Characterization of a sponge microbiome using an integrative genome-centric approach

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Abstract

Marine sponges often host diverse and species-specific communities of microorganisms that are critical for host health. Previous functional genomic investigations of the sponge microbiome have focused primarily on specific symbiont lineages, which frequently make up only a small fraction of the overall community. Here, we undertook genome-centric analysis of the symbiont community in the model species *Ircinia ramosa* and analyzed 259 unique, high-quality metagenome-assembled genomes (MAGs) that comprised 74% of the *I. ramosa* microbiome. Addition of these MAGs to genome trees containing all publicly available microbial sponge symbionts increased phylogenetic diversity by 32% within the archaea and 41% within the bacteria. Metabolic reconstruction of the MAGs showed extensive redundancy across taxa for pathways involved in carbon fixation, B-vitamin synthesis, taurine metabolism, sulfite oxidation, and most steps of nitrogen metabolism. Through the acquisition of all major taxa present within the *I. ramosa* microbiome, we were able to analyze the functional potential of a sponge-associated microbial community in unprecedented detail. Critical functions, such as carbon fixation, which had previously only been assigned to a restricted set of sponge-associated organisms, were actually spread across diverse symbiont taxa, whereas other essential pathways, such as ammonia oxidation, were confined to specific keystone taxa.

Introduction

Marine sponges are a ubiquitous and functionally important component of aquatic ecosystems, providing valuable

habitat for a variety of species and coupling the benthic and pelagic zones through the filtration of seawater and recycling of (in)organic nutrients [1–3]. Sponges frequently harbor dense, diverse, and stable microbial communities, which can comprise up to 35% of the biomass of high microbial abundance (HMA) sponges [4, 5]. These sponges contain 10^8 – 10^{10} microbial cells g^{-1} sponge wet weight in contrast to low microbial abundance sponges that contain 10^5 – 10^6 microbes g^{-1} sponge wet weight [6]. The microorganisms are often host species-specific [7], distinct from those in the surrounding environment [8] and are hypothesized to contribute to many aspects of the sponge's physiology and ecology [4]. Considering the diversity, abundance, and potential functional importance of their microbiome, sponges are often described as “holobionts”—a complex and interdependent consortium comprising the sponge host and the entire microbiome [4].

Recent studies have suggested that sponge-associated microbial symbionts have the capacity to fix carbon dioxide, metabolize nitrogen, sulfur, and phosphate, and synthesize essential B-vitamins [5]. However, these insights are primarily inferred from 16S rRNA or functional marker gene

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surveys that either give no information about the functional potential of the symbionts or are limited to analyzing a few discrete pathways that were decided a priori to be of interest. In addition, gene-centric analysis has provided critical functional insights into the sponge microbiome, but could not link function with phylogeny [9]. Only a limited number of symbiont genomes have been retrieved from multiple sponge species [10–13]. However, the majority of these studies focused on specific lineages that make up a small fraction of the complex sponge microbiome. With sponge microbial richness estimates exceeding 40,000 genetically distinct taxa [7], spread across 50 microbial phyla [5], the majority of sponge symbiont genomes and their functional potential remains undescribed. Here, we used genome-centric metagenomics to characterize 259 high-quality microbial metagenome-assembled genomes (MAGs) retrieved from *I. ramosa*, an HMA sponge from the Great Barrier Reef (GBR), Australia. The MAGs represent 74% of the microbial community, providing an unprecedented opportunity to characterize the metabolic potential of all major taxa within a sponge microbiome.

Materials and methods

Sample collection, processing, and sequencing

Six *Ircinia ramosa* (Porifera: Demospongiae, Fig. S13) individuals were collected using SCUBA at 5–7 m water depth at Davies Reef (18°49' S, 147°38' E) on the GBR Australia, in October 2016. Samples were held in flow-through aquaria for 2 h during transportation to the Australian Institute of Marine Science Sea Simulator, where the sponges were transferred to a 3600 L mesocosm and kept for 6 months. Directly after transfer to the mesocosm, ~10 cm² fragments were excised from each specimen (T0) and snap frozen in liquid nitrogen. The six individual sponges were again subsampled after 3 (T3) and 5 (T5) months in the aquaria to determine the composition of the microbiome over time. All excised tissue, comprising both external and internal body tissue, was immediately snap frozen in liquid nitrogen.

