ORIGINAL ARTICLE

Bacterial diversity in the *clarki* ecotype of the photosynthetic sacoglossan, *Elysia crispata*

Padmanabhan Mahadevan [iD] | Michael L. Middlebrooks

Department of Biology, The University of Tampa, Tampa, Florida

Correspondence
Padmanabhan Mahadevan and Michael L. Middlebrooks, Department of Biology, The University of Tampa, 401 W. Kennedy Blvd, Tampa, FL 33606.
Emails: pmahadevan@ut.edu (PM); mmiddlebrooks@ut.edu (MLM)

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Abstract

Few studies have examined the bacterial communities associated with photosynthetic sacoglossan sea slugs. In this study, we determined the bacterial diversity in the clarki ecotype, *Elysia crispata* using 16S rRNA sequencing. Computational analysis using QIIME2 revealed variability between individual samples, with the Spirochaetes and Bacteroidetes phyla dominating most samples. Tenericutes and Proteobacteria were also found, among other phyla. Computational metabolic profiling of the bacteria revealed a variety of metabolic pathways involving carbohydrate metabolism, lipid metabolism, nucleotide metabolism, and amino acid metabolism. Although associated bacteria may be involved in mutually beneficial metabolic pathways, there was a high degree of variation in the bacterial community of individual slugs. This suggests that many of these relationships are likely opportunistic rather than obligate and that many of these bacteria may live commensally providing no major benefit to the slugs.

KEYWORDS
bacteria, diversity, metagenomics, photosynthetic, sacoglossan, sea slug

1 | INTRODUCTION

Sacoglossan sea slugs are a group of herbivorous marine gastropods that feed suctorially on siphonaceous green algae. The close relationship that these slugs have with their algal food has caused them to receive some attention for a variety of reasons including their ability to sequester chemical secondary metabolites from host algae (Marin & Ros, 2004), convergent ecological roles similar to terrestrial insects (Rasher et al., 2015) and due to the unusual ability found in some species to sequester algal chloroplasts known as kleptoplasty (reviewed by Pierce & Curtis, 2012; Pierce, Curtis, & Middlebrooks, 2015). Despite interest in some areas of their biology, much about the sacoglossans generally remains poorly studied.

One aspect of the biology of sacoglossans, which has received little attention, is their microbiome. Other photosynthetic animals such as corals and sponges, which photosynthesize through a mutualistic relationship with algae, may rely on bacteria for nitrogen fixation (Shashar, Walter Cohen, Loya, & Sar, 1994; Wilkinson & Fay, 1979). Furthermore, microbial communities have been described living on the mucus coating the epidermis of corals (Ritchie, 2006). These bacteria likely play an important role by producing antifouling, antifungal, and antibiotic compounds and secondary metabolites protecting their larger invertebrate hosts (Shnit-Orland & Kushmaro, 2009). Other bacteria may form important mutualisms with mollusk hosts such as chemoautotrophic bacteria supplementing nutrition in bivalves (Taylor & Glover, 2010) or light-producing bacteria in cephalopods (Ruby, 1996).

Only a few sacoglossan sea slugs, however, have had their microbiome described. *Elysia chlorotica*, which can complete an up to 9-month life cycle relying only on retained chloroplasts (Mondy &

Pierce, 2003; Pierce, Biron, & Rumpho, 1996), was found to have a diverse microbiome that varied between wild-caught and laboratory-reared slugs (Devine, Pelletreau, & Rumpho, 2012). Several potential roles of the bacteria found in association with _E. chlorotica_ include nitrogen fixation and vitamin B_{12} production although these roles are currently only speculative (Devine et al., 2012). The microbiome of _Elysia rufescens_, first characterized by Davis et al. (2013), is associated with the defensive compound kahalalide F (Becerro, Goetz, Paul, & Scheuer, 2001). This compound is synthesized by a bacterium found in the _Bryopsis_ sp. algal diet of _E. rufescens_ (Zan et al., 2019). Kurahashi and Yokota (2007) described a gamma-proteobacterium with unknown roles isolated from _Elysia ornata_, although it should be noted that recent phylogenetic analysis does suggest that this slug native to Japan is likely an undescribed _Elysia_ species, as _E. ornata_ is endemic to the greater Caribbean region (Krug, Vendetti, & Valdés, 2016).

Elysia crispata is a large sacoglossan with a Caribbean wide range and the ability to retain functional chloroplasts for several months after feeding (Curtis, Middlebrooks, Schwartz, & Pierce, 2015; Krug et al., 2016; Middlebrooks, Pierce, & Bell, 2011, 2012). This slug has a wide-ranging diet, capable of feeding on many different species of algae and retaining their chloroplasts (Middlebrooks, Bell, Curtis, & Pierce, 2014; Middlebrooks, Curtis, & Pierce, 2019). Mangrove-dwelling slugs from the Florida Keys with a distinct morphology, when compared to the reef inhabiting _E. crispata_, were previously described as _E. clarki_ (Pierce et al., 2006). However, recent molecular analysis has synonymized _E. clarki_ with _E. crispata_ while retaining _clarki_ as an ecotype (Krug et al., 2016). Davis (2015) described the bacterial community of two populations of _E. crispata_ from the Bahamas and Puerto Rico; however, the bacterial community of the _clarki_ ecotype has not yet been examined. Here, we provide a preliminary description of the bacterial community associated with _clarki_ ecotype _E. crispata_ from the Florida Keys.

