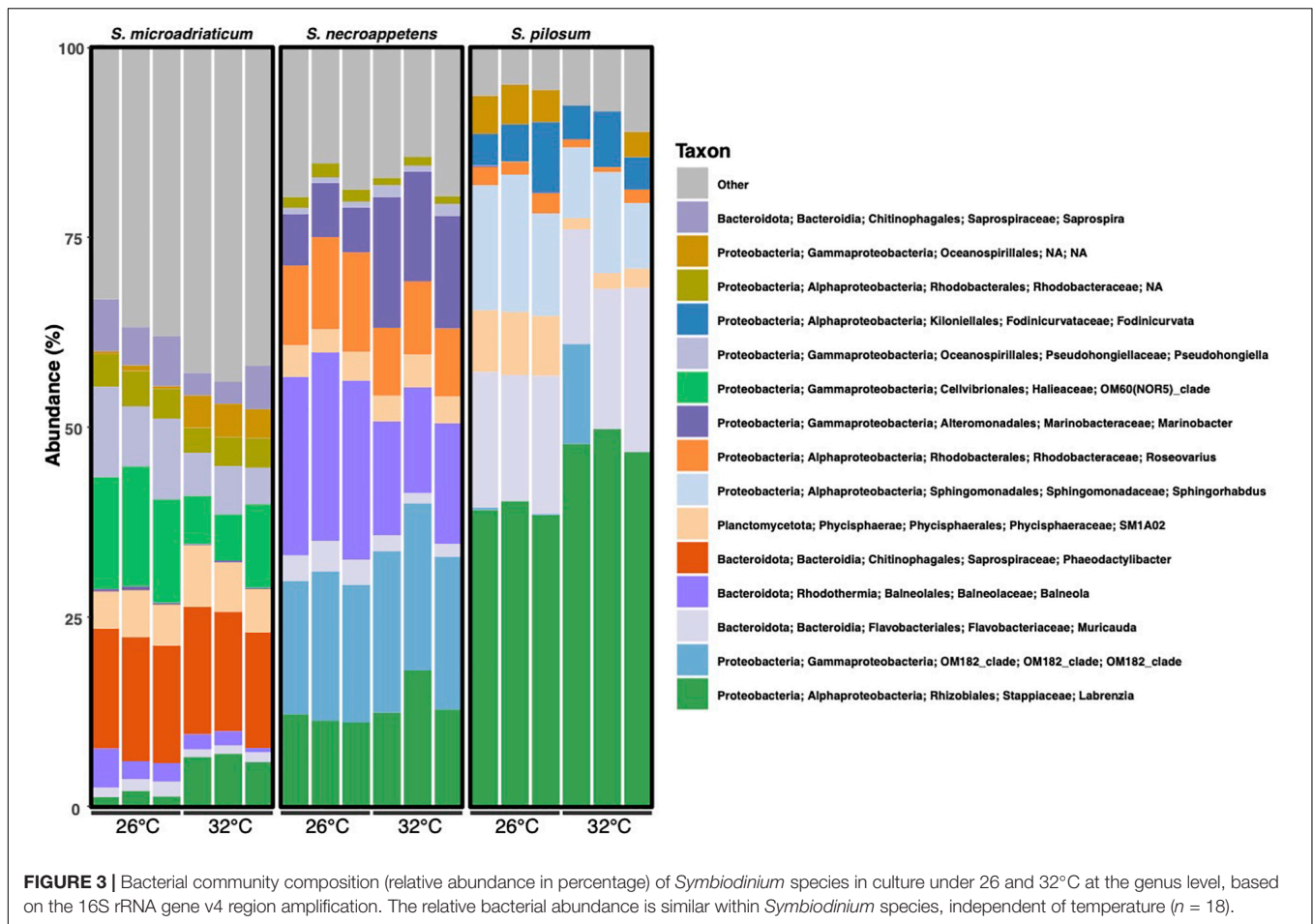


K. Trench at least 20 years ago (LaJeunesse, 2001), *S. pilosum* was isolated at least 27 years ago (Iglesias-Prieto and Trench, 1994), and both were isolated from the Caribbean by the same laboratory with protocols which included adding antibiotics and using a low nutrient culture media to remove bacteria (Schoenberg and Trench, 1980). This may explain the low bacterial diversity we observed in *S. pilosum*. In contrast, *S. microadriaticum* was isolated at least 35 years ago by a different laboratory using f/2 media (Taguchi and Kinzie, 2001). It is therefore unknown whether grouping of bacterial communities is due to long-term culturing artifacts or true symbiotic associations, as these differing protocols may have resulted in very different founder effects, leading to the differences we observed. Maire et al. (2021) found *in situ* samples of Symbiodiniaceae differed greatly from corresponding cultures, suggesting that co-culturing may have influenced our results rather than true symbiosis. The same study also found the presence of intracellular bacteria in Symbiodiniaceae to be common, diverse, and highly distinct from extracellular bacteria. We are unable to separately characterize the responses of intracellular and extracellular communities to thermal stress because we did not distinguish these communities. With these considerations, our results provide insight into the potential connection between *Symbiodinium* thermotolerance and

associated bacterial communities, and point toward future studies on *in situ Symbiodinium* that distinguish between closely- and loosely-associated bacteria.