Microbial cells were separated from frozen sponge tissue as described previously [14], with the exception that tissue was initially cut into 2 mm² pieces prior to 1 min homogenization, and cells were filtered twice through 8 µm filters and twice through 5 µm filters. Bacterial pellets were collected after a final centrifugation at 8800 × g for 20 min and cells were frozen at −20 °C. DNA was extracted from cell pellets using the UltraClean Microbial kit (MO BIO, San Diego, USA) according to the manufacturer's instructions with an additional incubation step of 10 min at 65 °C before bead-beating for 40 s at 5 m s^{−1} in a FastPrep24 instrument

(MP Biomedicals, Santa Ana, CA, USA) to assist lysis. Extracted DNA was further purified using the ZymoResearch (California, USA) DNA Clean and Concentrator kit. DNA was sent to the Australian Centre for Ecogenomics (ACE), Brisbane, libraries were prepared with Illumina Nextera XT, and samples were sequenced on the NextSeq 500 High Output using 2 × 150 bp paired end chemistry. All samples ($n = 18$) were first sequenced at an average depth of 1.7 Gbp for differential coverage binning and replicates 1, 2, and 4 from T0 were selected for deeper sequencing, with an average depth of 20.6 Gbp, to obtain better coverage of lower abundance members of the microbiome.

Metagenome assembly, taxonomic assignment, and functional annotation

Sequencing adapters were removed using SeqPurge v0.1-852-g5a7f2d2 [15] and all reads were assembled using metaSPades v3.9.0 [16]. Reads were mapped to the resulting assemblies with bamM v1.7.3 (<https://github.com/Ecogenomics/BamM>), which leverages the Burrows-Wheeler Aligner [17], aligning sequences based on a backward search with the Burrows-Wheeler transformation. Binning was performed with UniteM v0.0.15 (<https://github.com/dparks1134/UniteM>). In brief, UniteM uses the binning algorithms maxbin v2 v2.2.4, metabat v1 v0.32.4 using all parameter sets (--verysensitive --mb_sensitive --mb_specific --mb_veryspecific --mb_superspecific), metabat v2 v2.12.1, and groopM2 v2 v2.0.0-1, and selects the best MAGs by their CheckM quality scores [18–22].

Taxonomy was assigned to each MAG using GTDB-Tk v0.2.2 (<https://github.com/Ecogenomics/GtdbTk>), which classifies MAGs based on placement in a reference concatenated gene tree based on a set of 120 bacterial and 122 archaeal markers using a combination of FASTANI and pplacer [23, 24]. GTDB-Tk v0.2.2 annotation is based on the Genome Taxonomy Database (GTDB, <http://gtdb.ecogenomic.org>) taxonomy R86.3 [25]. MAGs obtained from sub-sampling the same sponge individuals over three time points were de-replicated at 99% identity with Drep v1.0.0 using default parameters [26].

To determine the percentage of the total community captured by the MAGs, the “pipe” and “appraise” functions of SingleM v0.9.0 were used to search for single copy marker genes in both the MAGs and the shallow sequenced raw reads to assess the fraction of marker genes recovered in the MAGs (<https://github.com/wwood/singlem>). The relative abundance of each MAG from the separate individuals was calculated with coverM v0.2.0 (<https://github.com/wwood/CoverM>), after de-replicating all 559 MAGs at 95% identity with Drep v2.2.4 [26] to avoid arbitrary mapping between representatives of highly similar genomes. MAGs with a cumulative abundance >2% (sum of

MAG abundance across replicates) were included in the heatmap visualized with R v3.5.1 [27]; Fig. S1). To visualize the composition of the *I. ramosa* microbiome across the three time points, a relative abundance heatmap was created in R v3.5.1 [27], based on 16S rRNA gene sequences recovered from the metagenomics reads using GraftM v0.11.1 [28]. The recovered 16S rRNA gene sequences are representative of all data, including unbinned lineages. Taxonomy was assigned using a GraftM package based on the Silva release NR99 132 [29] after clustering sequences at 97% identity with cd-hit-est v4.8.1 [30, 31]. Relative abundance is calculated as the percentage of reads that match a specific taxonomy string (Fig. S2). GraftM packages can be accessed via <https://data.ace.uq.edu.au/public/graftm/7/>.

Phylogenetic tree building and calculation of phylogenetic distance and gain

Bacterial and archaeal trees were inferred with GTDB v2.2.2 [25], filtering out MAGs below 40% MAG quality, defined as percentage genome completeness minus five times contamination [25]. Of the 259 de-replicated MAGs produced in this study, 15 were filtered out based on quality and an insufficient amount of amino acids in the multiple

sequence alignment (Table S1). A concatenated marker gene tree was inferred using the 244 de-replicated MAGs, together with all publicly available sponge symbiont genomes (MAGs, SAGs, single-cell amplified genomes, and isolate genomes) that were $\geq 50\%$ complete and possessed $\leq 10\%$ contamination. For comparison, taxonomy was reassigned to the previously published sponge symbiont genomes using GTDB-Tk v0.2.2 (<https://github.com/Ecogenomics/GtdbTk>). Phylogenetic distance, defined as the “total branch length spanned by a set of taxa” [32] and phylogenetic gain, defined as the “additional branch length contributed by a set of taxa” [32], were calculated for both the archaeal and bacterial tree with GenomeTreeTk v0.0.41 (<https://github.com/dparks1134/GenomeTreeTk>) to determine how much additional phylogenetic diversity the MAGs from the current study added to the trees. The tree files were also visualized with Itol v4.2.3 [33] and refined in Inkscape v0.92.3 (<https://inkscape.org/>) for Fig. 1.