2 | METHODS

Eight individual _clarki_ ecotype _E. crispata_ were hand-collected on a snorkel in August 2015 from a borrow pit in Crawl Key, Florida (24°44956.070N, 80°58942.770W), a site used for several studies on this species before the slug population at this location being eliminated by Hurricane Irma (Clark, 1994; Middlebrooks et al., 2014; Middlebrooks, Curits, & Pierce, 2020; Pierce et al., 2006). Specimens were immediately preserved in 95% ethanol until DNA extraction. The Zymo Research Quick-DNA MiniPrep kit was used to isolate genomic DNA from the _E. crispata_ samples according to the manufacturer's instructions. Eight samples of DNA isolated from _E. crispata_ were sent for 16S rRNA sequencing to MRDNA (Shallowater). Briefly, the 16S rRNA V4 variable region was amplified using 515/806 PCR primers in a 30 cycle PCR with the HotStarTaq Plus Master Mix Kit (Qiagen) using the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s, and

72°C for 1 min, and a final elongation step at 72°C for 5 min. DNA libraries were prepared using the Illumina TruSeq DNA library preparation protocol, and sequencing was performed on a MiSeq following manufacturer's guidelines.

QIIME2 (Bolyen et al., 2019) was used to analyze the sequence data. The data were imported using the _import_ command with _EMPPairedEndSequences_ type and demultiplexed using the _demux_ command. DADA2 (Callahan et al., 2016) was used to denoise, trim the sequences, and remove chimeras, and the resulting sequences were classified using the _feature-classifier_ command (Bokulich et al., 2018) with a pre-trained Naïve Bayes classifier trained on the SILVA v.132 99% OTUs full-length sequences (Quast et al., 2013). The phylogenetic tree was constructed in QIIME2 using MAFFT (Katoh, Asimenos, & Toh, 2009) and FastTree (Price, Dehal, & Arkin, 2010). The tree was visualized using the iTOL (Interactive Tree of Life) online tool (Letunic & Bork, 2019). The Piphillin server (Iwai et al., 2016) was used to predict functional and metabolic information for the bacteria present in the samples using both the October 2018 version of the KEGG (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017) and BioCyc version 22.5 (Karp et al., 2019) databases, with a sequence identity cut-off of 97%. The resulting KEGG pathway output was interpreted using the KEGG Mapper (Kanehisa et al., 2017), and the BioCyc pathway output was interpreted using the Omics Dashboard (Paley et al., 2017).

3 | RESULTS

The rarefaction curve (Figure 1) shows that at a sequencing depth of approximately 2,500, the Shannon diversity index which measures species diversity plateaus, indicating that species richness has been captured by our sequencing. Samples 2 and 4 show a higher Shannon diversity index than the other samples. Initially, the taxonomic analysis showed an abundance of chloroplasts most closely matching the algae _Halimeda discoidea_ in all samples, ranging from a relative frequency of 70% to 98%. This is not surprising since _E. crispata_ feeds on these algae and sequesters their chloroplasts. To obtain better resolution of the other bacterial taxa, chloroplasts were filtered out from the data. Figure 2 shows a bar plot of the various bacterial taxa corresponding to the phylum level in the eight _E. crispata_ samples. There is a good degree of inter-individual variability of bacterial taxa in the _E. crispata_ samples. These are discussed by taxonomic rank below:

3.1 | Phylum

Samples 2, 4, and 7 were dominated by Spirochaetes, ranging from 69% to 95%, while samples 5 and 6 had low relative frequencies of Spirochaetes with 12% and less than 1%, respectively (Figure 2). Bacteroidetes were present in all samples, with relative frequencies ranging between 2% and 90%. Indeed, samples 1, 3, 5, 6, and 8 were dominated by Bacteroidetes. The Tenericutes were present in
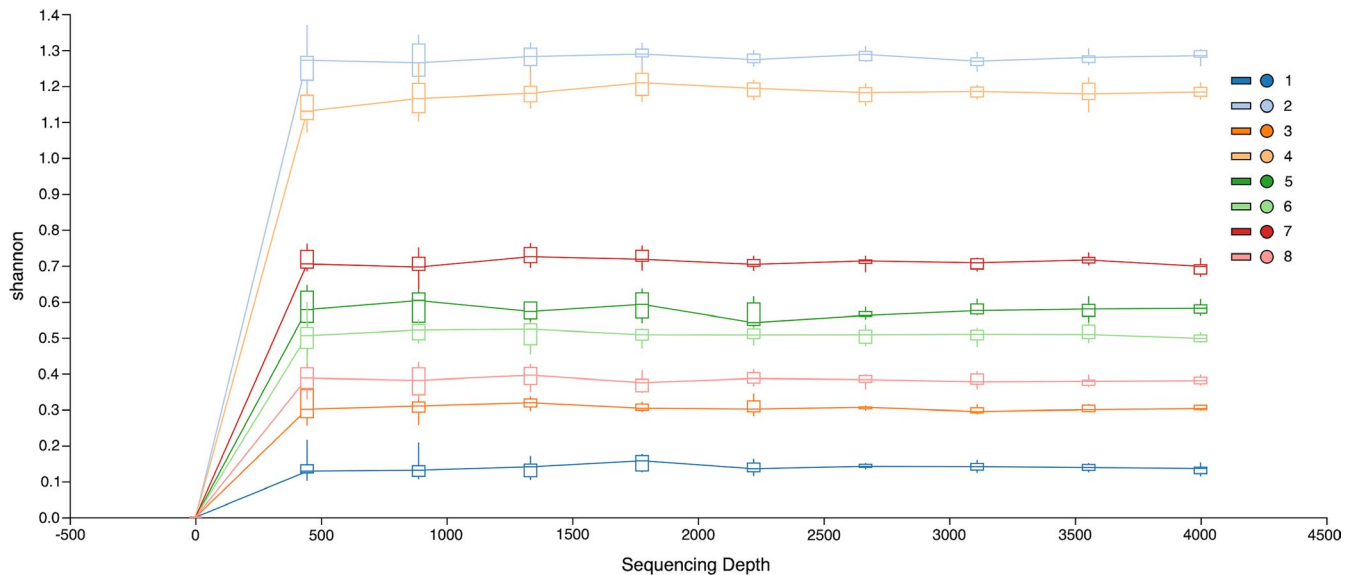
FIGURE 1 Rarefaction curve showing sequencing depth vs. Shannon diversity for 8 samples of *Elysia crispata*