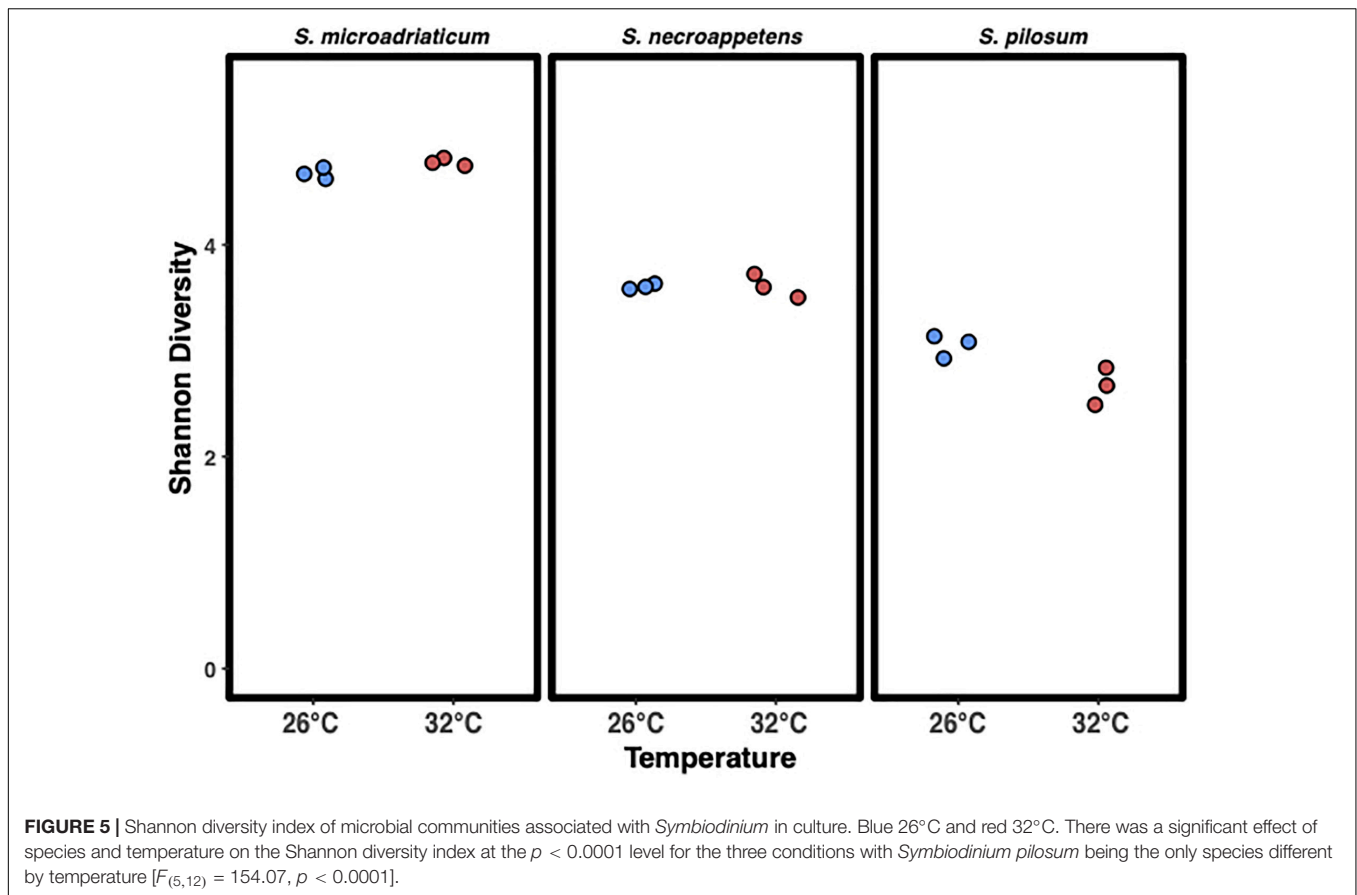
Microbial Community Response to Temperature Stress May Correlate With Host Thermotolerance