Metabolic reconstruction of the MAGs

MAGs were annotated with the PFAM and Kyoto Encyclopedia of Genes and Genome (KEGG) Orthologies (KOs) databases [34, 35] using the “annotate” function of EnrichM v0.2.1 (<https://github.com/geronimp/enrichM>). Genes not

Fig. 1 Combined archaeal and bacterial phylogenetic tree based on single copy marker proteins (inferred with GTDB). Labels colored in red are MAGs retrieved from this study. Black labels are previously published sponge symbiont genomes (MAGs, SAGs, and isolate genomes). Branch labels display taxonomy at the lowest inferred level. Genomes “GB_GCA_000522425.1” and

“RS_GCF_900079095.1” were taxonomically uncharacterized by GTDB-Tk v0.2.2 (<https://github.com/Ecogenomics/GtdbTk>) and published taxonomy was used (*Ca. Entothionella factor* [97] and *Ca. Entothionella palauensis* [43], respectively). The bacterial and archaeal tree are rooted to LS-NOB (GB_GCA_001542995.1) and Nitrosopumilus (GCA_001543015.1), respectively.

annotated by KEGG were considered to encode “hypothetical proteins.” EnrichM’s “classify” function was then used to calculate the completeness of KEGG modules, which are groups of genes organized by steps in a metabolic pathway as defined by KEGG [35]. As we are working with partial genomes, for a function (e.g., carbon fixation) to be assigned to a MAG, the appropriate KEGG module was required to be $\geq 60\%$ complete and all key enzymes (as defined by the literature) had to be present. A separate module was created for cobalamin because the KEGG module contained only part of the full pathway (Note S1). In addition, ABC transporters were positively assigned if genes for both the substrate specific component and the integral membrane protein were present in the MAG. Bacterial and archaeal trees described in the phylogenetic tree building section (only including MAGs retrieved from this study) were decorated with positively assigned functions from KEGG for Figs. S3–S12. Although we recovered a large proportion of the community (74.2%), the remaining 25.8% of unbinned genomic material may contain functionality we have not described and bioinformatics-based predictions could include incomplete pathways, due to the analysis of incomplete genomes.

GraftM v0.11.1 [28] was additionally used to validate KO annotations in the MAGs and the reads for the key enzymes dimethyl sulfoxide reductase (*dmsA*), dimethylsulfoniopropionate-dependent demethylase (*dmdA*), ammonia monooxygenase (*amoA*), ATP-citrate lyase (*acl*), Rubisco (*rbc*), and hydroxylamine dehydrogenase (*hao*). In brief, GraftM searches for defined key enzymes in MAGs or reads using custom HMMs and inserts them into a reference tree with functional clades [28].

Results and discussion

In total, 18 metagenomes from six *Ircinia ramosa* biological replicates sampled over three time points were sequenced to produce 559 MAGs ($85.97 \pm 13.40\%$ completeness, $1.47 \pm 1.73\%$ contamination), including 13 archaea and 546 bacteria (Tables S1 and S2). The MAGs contained on average 2974 ± 887 coding sequences, of which $33.45 \pm 6.71\%$ were hypothetical proteins. De-replication of the MAGs within each time point reduced the total number to 259, including six archaeal MAGs belonging the phylum Thaumarchaeota (Crenarchaeota as per GTDB) and 253 bacterial MAGs belonging to 14 phyla, including the Acidobacteriota (25 MAGs), Actinobacteriota (43), Bacteroidota (8), Bdellovibrionota (1), Chloroflexota (63), Cyanobacteriota (4), Dadabacteria (6), Gemmatimonadota (21), Latescibacterota (15), Nitrospinota (1), Nitrospirota (5), Patescibacteria (2), Poribacteria (9), and Proteobacteria (50). This community profile is generally consistent with previously described

Irciniidae spp. [7, 36, 37] and other HMA sponge microbial surveys [7], though some lineages identified in previous studies have been reclassified in the GTDB taxonomy. For example, Nitrospinota were previously part of the Proteobacteria and the Latescibacterota include the SAUL lineage, also known as PAUC34f (see Table S1 for further taxonomic comparison). However, in contrast to dominant lineages commonly reported in other HMA sponges [38], the microbiome of *I. ramosa* was dominated by the orders Rhodothermales (Bacteroidota) and Microtrichales (Actinobacteriota; Figs. S1 and S2). Analysis of single copy marker genes present in the reads and MAGs using SingleM showed that the de-replicated MAGs represented 74.2% of the *I. ramosa* microbial community. Over 60% of the taxa were present in $>50\%$ of the replicates across time points (Fig. S2), facilitating determination of the metabolic potential of all dominant taxa.