samples 1, 2, 6, 7, and 8, with sample 1 having the highest relative frequency of 37%. The Proteobacteria were present in samples 1, 2, 3, 4, 5, 7, and 8, with sample 1 having the highest relative frequency at 16%, with the others having low relative frequencies of 7% or much less. The Patescibacteria were only found in sample 4, with a very low relative frequency of less than 1%. Similarly, the Firmicutes were only found in samples 1 and 8, with low relative frequencies of 2% and less than 1%, respectively. Sample 3 was the only one that contains Cyanobacteria, with a relative frequency of approximately 6%. The Planctomycetes were only found in sample 3 with a relative frequency of 4%.

3.2 | Class, order, family, genus, and species

The Spirochaetia (belonging to Spirochaetes phylum) were found in samples 2, 4, 5, 6, and 7. All samples had the Bacteroidea class (belonging to Bacteroidetes phylum). The Mollicutes (belonging to Tenericutes phylum) were found in samples 1, 2, 6, 7, and 8. Three classes of Proteobacteria were found in the samples which are Deltaproteobacteria, Gammaproteobacteria, and Alphaproteobacteria. The Deltaproteobacteria were found in samples 1, 4, 5, and 7 with relative frequencies ranging between less than 1% and 16%. The Gammaproteobacteria were found in samples 2, 3, and 8 at relative frequencies between less than 1% and 6%. The Alphaproteobacteria were only found in sample 8 at a relative frequency of 2%. The Gracilibacteria (belonging to Patescibacteria phylum) was only present in sample 4. The Bacilli (belonging to Firmicutes phylum) were only found in samples 1 and 8. The Oxyphotobacteria (belonging to Cyanobacteria phylum) were only found in sample 3, as were the Planctomycetacia (belonging to Planctomycetes phylum).

Several different orders were present in the samples including the Spirochaetales (belonging to Spirochaetia class), Cytophagales (belonging to Bacteroidia class), Mycoplasmatales

(belonging to Mollicutes class), Lactobacillales and Bacillales (belonging to Bacilli class), Pirellulales (belonging to Planctomycetacia class), Synechoccoles (belonging to Oxyphotobacteria class), JGI 0000069-P22 (belonging to Gracilibacteria class), SAR324 clade-Marine Group B (belonging to Deltaproteobacteria class), Oceanospirillales (belonging to Gammaproteobacteria class) Enterobacteriales (belonging to Gammaproteobacteria class), and Rhizobiales (belonging to Alphaproteobacteria class).

The bacterial families that were present in the samples include the Spirochaetaceae (belonging to Spirochaetales order), Cyclobacteriaceae (belonging to Cytophagales order), Mycoplasmataceae (belonging to Mycoplasmatales order), Endozoicomonadaeae (belonging to Oceanospirillales order), Rhizobiaceae (belonging to Rhizobiales order), Eneterobacteriaceae (belonging to Enterobacteriales order), Cynaobiacea (belonging to Synechococcales order), Leuconostocaceae (belonging to Lactobacillales order), Pirellulaceae (belonging to Pirellulales order), Alcanivoracaceae (belonging to Oceanospirillales order), and Planococcaceae (belonging to Bacillales order).

Several genera (Figure 3) were identified in the samples including *Ekhidna*, *Mycoplasma*, *Kistimonas*, *Phyllobacterium*, *Enterobacter*, *Synechococcus CC9902*, *Weissella*, *Alcanivorax*, and *Kurthia*. Species identification could only be made for the *Weissella* genus, with *Weissella paramesenteroides* being present in sample 8 at a very low relative frequency of less than 1%.