Bacterial diversity of *S. microadriaticum* and *S. necroappetans* remained stable at high temperatures while that of *S. pilosum* was significantly lower at 32°C. Stable *H'* values across different environmental conditions can indicate a lack of substantive change in community composition, or that the combined richness and evenness of the community is relatively stable (Kimbrow and Grosholz, 2006; Willis, 2019). The flexibility that we observed in *S. pilosum* may be related to its higher thermotolerance, as acclimating to rapid changes in environmental conditions has been associated with dramatic shifts in bacterial community composition (Vargas et al., 2020; Voolstra and Ziegler, 2020). In contrast to our results, Camp et al. (2020) found that the microbiome of *Durusdinium trenchii*, a thermotolerant Symbiodiniaceae, remained stable at high temperatures.



The bacterial communities of *S. microadriaticum* and *S. pilosum* also clustered by temperature. *S. necroappetens* samples clustered together but not separately by temperature, most likely due to experiencing a much smaller change with temperature. These results support the idea that microbiome responses to heat stress are unique to *Symbiodinium* species, perhaps regardless of dinoflagellate thermotolerance. Our results also suggest that increased thermotolerance may correlate with lower levels of microbial diversity and greater individual variability in community composition. According to the “Anna

Karenina principle,” the increased prevalence of environmental stressors may reduce a host’s ability to regulate its microbiome composition (Zaneveld et al., 2017). While our results did not offer evidence for this principle, if well-supported with future research, the Anna Karenina principle has strong implications for coral reefs in the context of climate change, as a strong link between rising ocean temperatures and abundance of coral pathogens within communities of coral-associated microbes has been described (Tout et al., 2015), and microbial communities without functional redundancy can be subject to environmental



stressors such as ocean warming (Voolstra and Ziegler, 2020). Further research using controlled studies to investigate this relationship may find a link between the thermotolerance of a microbial community and instability of the holobiont under high temperatures. The potential benefits of greater flexibility and diversity in microbiome community composition for thermotolerance should be further explored in future studies using larger sample sizes and other *Symbiodinium* species. Additionally, while thermotolerance is known to be affected by other factors such as genetics, environmental factors, and host-symbiont type mismatches (Brown et al., 2002; Mieog et al., 2009), the degree of contribution (positive or negative) by certain microbial taxa, their potential symbiotic interactions with *Symbiodinium*, and their relationships to *Symbiodinium* thermotolerance remain unexplored and should be considered for future studies.

Notable ASVs and Their Potential Roles in *Symbiodinium* Thermotolerance

No ASVs consistently increased with temperature across all *Symbiodinium* species. Rather, different ASVs were differentially abundant by temperature for each *Symbiodinium* species. While some or all of these differences may be a result of long-term culturing, we offer insights on the potential importance of key ASVs for *Symbiodinium* thermotolerance.

We found *Labrenzia* to be particularly abundant across all *Symbiodinium* species, but especially in *S. pilosum*, the most thermotolerant species. Additionally, *Labrenzia* were not differentially abundant between temperature treatments in *S. pilosum* but were significantly less abundant at 32°C in the two intermediately thermotolerant *Symbiodinium*. *Labrenzia* are known for their ability to produce dimethylsulfoniopropionate, which scavenges reactive oxygen species and thus may play a role in increasing *Symbiodinium* stress tolerance (Díaz et al., 2016; Curson et al., 2017). It is also plausible that these bacteria could be directly reducing pathogenic microbes, which might opportunistically take advantage of heat stressed *Symbiodinium*. An oyster-colonizing *Labrenzia* sp. exhibited broad antimicrobial activity and reduced the growth of a pathogenic *Roseovarius* (Amiri Moghaddam et al., 2018), and labrenzbactin, a siderophore produced by a species of *Labrenzia*, has been shown to exhibit antimicrobial activity *in vitro* (Sharma et al., 2019). However, its effect on *Symbiodinium*-specific pathogens has not yet been explored. The increased prevalence of *Labrenzia* in *S. pilosum* and its relatively stable abundance with temperature stress may be related to thermotolerance, or it may be a result of having lower levels of diversity in general. Research on the ecological and physiological roles of *Labrenzia* for *Symbiodinium* fitness is necessary to discern whether these bacteria are generally mutualistic, parasitic, or commensal partners in heat-stressful conditions.

