

# Validation of key sponge symbiont pathways using genome-centric metatranscriptomics

Paul A. O'Brien<sup>1,2,3,4</sup>  | Shangjin Tan<sup>5</sup>  | Pedro R. Frade<sup>6</sup>  |  
Steven J. Robbins<sup>4</sup> | J. Pamela Engelberts<sup>4,7</sup>  | Sara C. Bell<sup>2</sup> |  
Inka Vanwonterghem<sup>4</sup> | David J. Miller<sup>8,9</sup> | Nicole S. Webster<sup>2,3,4,10</sup>  |  
Guojie Zhang<sup>11,12</sup>  | David G. Bourne<sup>1,2,3</sup> 

<sup>1</sup>College of Science and Engineering, James Cook University, Townsville, Queensland, Australia

<sup>2</sup>Australian Institute of Marine Science, Townsville, Queensland, Australia

<sup>3</sup>AIMS@JCU, Townsville, Queensland, Australia

<sup>4</sup>Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Queensland, Australia

<sup>5</sup>BGI-Shenzhen, Beishan Industrial Zone, Shenzhen, China

<sup>6</sup>Natural History Museum Vienna, Vienna, Austria

<sup>7</sup>Centre for Microbiome Research, Translational Research Institute, Queensland University of Technology, Brisbane, Queensland, Australia

<sup>8</sup>ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia

<sup>9</sup>Centre for Tropical Bioinformatics and Molecular Biology, James Cook University, Townsville, Queensland, Australia

<sup>10</sup>Australian Antarctic Division, Department of Climate Change, Energy, Environment and Water, Kingston, Tasmania, Australia

<sup>11</sup>Centre for Evolutionary & Organismal Biology and Women's Hospital, Zhejiang University, School of Medicine, Hangzhou, China

<sup>12</sup>Liangzhu Laboratory, Zhejiang University Medical Center, Hangzhou, China

## Correspondence

David G. Bourne, College of Science and Engineering, James Cook University, Townsville, QLD, Australia.

Email: [david.bourne@jcu.edu.au](mailto:david.bourne@jcu.edu.au)

Guojie Zhang, Centre for Evolutionary & Organismal Biology and Women's Hospital, Zhejiang University, School of Medicine, Hangzhou 310058, China.

Email: [guojiezhang@zju.edu.cn](mailto:guojiezhang@zju.edu.cn)

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## Abstract

The sponge microbiome underpins host function through provision and recycling of essential nutrients in a nutrient poor environment. Genomic data suggest that carbohydrate degradation, carbon fixation, nitrogen metabolism, sulphur metabolism and supplementation of B-vitamins are central microbial functions. However, validation beyond the genomic potential of sponge symbiont pathways is rarely explored. To evaluate metagenomic predictions, we sequenced the metagenomes and metatranscriptomes of three common coral reef sponges: *Ircinia ramosa*, *Ircinia microconulosa* and *Phyllospongia foliascens*. Multiple carbohydrate active enzymes were expressed by Poribacteria, Bacteroidota and Cyanobacteria symbionts, suggesting these lineages have a central role in assimilating dissolved organic matter. Expression of entire pathways for carbon fixation and multiple sulphur compound transformations were observed in all sponges. Gene expression for anaerobic nitrogen metabolism (denitrification and nitrate reduction) were more common than aerobic metabolism (nitrification), where only the *I. ramosa* microbiome expressed the nitrification pathway. Finally, while expression of the biosynthetic pathways for B-vitamins was common, the expression of additional transporter genes was far more limited. Overall, we highlight consistencies and disparities between metagenomic and

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metatranscriptomic results when inferring microbial activity, while uncovering new microbial taxa that contribute to the health of their sponge host via nutrient exchange.

## INTRODUCTION

Marine sponges are key members of coral reef ecosystems. By filtering large volumes of seawater, sponges make dissolved organic matter (DOM) available to higher trophic levels, thereby providing a nutrient source in an otherwise nutrient poor environment (De Goeij et al., 2013). Part of that trophic link is performed by sponge-associated microbial communities (Hudspith et al., 2021), where up to 87% of DOM can be assimilated by the microbiota in high-microbial abundance (HMA) sponges (Rix et al., 2020), likely performed by specific lineages within the community (Campana et al., 2021; Robbins et al., 2021). This ability to break down and cycle nutrients demonstrates the importance of the microbial community in underpinning the ecological function of sponges.