3.3 | Phylogenetic tree

A phylogenetic tree of the OTUs in the *E. crispata* samples is shown in Figure 4. This tree shows the chloroplasts as well, belonging to *H. discoidea* and *Rhipocephalus phoenix*. *Elysia crispata* is known to feed upon and sequester chloroplast from multiple species of *Halimeda*, but has not previously been reported as feeding upon *Rhipocephalus* (Middlebrooks et al., 2019). However, the slug does
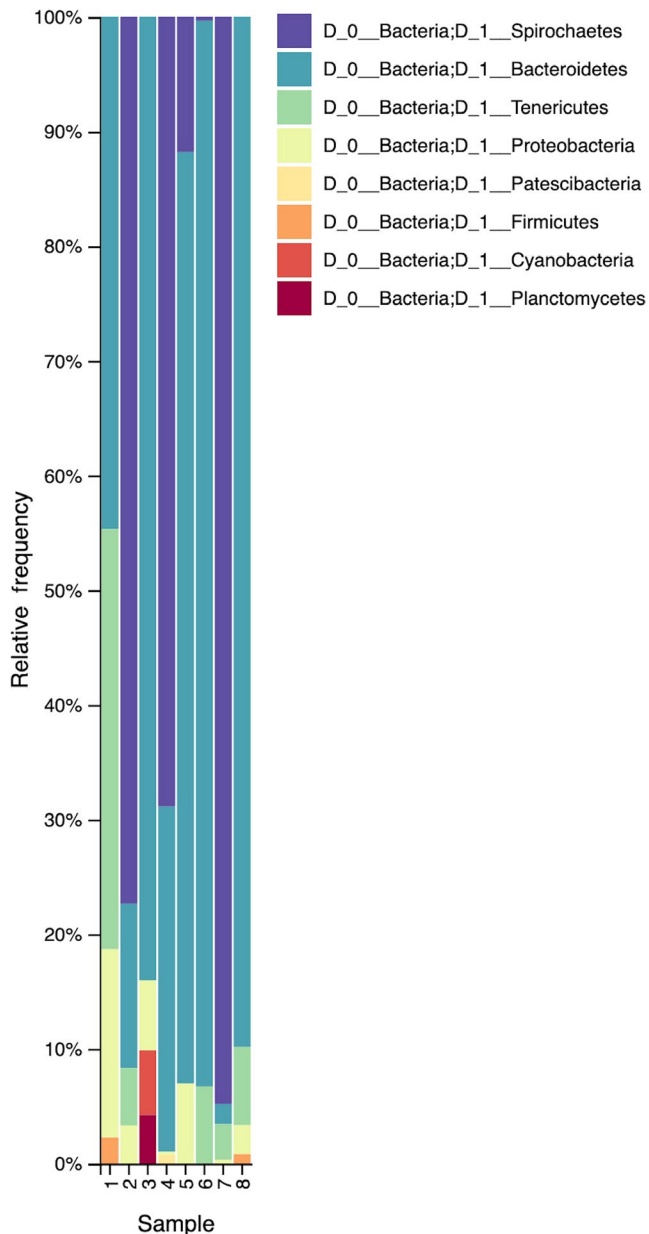
FIGURE 2 Phylum level taxonomic bar plot for the 8 *Elysia crispata* samples

feed upon several species of closely related algae, so it would not be surprising if these algae were also part of its diet. One more OTU matched a chloroplast from *E. cornigera*, another sacoglossan. *E. cornigera* has a specialized diet, only feeding upon the algae *Acetabularia crenulata*, so that it is most certainly the source of that chloroplast (Krug, Händeler, & Vendetti, 2011) and *E. crispata* also sometimes feeds upon this alga (Middlebrooks et al., 2019). Six similar OTUs corresponding to the Ekhidna family were found in several samples. That is, one OTU was found in samples 1, 2, 4, 5, 6, and 7. The second OTU was found in samples 2, 3, 5, and 8. The third OTU was found in sample 5, the fourth OTU was found in sample 1, the fifth OTU was found in sample 8, and the sixth OTU was found in sample 6. Three OTUs corresponding to the Kistimonas family were found in sample 2. Two OTUs

corresponding to the Spirochaetaceae family were found in several samples. That is, one OTU was found in samples 2, 4, 5, and 7. The other OTU was found in samples 5 and 6. Two OTUs corresponding to the Mycoplasma genus were found in the samples as well. One OTU was found in samples 1, 2, 6, and 7. The other OTU was found in samples 2 and 8. The similar OTUs corresponding to these families all clustered together in their respective clades on the phylogenetic tree.

3.4 | Computational metabolic characterization

Metabolic profiles generated of the bacteria in the samples using the Piphillin server (Iwai et al., 2016), KEGG mapper (Kanehisa et al., 2017) and Omics Dashboard (Paley et al., 2017) showed numerous categories of KEGG and BioCyc pathways such as amino acid metabolism, purine and pyrimidine metabolism, lipid metabolism, carbohydrate metabolism, and xenobiotics biodegradation and metabolism. The visualization of the BioCyc pathways is shown in Figure 5. Several broad categories were found such as amino acid synthesis and degradation, carbohydrate synthesis and degradation, fermentation, photosynthesis, detoxification, and macromolecule modification. Interestingly, for most of these categories, sample 3 had the most abundant pathways.

4 | DISCUSSION

There was a high degree of variation in the bacterial community between individual specimens of *clarki* ecotype *E. crispata* despite them all coming from the same location and collection date. This was an unexpected result and may suggest that many of these variable bacterial groups are not obligate symbionts for the slugs. However, that does not rule out a potential facultative role. The only bacteria found in all individuals were the Bacteroidetes, which was also the second most abundant bacteria found by Davis (2015) on reef-dwelling *E. crispata*. Bacteroidetes are very abundant in the marine environment (Kirchman, 2002) so their dominance in the samples is perhaps not so surprising. Marine Bacteroidetes have a high number of polymer-degrading enzymes such as peptidases and glycoside hydrolases, and the ones that possess proteorhodopsin also have a high number of genes involved in CO_2 fixation (Fernández-Gómez et al., 2013). These bacteria may help *E. crispata* break down polymers such as cellulose and help in the process of photosynthesis.