Roseovarius were also present in all three host species, in much lower relative abundances than *Labrenzia*, and was highest in *S. necroappetens*. Other studies have similarly found *Roseovarius* in relatively low abundance with Symbiodiniaceae (Camp et al., 2020; Maire et al., 2021). Notably, Pootakham et al. (2019) found that *Roseovarius* were one of the two most prevalent genera associated with heat-stressed corals. Members of *Roseovarius* have also been implicated in coral diseases such as black band disease (Ng et al., 2015). However, we did not find that *Roseovarius* significantly increased in relative abundance with increased temperature in *Symbiodinium* (Figure 2). Our results therefore suggest that *Roseovarius* may not play a similar role in heat stressed *Symbiodinium*, or perhaps only in more thermosensitive species. Further research on the functional ecology of *Roseovarius* with *Symbiodinium* under heat stress is necessary to investigate these hypotheses.

Muricauda was also shared by all three *Symbiodinium* species we cultured and were differentially abundant by temperature in the two intermediately thermotolerant *Symbiodinium*, but not in *S. pilosum*. Several studies have demonstrated that *Muricauda* are intimately associated with Symbiodiniaceae (Han et al., 2016; Lawson et al., 2018). In the first comparison of intracellular, closely-associated, and loosely-associated bacterial communities, Maire et al. (2021) found *Muricauda* present in most *in situ* samples of different Symbiodiniaceae species, but consistently in less than 1% abundance. We found *Muricauda* in greater than 1% abundance in all *Symbiodinium*, but especially in *S. pilosum*, in which *Muricauda* consistently accounted for over 15% of each sample. This discrepancy may be the result of culturing, as cultured samples have previously been found to differ substantially from *in situ* Symbiodiniaceae samples (Maire et al., 2021). Regardless of its potentially low abundance in wild Symbiodiniaceae, *Muricauda* is one of 12 bacterial genera ubiquitously associated with various reef-building corals across the globe, suggesting its functional importance not only to Symbiodiniaceae but also to the coral holobiont (Bernasconi et al., 2019). Furthermore, *Muricauda* may serve a functional role in *Symbiodinium* heat resistance. A close relative to *Muricauda lutaonensis* enhanced thermal and light stress resistance of cultured Symbiodiniaceae (Motone et al., 2020). We did not find significant differences in *Muricauda* by temperature for any *Symbiodinium* species, but *in situ* sequencing may indicate whether they play a key role in *Symbiodinium* thermotolerance.

Sphingorabdus were one of the dominant taxa in *S. pilosum* but were not present in greater than 1% abundance in the intermediately thermotolerant host species (Figure 3). *Sphingorabdus* have been isolated from Gorgonian coral and have been successfully cultivated (Keller-Costa et al., 2017; Silva et al., 2018), but otherwise are not well-known, so further research may reveal their importance for *Symbiodinium* thermotolerance.

In *S. pilosum*, a member of Phycisphaeraceae (SM1A02) accounted for the greatest amount of community dissimilarity between temperature treatments, displaying greater abundance at 26°C. This may be due to strong competitive pressure from the highly dominant *Labrenzia* in thermally-stressed *S. pilosum*, as ASVs from Phycisphaeraceae did not significantly decrease

in abundance in the other two *Symbiodinium* species. Kellogg (2019) found three OTUs belonging to Phycisphaerales to be bacterial associates across seven deep-sea corals (Eloe et al., 2011; Allers et al., 2013). Sequences similar to those three OTUs were not associated with tropical corals, possibly suggesting a role specific to cold-water corals (Kellogg, 2019). Contrarily, members of Phycisphaerales were associated with tropical stony corals, and in greater abundance under highly variable environmental conditions by Ziegler et al. (2017). Together with our results, these findings suggest that the observed decrease in Phycisphaeraceae with temperature may not be indicative of thermotolerance, but rather a consequence of the increase in relative abundance of *Labrenzia* in our most thermotolerant host.

In total, no ASVs were indicative of thermotolerance across all *Symbiodinium* species. Rather, we found ASVs that were differentially abundant by temperature differed by *Symbiodinium* species. These differences may be explained by functional redundancy of thermotolerance within and between bacterial communities (Stilianos et al., 2018). While *Symbiodinium*-associated bacterial communities may differ substantially, even among similar *Symbiodinium* species, these communities are likely to converge on key functional traits. For example, members of Rhodobacteraceae have generally been found to associate with other algae and provide vitamins and other amino acids to their hosts (Rosales et al., 2020), and play important roles in marine sulfur and nitrogen cycles (Chen et al., 2011). In microbial communities, many species fulfilling similar functions often coexist (Louca et al., 2018). Since all our cultures contained diverse members of Rhodobacteraceae, functional stability with stress could theoretically be maintained despite each culture containing different representatives of this family. For example, soil microbial communities showed high functional stability in long-term drought (Yuste et al., 2014). Future research on the relationship between phylogenetic diversity and functional stability may evidence similar consistencies in functional stability of dinoflagellate microbial communities under long-term increases in heat stress driven by climate change.