Ircinia MAGs increase phylogenetic diversity by 32% for archaea and 41% for bacteria

A concatenated genome tree was constructed from 95 previously published sponge symbiont genomes: 27 bacterial isolate genomes, 3 bacterial SAGs, and 65 MAGs (5 archaeal and 60 bacterial; Table S3) and 244 MAGs from this study (6 archaeal and 238 bacterial), that passed the quality criteria (Fig. 1). The MAGs from this study provided a phylogenetic gain (the additional branch length contributed by a set of taxa) of 31.74% for the archaea and 40.51% for the bacteria (Table 1). Although previously published genomes, originating from 24 sponge species, were widely distributed across the phylogenetic tree (Fig. 1), sequencing the microbiome of only one sponge species out of the 9209 presently described sponge species considerably expanded the sponge microbiome phylogenetic tree [39].

Three autotrophic carbon fixation pathways

Sponges obtain nutrients through active filter feeding on organic carbon, but some species have been shown to fix carbon via their microbial photosymbionts [40] or live in symbiosis with methane-oxidizing bacteria [41]. Relatively few sponge symbionts have been identified with autotrophic carbon fixation pathways [42–45], although carbon exchange between microbes and their host has been confirmed in several species [40, 41, 46, 47]. As microbial carbon acquisition could contribute to sponge metabolism, we searched the *I. ramosa* MAGs for the six known prokaryotic autotrophic carbon fixation pathways: the Wood-Ljungdahl (WL) pathway, reductive citric acid (rTCA) cycle, 3-hydroxypropionate/4-hydroxybutyrate (HP-HB) cycle, 3-hydroxypropionate (3-HP) bicycle, dicarboxylate/4-hydroxybutyrate (DC-HB)

Table 1 Calculated phylogenetic distance (PD) and phylogenetic gain (PG) for the archaeal and bacterial genome trees, per taxon.

	No. taxa	PD	PD (%)
Archaea			
Full tree	11	1.77	100.00
Outgroup taxa (PD)	5	1.21	68.26
Ingroup taxa (PD)	6	0.20	11.22
Ingroup taxa (PG)	6	0.56	31.74
Bacteria			
Full tree	328	49.66	100.00
Outgroup taxa (PD)	90	29.55	59.50
Ingroup taxa (PD)	238	33.83	68.11
Ingroup taxa (PG)	238	20.12	40.51

Outgroup taxa include previously published sponge symbiont genomes (MAGs, SAGs, and isolate genomes) and ingroup taxa include MAGs retrieved from this study.

cycle, and the Calvin–Benson–Bassham (CBB) cycle [48]. Metabolic potential for three autotrophic carbon fixation pathways was identified in the *I. ramosa* microbiome (Figs. 2 and S3, Table S4), with the relative abundance of these MAGs in the six *I. ramosa* individuals ranging from 0.65 to 5.98%. Consistent with Nitrospirota recovered from other environments, key enzymes of the rTCA cycle, ATP-citrate lyases (*aclA* and *aclB*), were identified in all five *I. ramosa* Nitrospirota MAGs (family UBA8639), with an rTCA module completeness of 90% (Figs. 2 and S3, Table S4). The HP–HB cycle was identified in sponge-associated Nitrosopumilaceae (5 MAGs; Figs. 2 and S3, Table S4), similar to what has been reported for Thaumarchaeota recovered from other sponge species [44, 45]. Furthermore, more than 70% of the genes necessary for carrying out the CBB cycle, including both subunits of Rubisco (*rbcS* and *rbcL*) were found in the genus *Synechococcus* (4 MAGs) and Gammaproteobacteria (3 MAGs), including the family LS-SOB (2 MAGs; Figs. 2 and S3, Table S4). These data show evidence for the presence of multiple carbon fixation pathways within a sponge microbiome and expands our view on the distribution of genes encoding the CBB cycle, which previously had only been identified in sponge-derived MAGs from Cyanobacteria [42] and Entotheonella [43].