Notably, the bacteria that were most common in the *crispata* ecotypes from the Bahamas and Puerto Rico, Verrucomircobia, (Davis, 2015) were completely absent from the *clarki* ecotypes in the present study. While both ecotypes were found to have Bacteroidetes in high abundance, the *crispata* ecotypes had an abundance of <1% of the Spirochaetes, which was other highly abundant bacteria in *clarki* ecotypes. These clear differences in bacterial communities add to the considerable habitat, morphological (Pierce et al., 2006),
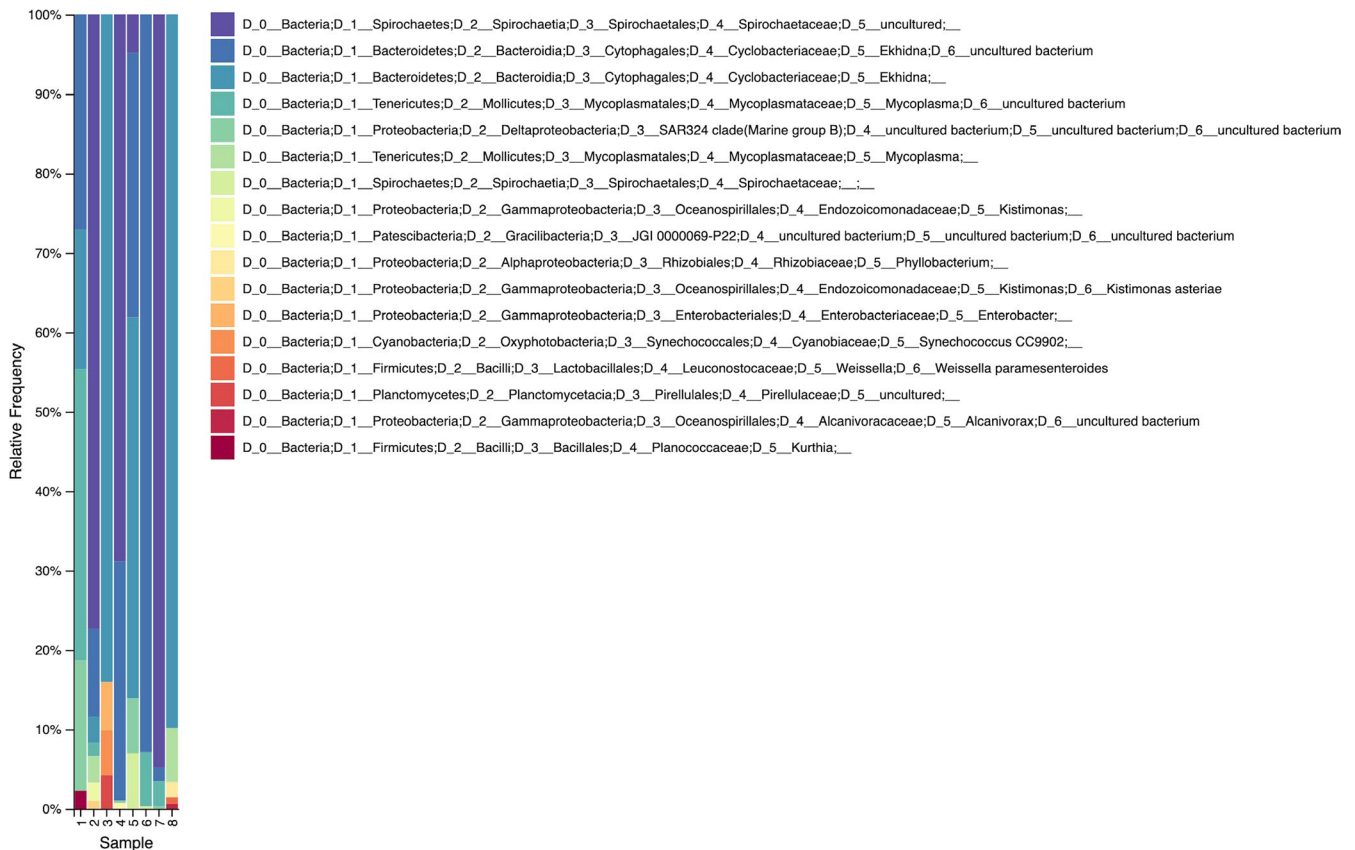
FIGURE 3 Taxonomic bar plot including the genus level for the 8 *Elysia crispata* samples showing inter-individual variability between them

and differences (Middlebrooks et al., 2014, 2019) which distinguish these ecotypes.

Spirochaetes, the other highly abundant bacteria found in this study, were found in most, but not all specimens. Some spirochaetes are endosymbionts in termite guts where they are involved in carbon and nitrogen fixation (Leadbetter, Schmidt, Graber, & Breznak, 1999; Lilburn et al., 2001). Also, they are involved in the breakdown of hemicellulose (Tokuda et al., 2018) and lignocellulose (Brune, 2014) in termites. Devine et al. (2012) postulated that nitrogen-fixing bacteria may be a source of this element for sea slugs to use in nitrogen-limited environments when slugs are relying entirely on photosynthesis and they may be serving a similar role for *E. crispata*. Although the Rhizobiaceae and Enterobacteriaceae were only present in sample 8 and sample 3, respectively, these bacteria have also been implicated in nitrogen-fixing (Bentzon-Tilia, Severin, Hansen, & Riemann, 2015; Carareto Alves, de Souza, Varani, & Lemos, 2014; Chen et al., 2016).