Additionally, high rates of horizontal gene transfer in long-term co-cultures could result in changes in the functional roles of different taxa, including those that may affect *Symbiodinium* thermotolerance (Cairns et al., 2018). This could have led to the development of similar thermotolerance-related functions in different taxa, leading to dissimilar patterns in bacterial community change with heat stress across cultures. While shifts in the functional roles of different community members have not been well-documented in long-term co-cultures, research on the human gut microbiome suggests that high rates of horizontal gene transfer and selective pressure have promoted functional redundancy in gut microbial communities despite wide diversity in microbiome community composition (Tian et al., 2020). More similar to our study system, biofilm bacterial communities of the kelp *Ecklonia radiata* commonly had horizontal gene transfers between bacterial members of the same class or order, and these transfers often included genes involved in stress response (Song et al., 2021). More research on Symbiodiniaceae is necessary to determine whether functional redundancy may be important for thermotolerance rather than any one bacterial taxa in particular.

CONCLUSION

In conclusion, we show that closely related species of *Symbiodinium* cultures with similar thermotolerance have specific bacterial assemblages that remain relatively stable under high temperature conditions. While differences in relative abundance of key bacterial genera were not consistent across *Symbiodinium* species, significant differences in certain genera by temperature treatment within *Symbiodinium* species suggest functional redundancy within bacterial communities. Future studies should focus on isolating symbiotic bacteria and assessing their interactions with *Symbiodinium* and with each other. Experimenting with axenic *Symbiodinium* isolates in culture with specific bacterial isolates can allow us to determine the effects of the specific microbes, which species might be essential, and whether those species can influence the physiology of *Symbiodinium*. Since multiple strains of *Symbiodinium* can be cultured and their physiology is well-known, this phylogenetic group can aid in characterizing the role of microbes in functional and physiological diversity of their symbionts. Additionally, metagenomes of bacteria associated with *Symbiodinium* can inform about the metabolic capabilities to hypothesize functional roles and if the interactions between these bacteria and *Symbiodinium* are mutualistic, neutral or pathogenic.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, PRJNA758393.

AUTHOR CONTRIBUTIONS

ED-A and MMe contributed to conception and design of the study. All authors contributed to the data analysis. AHO, TR, and FP performed the statistical analysis. ED-A wrote the first draft of the manuscript. All authors wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.764086/full#supplementary-material>

Supplementary Figure 1 | Cultures were grown on top of a glass shelf under lights facing up to prevent shading. **(A)** Cultures in flasks were acclimated for 2 months at $100 \mu\text{M}$ quanta $\text{m}^{-2} \text{s}^{-1}$ in a 12:12 hr/light:dark cycle. Cell densities were adjusted to 1×10^5 cells/ml before starting experiments. **(B)** Cultures were moved to their experimental conditions with the previous light and circadian rhythm into their respective temperatures in glass tubes. Cultures started at 1×10^5 cells/ml, F_v/F_m was measured for $n = 5$ on day 27, cells for DNA extraction were collected for $n = 3$ on day 27.

Supplementary Figure 2 | Shared number of genera present in each species core microbiome of *Symbiodinium* under two temperatures (26 and 32°C). Each treatment had three replicates per *Symbiodinium* species per temperature.

Supplementary Figure 3 | Shared number of genera present in each species core microbiome of *Symbiodinium* **(A–C)** over two temperatures (26 and 32°C). The shared core microbiome at 26 and 32°C, independent of *Symbiodinium* species is also represented **(D)**. To be considered part of the core microbiome at each temperature, the genera must be present in all samples ($n = 18$).

Supplementary Figure 4 | Heatmap of relative abundances of bacteria. At the top, a Euclidean distance cladogram shows samples grouped by *Symbiodinium* species.

Supplementary Table 1 | Differential abundances calculated with DESeq2.

Supplementary Table 2 | Differential abundances calculated with SIMPER.

Supplementary Table 3 | Permanova comparisons per species **(A)** and per treatments **(B)**.

Supplementary Table 4 | Number of observed features per sample after alpha rarefaction. Reads were rarefied using the Qiime2 alpha-diversity function. Rarefaction was performed to a maximum depth of 30,000 reads over 20 steps. Feature tables were generated over 10 iterations. The variability in observed features for each iteration are presented as box plots for each sampling depth.

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