The WL pathway, 3-HP bicycle, and DC–HB cycle were absent in the *I. ramosa* MAGs (Fig. S3, Table S4). The WL pathway has previously been reported in Poribacteria [49] and the so-called SAUL lineage [50]. However, the contamination of the poribacterial SAG presented in that study was 74.02% as shown in Table S1 from Kamke et al. [51]. Further, key enzymes of the bacterial WL pathway, acetyl-CoA synthase/carbon monoxide dehydrogenase (ACS/CODH) subunits B, C, D, and E, were absent from the SAUL lineage (Note S2). Bacterial and archaeal key

enzymes for the WL pathway (*acsABCDE* and *cdhABCDE*, respectively) were also absent in our MAGs. Hence evidence for the WL pathway in sponge symbionts is lacking, as is evidence for the DC–HB cycle.

Both radio-labeled and stable isotope-tracer experiments have confirmed that photosymbiont fixed carbon through the CBB pathway can be taken up by the host [40, 46]. However, there is currently no data regarding the fate of carbon from other autotrophic fixation pathways. If symbiont-fixed carbon from each of the pathways gets translocated to the sponge host, the acquired autotrophic carbon could be contributing to the overall high environmental tolerance and plasticity of sponges, particularly to ocean warming, which can cause loss of photosymbionts [52].

First step of nitrification is confined to the Thaumarchaeota

Through their excretion of ammonia, sponges would provide an attractive niche for microorganisms, particularly in ammonium-limited oligotrophic seas [53]. Sponge symbionts are thought to use ammonia as an energy source, thereby detoxifying their host through nitrification [54, 55]. During nitrification, ammonia is consecutively oxidized to hydroxylamine, nitrite, and nitrate [56], catalyzed by ammonia monooxygenases (*amoABC*), hydroxylamine dehydrogenase (*hao*), and nitrate oxidoreductases (*nirAB*), respectively. The *amoA*, *B*, and *C* subunits were identified in four *I. ramosa* Thaumarchaeota (family Nitrosopumilaceae) MAGs via KO annotation and placement into a phylogenetic reference tree using an *amo*-specific GraftM package (Figs. 2 and S4, Table S4). In addition, a search for *amoA* amongst the raw reads using GraftM showed that *amoA* was exclusively assigned to archaea. Ammonia oxidizing bacteria and archaea were previously reported to co-occur in a single sponge species [9, 57, 58]. However, as we did not find any evidence for ammonia oxidizing bacteria in *I. ramosa*, our data suggest that Thaumarchaeota are solely responsible for ammonia oxidation, representing a keystone taxa within the *I. ramosa* microbiome.

Although graftM identified 13 bacterial MAGs that contained putative *haos*, these were only distantly related to known *haos*. As the MAGs containing these *hao*-homologs did not have the *amoA* gene necessary for conversion of ammonium to hydroxylamine, we suspect that the *hao*-homologs do not serve in nitrification, but might rather be involved in the detoxification of hydroxylamine [59] originating from other cellular processes. This finding supports our assertion that the Thaumarchaeota are the only oxidizers of ammonia, as they are able to convert ammonia directly to nitrite themselves [47, 60, 61]. Nitrospirota (3 MAGs), Alphaproteobacteria (9), and Gammaproteobacteria (5)

Fig. 2 Metabolic reconstruction and proposed exchange of carbon, nitrogen, sulfur, and vitamins between *Ircinia ramosa* symbionts and the host. HP-HB cycle: 3-hydroxypropionate/4-hydroxybutyrate cycle. Lineages in bold within the taurine metabolism box contain both genes encoding for the taurine transporter (*tauACB*) and taurine

dioxygenase (*tauD*). Numbers in brackets within the vitamin metabolism stand for the metabolic potential to synthesize one or more of the following B-vitamins: 1: Biotin, 2: Cobalamin, 3: Pantothenate, 4: Pyridoxine, 5: Riboflavin, 6: Thiamine. See Fig. S13 for *I. ramosa* morphology.

encoded for the genes *nxrA* and *nxrB*, that catalyze the conversion of nitrite to nitrate.