The bacteria found in *clarki* ecotype *E. crispata* were also quite different from those found in other species of sacoglossan. A previous study on bacterial diversity in the sea slug *E. chlorotica* (Devine et al., 2012) revealed that the samples were dominated by Proteobacteria, which were present in some, but not all of our samples and in much lower numbers. Another study that looked at bacterial diversity in the sea slug *E. rufescens* found that Tenericutes and Gammaproteobacteria were most abundant in the sea slugs and

their mucus, respectively (Davis et al., 2013). This is in contrast to this present study in *E. crispata* where the samples were dominated by Spirochaetes and Bacteroidetes.

In this study, Tenericutes (Mycoplasmataceae order) was present in multiple samples. Mycoplasmas were also found in the sea slug *E. rufescens* (Davis et al., 2013), but were found in <1% of *crispata* ecotype *E. crispata* (Davis, 2015). These frequently facultative intracellular symbionts infect humans, plants, animals, and insects (Citti, Dordet-Frisoni, Nouvel, Kuo, & Baranowski, 2018). Mycoplasma has been found in the green algae *Bryopsis* (Hollants et al., 2011) and perhaps the sea slugs have acquired them from their algal diets as suggested by Davis et al. (2013). In the abalone *Haliotis tuberculata*, a generalist marine herbivore, it was proposed that polysaccharides are converted to pyruvate or into solubilized monosaccharides by Flavobacteria and Alpha and Gammaproteobacteria, which are then fermented into short-chain fatty acids by anaerobic bacteria such as Mycoplasma which are then used by the host (Gobet et al., 2018).

Other bacteria that were found less frequently in *clarki* ecotypes are less likely to be playing critical roles for the slugs. However, potential facultative relationships are worth discussing. It should be kept in mind that these are speculative functions and the bacteria may simply be living opportunistically on the slugs. The bacteria such as the SAR324 clade (Marine Group B), Synechococcus CC9902, and Oceanospirillales may play a role in photosynthesis. Synechococcus is an abundant marine cyanobacterium (Flombaum et al., 2013) and members of the
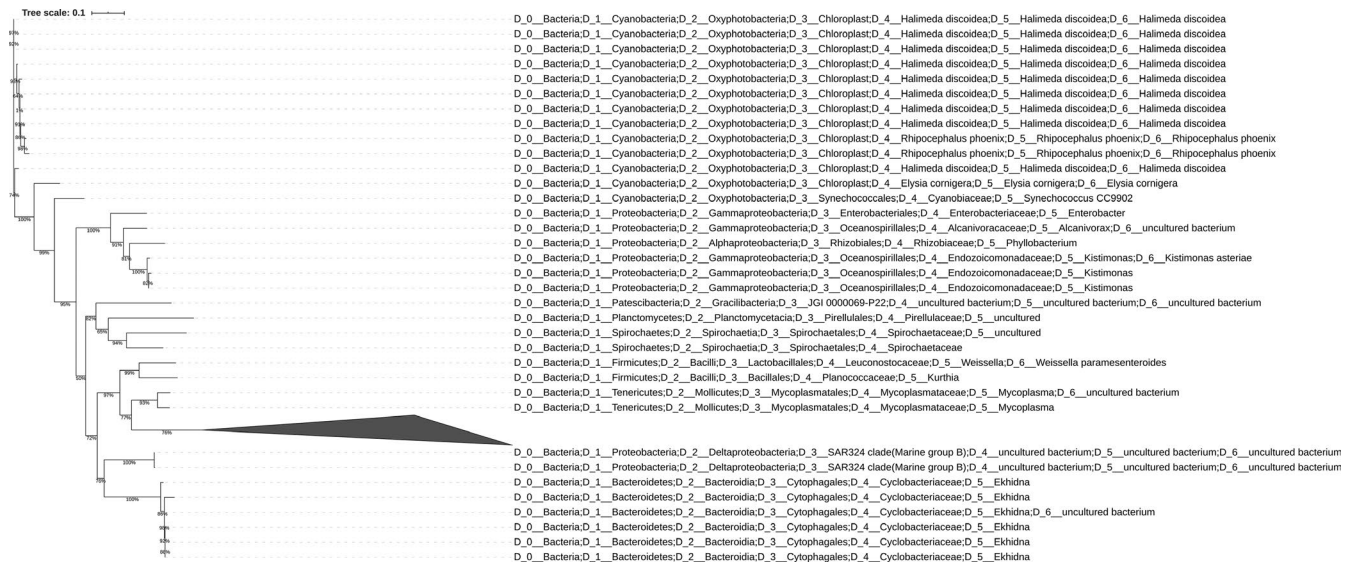
Tree scale: 0.1