Nitrate resulting from nitrification can be recycled through denitrification [62], which is carried out in four steps: nitrate and nitrite reduction (catalyzed by *narGHI* or *napAB* and *nirK* or *nirS*, respectively), nitric oxide reduction (*norBC*), and nitrous oxide reduction into nitrogen (*nosZ*). In the *I. ramosa* microbiome, *narGHI* and *napAB* were identified in the Alphaproteobacteria (10 MAGs), Gammaproteobacteria (6), and Nitrospirota (3; Figs. 2 and S4, Table S4). One Gemmatimonadota (class Gemmatimonadetes) and 18 Gammaproteobacteria MAGs encoded both the *norB* and *norC* genes, suggesting that only members of these phyla can convert nitric oxide into nitrous oxide. Absent from all MAGs was *nosZ*, suggesting that nitrous oxide is not reduced. Full denitrification has so far only been found in sponge-associated Alphaproteobacteria [63] and Entotheonella [43]. Previous studies have shown that only enzymes responsible for the first steps of denitrification (i.e. *narGHI*, *napAB*, *nirK*, and *nirS*) are enriched in sponge symbionts, while enzymes for the final two steps (i.e., *norBC* and *nosZ*) are absent [9, 64, 65], suggesting an incomplete pathway, which would result in the release of

nitrous oxide into the surrounding seawater. KOs associated with annamox were absent from all MAGs, as were KOs involved in nitrogen fixation.

Sponge symbionts can translocate nitrogen to the host [46, 66], but the sponge microbiome also contains a diverse array of microbes that assist the host with efficient removal of nitrogenous waste products including ammonium and nitrite [67, 68]. Congruently, the pathways identified in the *I. ramosa* microbiome were mainly involved in nitrogen removal from the system.

Widespread potential for sulfur metabolism

Taurine is a naturally occurring compound in a wide variety of organisms and taurine-conjugated fatty acids have been identified in *Ircinia* sp. [69]. The potential of sponge-associated microbes to use taurine via ABC transporters (*tauACB*) and subsequently convert it to sulfite via taurine dioxygenases (*tauD*) has been described in a number of lineages, including the Proteobacteria [64, 70] and *Ca. Entotheonella* [71]. Taurine dioxygenases were identified in *I. ramosa* MAGs belonging to the Acidobacteriota (21 MAGs), Actinobacteriota (24), Alphaproteobacteria (6),

Chloroflexota (10), Gammaproteobacteria (26), Gemmatimonadota (20), Latescibacterota (14), Nitrospina (1), and Poribacteria (2), of which the *tauACB* transporter was also identified in the Actinobacteriota (13), Alphaproteobacteria (4), Gammaproteobacteria (4), and Nitrospina (1), suggesting widespread potential for sponge symbionts to use host derived taurine (Figs. 2 and S5, Table S4). In addition to the cleavage of taurine, sulfite may also originate from the dissimilatory oxidation of sulfide by dissimilatory sulfite reductases, *dsrAB* and *dsrEFH*. Interestingly, members of the Gammaproteobacteria (6 MAGs, of which four from the family LS-SOB) are the only taxa capable of dissimilatory sulfide oxidation in the *Ircinia* holobiont (Figs. 2 and S5, Table S4). Sulfite can in turn be oxidized to sulfate via sulfite oxidase (*suox*). Genes encoding *suox* were identified in all phyla except Thaumarchaeota, Cyanobacteriota, Bdellovibrionota, Dadabacteria, and Patescibacteria. Genes involved in dissimilatory sulfate reduction to sulfite (*sat* and APS reductases *aprAB*) were identified in Alpha- (3 MAGs) and Gammaproteobacteria (5), and Chloroflexota (5; Figs. 2 and S5, Table S4).

Incomplete oxidation of sulfides can produce thiosulfate, which can be oxidized to sulfate through sulfur-oxidizing proteins (*soxAB*, *soxXYZ*), called the SOX complex [72]. Two Alpha- and five Gammaproteobacteria MAGs (similar to the Gammaproteobacteria MAGs capable of sulfide oxidation) were identified with *soxAB* and *soxXYZ*, confirming potential to oxidize sulfur through the entire SOX system (Figs. 2 and S5, Table S4). This study demonstrates widespread potential for sulfur metabolism in *I. ramosa*, which is likely beneficial for both microbes and host. Host derived taurine would provide a source of sulfur for the microbial community while sulfur-oxidizing bacteria could balance sulfide generation from sulfate-reducing bacteria in the sponge [73], providing a detoxifying function and increasing the overall stability of the holobiont.

Metabolic potential for synthesis of six essential B-vitamins

B-vitamins and their cofactors are essential compounds for organismal central metabolism [74, 75]. However, animals cannot synthesize these vitamins themselves and must therefore acquire them through feeding or symbiotic relationships with microorganisms [76, 77]. Previous genomic analyses of sponge microbiomes have highlighted the presence of thiamine (vitamin B1), riboflavin (B2), pyridoxine (B6), biotin (B7), and cobalamin (B12) biosynthesis genes, suggesting a possible role in provisioning vitamins to the sponge host. However, gene-centric analyses could not link these pathways with taxonomy and genomic analyses were focussed on just a few lineages able to metabolize these B-vitamins. Here we show that the potential for biosynthesis

of B1, B2, pantothenate (B5), B6, B7, and B12 is widely distributed within the microbiome, with 13 out of the 18 *I. ramosa* associated microbial phyla containing pathways to synthesize thiamine: Acidobacteriota (17 MAGs), Actinobacteriota (8), Bacteroidota (1), Chloroflexota (21), Dadabacteria (6), Gemmatimonadota (18), Latescibacterota (9), Nitrospina (1), Nitrospirota (5), Poribacteria (9), and Proteobacteria (22). In addition, the Actinobacteriota (34), Chloroflexota (16), and Proteobacteria (7) encoded for genes necessary for thiamine transport (Figs. 2 and S6, Table S4) and all phyla apart from the Patescibacteria were able to synthesize riboflavin and convert it into the functional coenzymes flavin mononucleotide and flavin adenine dinucleotide (Figs. 2 and S7, Table S4). In addition, the genes necessary to synthesize pantothenate were only absent in Bdellovibrionota and Thaumarchaeota (Figs. 2 and S8, Table S4) and pyridoxine can be synthesized from D-Erythrose 4-phosphate by Acidobacteriota (12 MAGs), Bacteroidota (8), Nitrospirota (5), and Proteobacteria (43; Figs. 2 and S9, Table S4). Further, biotin metabolism was widespread and only absent from MAGs belonging to Bacteroidota, Gemmatimonadota, and Patescibacteria. The biotin transporter was identified in Actinobacteriota (3), Cyanobacteriota (4), Latescibacterota (5), and Proteobacteria (3; Figs. 2 and S10, Table S4). Potential for cobalamin biosynthesis was also identified in four phyla: Chloroflexota (7 MAGs), Cyanobacteriota (4), Proteobacteria (9), and Thaumarchaeota (5; Figs. 2 and S11, Table S4).

The potential for B-vitamin biosynthesis had previously only been reported for single symbiont lineages within any sponge species [14, 38, 78–81]. Here we reveal extensive taxonomic redundancy in vitamin provisioning within the complex sponge microbiome, including the metabolism of vitamin B5. Although sponges can obtain their B-vitamins through feeding, symbionts likely provide an alternative pathway for vitamin provision, which is supported by the presence of vitamin transporters in the sponge microbiome [45, 65, 82, 83], as well as the presence of vitamin transporters and vitamin catabolism [78] in the sponge host.

Secretion systems and eukaryotic-like proteins in the sponge microbiome

Many symbiotic microorganisms are known to interact with their host via microbial secretion systems, some of which are needle-like protein complexes (Type III, IV, and VI) used to inject effector proteins into an adjacent cell to modulate its metabolism [84]. Eukaryotic-like proteins, such as Ankyrin-repeat (Ank) and WD40 repeat-containing proteins [85, 86], are also thought to be used for bacterial establishment in the host [87, 88]. Surprisingly, only members of the Gammaproteobacteria (3 MAGs) in the *I.*

ramosa microbiome contained genes encoding Type III secretion systems, responsible for the transport of bacterial effector proteins into eukaryotic cells [89], including the order Pseudomonadales (2 MAGs; Fig. S12, Table S4). Secretion Type VI, used by bacterial symbionts to eliminate microbial competitors [90] and to translocate toxic effector proteins into eukaryotic cells [84], was also identified in Pseudomonadales (2 MAGs; Fig. S12, Table S4). KOs associated with Type IV secretion systems, which may play a role in delivering eukaryotic-like proteins into host cells [87], were identified in lineages belonging to the Acidobacteriota and Gemmatimonadota (Fig. S12, Table S4). While Type IV secretion systems have previously been reported to be abundant [9] and enriched [91] in sponge symbionts, overall module completeness was below 60% in *I. ramosa* MAGs. These data suggest that the majority of the *I. ramosa* microbiome does not interact directly with the host cells via needle-like secretion systems, but highlights specific lineages that may. Interestingly, the presence of the Type VI secretion system raises the possibility of bacteria–bacteria interactions or potentially parasitism/pathogenesis within the Pseudomonadales.