D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Rhipocephalus phoenix;D_5__Rhipocephalus phoenix;D_6__Rhipocephalus phoenix
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Rhipocephalus phoenix;D_5__Rhipocephalus phoenix;D_6__Rhipocephalus phoenix
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Halimeda discoidea;D_5__Halimeda discoidea;D_6__Halimeda discoidea
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;D_4__Elysia cornigera;D_5__Elysia cornigera;D_6__Elysia cornigera
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Synechococcales;D_4__Cyanobiaceae;D_5__Synechococcus CC9902
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Enterobacter
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Alcanivoracaceae;D_5__Alcanivorax;D_6__uncultured bacterium
D_0__Bacteria;D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Rhizobiaceae;D_5__Phyllobacterium
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Endozoicomonadaceae;D_5__Kistimonas;D_6__Kistimonas asteriae
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Endozoicomonadaceae;D_5__Kistimonas
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Oceanospirillales;D_4__Endozoicomonadaceae;D_5__Kistimonas
D_0__Bacteria;D_1__Patescibacteria;D_2__Gracilibacteria;D_3__JGI 0000069-P22;D_4__uncultured bacterium;D_5__uncultured bacterium;D_6__uncultured bacterium
D_0__Bacteria;D_1__Planctomycetes;D_2__Planctomycetacia;D_3__Pirellulales;D_4__Pirellulaceae;D_5__uncultured
D_0__Bacteria;D_1__Spirochaetes;D_2__Spirochaetia;D_3__Spirochaetales;D_4__Spirochaetaceae;D_5__uncultured
D_0__Bacteria;D_1__Spirochaetes;D_2__Spirochaetia;D_3__Spirochaetales;D_4__Spirochaetaceae
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Leuconostocaceae;D_5__Weissella;D_6__Weissella paramesenteroides
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Planococcaceae;D_5__Kurthia
D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mycoplasmatales;D_4__Mycoplasmataceae;D_5__Mycoplasma;D_6__uncultured bacterium
D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mycoplasmatales;D_4__Mycoplasmataceae;D_5__Mycoplasma

D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__SAR324 clade(Marine group B);D_4__uncultured bacterium;D_5__uncultured bacterium;D_6__uncultured bacterium
D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__SAR324 clade(Marine group B);D_4__uncultured bacterium;D_5__uncultured bacterium;D_6__uncultured bacterium
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhidna
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhidna
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhidna;D_6__uncultured bacterium
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhidna
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhidna
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Cytophagales;D_4__Cyclobacteriaceae;D_5__Ekhidna

FIGURE 4 Phylogenetic tree of the OTUs found in the _Elysia crispata_ samples. Bootstrap % values are shown. The collapsed node contains 2 OTUs, one of which was unassigned and one that may have been misclassified as a eukaryote
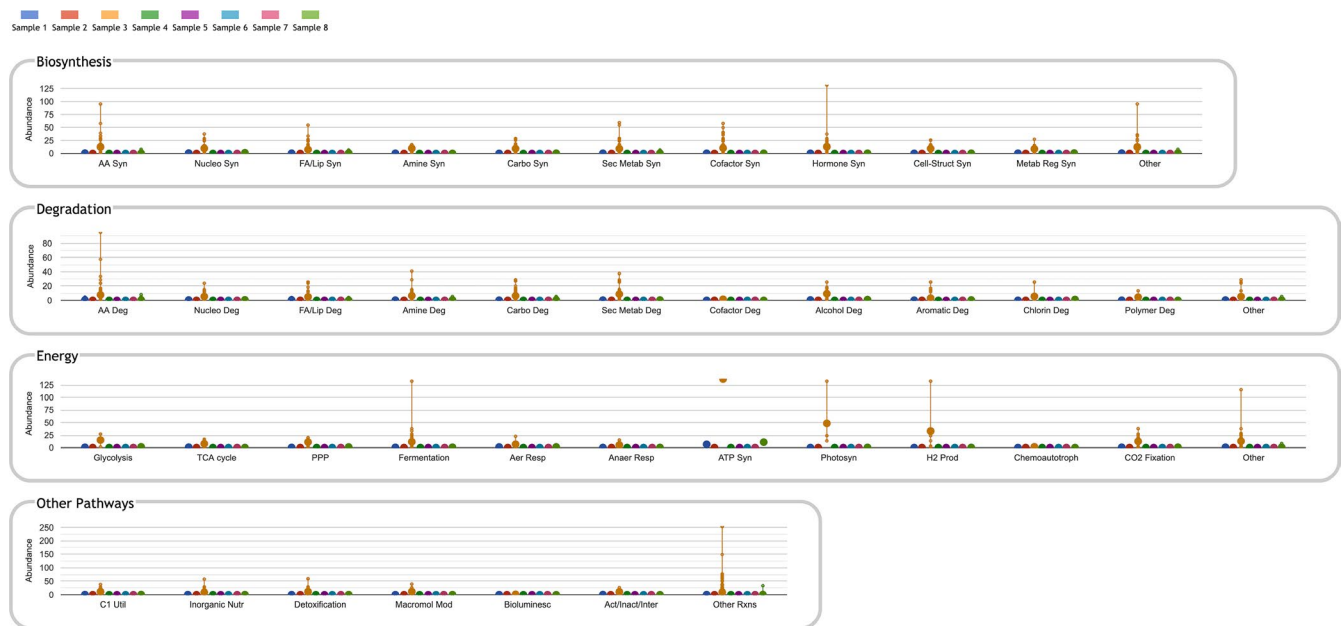

FIGURE 5 Abundance plot of metabolic pathways found in the _Elysia crispata_ samples. The large dots represent the average values for metabolic pathways in a particular subsystem belonging to a sample. The small dots represent value for an individual pathway belonging to a metabolic subsystem. Examples of pathways in the "Other" categories are butanediol biosynthesis, ʟ-citrulline biosynthesis and degradation, ʟ-ornithine degradation, C4 photosynthetic carbon assimilation cycle, and chitin degradation to ethanol. Here, sample 3 has the greatest abundance of metabolic pathways

SAR234 clade and some Oceanospirillales are chemolithoautotrophs. They contain ribulose-1,5-bisphosphate carboxylate-oxygenase (RuBisCO), a major carbon fixation enzyme in the Calvin–Benson–Bassham cycle (Swan et al., 2011). The Endozoicomonadaceae order of Oceanospirillales was only found only in sample 2 and has also been associated with marine hosts such as corals, where they may contribute to carbohydrate cycling, amino acid production, vitamins, cofactors, and pigments (Neave, Michell, Apprill, & Voolstra, 2017).