Ank repeat proteins (ARPs) mediate protein–protein interactions and are one of the most common domains present in eukaryotes, though they are much rarer in bacteria and archaea [89]. ARPs are commonly enriched and actively expressed in sponge symbionts [9, 14, 49, 82] and their presence in microorganisms has been suggested to mediate host–microbe interactions and protect sponge symbionts from host phagocytosis [14, 65, 85]. The average percentage of ARPs in the *I. ramosa* MAGs was 0.14% of all coding genes (Table S1), ranging from 0 to 0.89% (Acidobacteriota, order Luteitaleales). Of the total MAGs, 27% (including Chloroflexota, Cyanobacteriota, Gemmatimonadota, and Latescibacterota) had an ARP/gene percentage >0.2%, similar to known symbionts (Note S3, [88]) ARPs were absent in multiple lineages, including the Thaumarchaeota, Patescibacteria, Actinobacteriota (family TK06 and order UBA5794), and Chloroflexota (family UBA2991 and order UBA2979), suggesting that these taxa must use alternative mechanisms to evade digestion. Another potential mediator of protein–protein interactions, previously identified as necessary for the infection of rhizobium in root nodules [92], are WD40 repeat-containing proteins. Like ARPs, these proteins are prevalent in sponge symbionts [79, 80]. Of the *I. ramosa* MAGs, 40% encoded WD40 repeat-containing proteins, with an average WD40 count/gene of 0.08% (Table S1), ranging from 0 to 3.59%. The highest number of WD40 repeat-containing proteins were found in the orders WGA-4E (Poribacteria), Rhodothermales (Bacteroidota), and UBA8231 (Latescibacterota). These findings suggest a role for both ARPs and WD40 repeat-containing proteins in maintaining the stable

symbiotic associations evident in *I. ramosa*. Lineages lacking both ARPs and WD40 repeats must use alternative, yet to be identified, mechanisms for evading host phagocytosis.

Conclusions

Genomic recovery of most members of the *I. ramosa* microbiome has provided unprecedented insights into the metabolic potential of a complex sponge microbiome. Previous investigations of the sponge microbiome [12] have suggested that microbes can be partitioned into distinct groups based on broad-scale analysis of all metabolic pathways (Clusters of Orthologous Groups) present in the MAGs. One of these groups was shown to encode higher numbers of genes for carnitine metabolism while a second was enriched for genes for sulfated polysaccharide degradation. These findings suggest a degree of metabolic specialization between microbial groups but do not preclude the existence of other pathways that might be shared between lineages. Here, we reveal considerable redundancy in pathways for autotrophic carbon fixation, nitrogen and sulfur metabolism, and vitamin synthesis. Sponges are sessile organisms, residing in a dynamic environment. Changes in the pumping activity of the sponge can rapidly modify the environmental conditions of the microbiome—for instance, shifting the mesohyl region from oxic to anoxic and vice versa [73, 93]. In such an environment, a variety of metabolic traits from the taxonomically-diverse microbiome increases the physiological plasticity of the holobiont under changing environmental conditions. The identification of three autotrophic carbon fixation pathways (rTCA cycle, HP-HB cycle, and CBB cycle) raises questions about the extent to which the sponge host relies on microbial symbionts for carbon acquisition. Importantly, functional redundancy in carbon fixation could increase the stability of the sponge under changing environmental conditions [94]. Supporting this hypothesis are observations of sponge bleaching, which, in comparison to coral bleaching, is rarely lethal [95, 96]. In addition, elevated inorganic carbon availability appears to reduce the degree of stress in sponges exposed to ocean warming [95]. Future functional physiological studies should test the relative contribution of each carbon fixation pathway to host nutrition under different environmental conditions.

This study also identified potential keystone taxa within the *I. ramosa* microbiome, with Thaumarchaeota (family Nitrosopumilaceae) as the sole taxa able to perform the ammonia oxidation step of nitrification and Gammaproteobacteria (predominantly the family LS-SOB) as the only sulfide oxidizers. Assigning potential functions to specific taxa through metagenomic analysis will allow these taxa to

be targeted in functional physiological studies. Loss of keystone taxa, due to environmental stress or other perturbations, might have detrimental effects on the sponge host and its direct environment. For example, in the absence of ammonia oxidizers, toxic ammonia would not be converted into nitrate and cycled through the sponge holobiont.

Current genomic resources for reef invertebrate microbiomes are limited. Using an integrative metagenomic approach we reveal extensive metabolic potential and taxonomic and functional redundancy in the sponge microbiome. Establishment of a comprehensive genomic baseline for a sponge microbiome provides a valuable platform for future functional studies aimed at validating and quantifying the contribution of each of the putative symbiotic functions to host health. Studies employing (meta)transcriptomics, proteomics, immunohistochemical and stable isotope-tracer experiments should be undertaken to confirm expression and track the fate of specific compounds within the holobiont. Future studies aimed at understanding the sponge as a holobiont should also move towards the retrieval of host genomes so that the metabolic potential of the sponge and its symbionts can be assessed for metabolic complementarity.

Data availability

All data for this study can be found under the Bioproject ID PRJNA555144. The metagenomic reads for *I. ramosa* are available at NCBI under the accession numbers SRR9841427-SRR9841447 and the MAGs can be found under the accession numbers VXL100000000-VYGU00000000, which are also shown in Table S1.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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