The Pirellulaceae and Planococcaceae (genus _Kurthia_) were only found in samples 3 and 1, respectively. The Pirellulaceae oxidize ammonia and have been found in sponges and deep-sea corals (Kellogg, Ross, & Brooke, 2016; Mohamed, Saito, Tal, & Hill, 2010). These bacteria may play a role in nitrification, as hypothesized by Kellogg et al. (2016) in corals. One strain of the genus Kurthia has been shown to transform phenolic compounds into acids that are less toxic (Xie et al., 2018).

Weisella paramesenteroides was only found in low frequency in sample 8, but is intriguing because it was found to produce the bacteriocin weissellin A which was active against several different bacterial strains (Papagianni & Papamichael, 2011). This anti-bacterial activity may help defend against pathogenic bacteria.

Metabolic profiling of these bacteria revealed some broad categories of KEGG and BioCyc pathways involved in energy, biosynthesis, and degradation. These include carbohydrate digestion and absorption, lipid metabolism, nucleotide metabolism, and amino acid metabolism. More specific sub-categories include nitrogen metabolism, methane metabolism, porphyrin and chlorophyll metabolism, carbon fixation pathways in prokaryotes, carbon fixation in photosynthetic organisms, photosynthesis, oxidative phosphorylation, vitamin B6 metabolism, riboflavin metabolism, pantothenate and CoA biosynthesis, chloroalkane and chloroalkene degradation, benzoate and aminobenzoate degradation, metabolism of xenobiotics by cytochrome P450, ATP biosynthesis, arsenate detoxification, methylglyoxal detoxification, reactive oxygen species degradation, cyanate degradation, acid resistance, and amine degradation. It is certainly possible that the bacteria performing these processes exist symbiotically with *E. crispata*, helping it in photosynthesis, production of vitamins, and degradation of harmful chemicals.

Other studies that looked at bacterial communities in sacoglossans also compared the bacteria found on their algal food (Davis, 2015; Davis et al., 2013; Devine et al., 2012; Kurahashi & Yokota, 2007). *Elysia chlorotica* is a specialist feeding only *Vaucheria*, while both *E. rufescens* and the species identified by Kurahashi and Yokota (2007) as *E. ornata* are specialists on *Bryopsis*. For these species, it is relatively straightforward to know which algae to test. *Elysia crispata*, however, feeds on over 20 distinct species of algae including species from both the Bryopsidales and Dasycladales (Middlebrooks et al., 2014, 2019). Davis (2015) tested two algal species on which *E. crispata* was found. This slug is most commonly found on bare substrate or algae on which it does not feed (Middlebrooks et al., 2014). Given these highly variable feeding patterns and the behavior of *E. crispata*, it was reasonable to focus solely on the slug for this study as it is not possible to guess on which algae they were feeding, especially considering that they can go months without actively seeking out food (Middlebrooks, Pierce, & Bell, 2011). However, given the differences in the bacterial community between individual slugs, some of these bacteria could come from differences in algal diet among individual slugs.

5 | CONCLUSIONS

In conclusion, several types of bacteria comprise the microbiome of *E. crispata*, with inter-individual variability between samples. These bacteria may serve important metabolic roles in this sea slug, although they may not be obligate symbiotes. Although it is not possible to examine more slugs from this location as their population was destroyed (Middlebrooks et al., 2020), it will be of particular interest to sample more *E. crispata* individuals from different locations, especially reef-dwelling specimens from Florida, to determine whether

similar bacteria are present and whether there is also significant inter-individual variability between individuals. Shotgun metagenomics could also be performed to elucidate more in-depth metabolic function profiling and pathway analysis, as well as to detect novel microorganisms such as ones that are not able to be cultured or rare species.

CONFLICT OF INTEREST
None declared.

AUTHOR CONTRIBUTIONS
Padmanabhan Mahadevan: Conceptualization (equal); formal analysis (lead); funding acquisition (equal); investigation (equal); methodology (equal); software (lead); validation (equal); visualization (lead); writing – original draft (equal); writing – review & editing (equal). **Michael L. Middlebrooks:** Conceptualization (equal); formal analysis (supporting); funding acquisition (equal); investigation (equal); methodology (equal); software (supporting); validation (equal); visualization (supporting); writing – original draft (equal); writing – review & editing (equal).

ETHICS STATEMENT
Specimens were collected under permit SAL-17-0616-SR issued by the State of Florida Fish and Wildlife Conservation Commission.

DATA AVAILABILITY STATEMENT
The sequence data from this study are available in the NCBI repository under the BioProject ID PRJNA607610: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA607610.

ORCID
Padmanabhan Mahadevan https://orcid.org/0000-0001-7596-1306

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