Sponges are one of the earliest diverging animal lineages and the sponge-microbe symbiosis likely represents one of the most basal host-microbe symbioses, likely contributing to the species specific and stable microbial associations observed today (Thomas et al., 2016). For example, the sponge microbiota shows stability under environmental disturbances such as changes in water quality, temperature and salinity (Glasl et al., 2018; Luter et al., 2014; Strand et al., 2017). Furthermore, there is a strong signal of phylosymbiosis for HMA sponges, displaying relatively low community variation within a host species, which becomes more dissimilar as host species become more divergent (O'Brien et al., 2020; Sabrina Pankey et al., 2022; Thomas et al., 2016). Within the sponge microbiota, a number of lineages have shown strong cophylogenetic signals, indicating a tightly coupled host-microbe relationship that underpins the health of the host (Matcher et al., 2017; O'Brien et al., 2021; Sabrina Pankey et al., 2022). Additionally, in some cases sponges vertically transmit a proportion of their microbes, ensuring the symbiosis persists through generations (Björk et al., 2019; Engelberts et al., 2022; Schmitt et al., 2008). This well-established, deeply divergent and tightly coupled host-microbe relationship, along with the importance of their microbiota, make sponges an ideal choice for host-microbe symbiosis research.

A wealth of knowledge on putative sponge-associated microbial activities has been generated through metagenomic studies. For example, pathways involved in carbon fixation, carbohydrate metabolism, sulphur and nitrogen cycling as well as biosynthesis of many B-vitamins are encoded in sponge associated

microbial genomes and putatively supplement host metabolism (Engelberts et al., 2020; Kamke et al., 2013; Moreno-Pino et al., 2020; Robbins et al., 2021; Thomas et al., 2010). In addition, the genomes of many sponge symbionts are enriched in genes that potentially facilitate life in a host environment, such as eukaryote-like proteins and restriction enzymes, which help symbionts evade phagocytosis and defend against harmful mobile genetic elements that might otherwise inhibit survival within a host (Reynolds & Thomas, 2016; Robbins et al., 2021). Further, some sponge symbionts contain the genomic repertoire for secretion systems (Engelberts et al., 2020; Robbins et al., 2021), which can be used by both beneficial and pathogenic symbionts to interact with host cells (Costa et al., 2015).

Metagenomic studies are highly valuable for inferring functional potential from DNA coding sequences in the microbiome. However, the application of metatranscriptomics, which employs the sequencing and analysis of microbial mRNA to establish microbial activity, can be used to validate inferences based on metagenomic data. Metatranscriptomics has been applied to show expression of microbially mediated B-vitamin and amino-acid synthesis as well as nitrification and carbon fixation in sponges (Fiore et al., 2015; Radax et al., 2012). However, these earlier studies used a gene-centric approach, precluding a direct linkage between gene activity and microbial taxonomy. More recent studies use a genome-centric approach (Burgsdorf et al., 2021; El Samak et al., 2023), which can identify the microbial genome that is responsible for the observed transcriptomic activity and can elucidate potential syntrophy. For example, genome-centric metatranscriptomics has previously been used to link the oxidation of ammonia to nitrite by sponge-associated Thermoproteota (formerly Thaumarcheota; Rinke et al., 2021) symbionts and the subsequent oxidation of nitrite to nitrate by members of the bacterial genus *Nitrospira* (Moitinho-Silva et al., 2017).

Here, we sequenced the metagenome and metatranscriptome of three common Great Barrier Reef (GBR) sponges, *Ircinia ramosa*, *Ircinia microconulosa* and *Phyllospongia foliascens* (formerly *Carteriospongia*; Abdul Wahab et al., 2021) followed by genome-centric analyses to validate metabolic pathways potentially utilised by sponge symbionts and link pathways to specific microbial lineages. These sponge species were selected for their HMA, wherein microbes have been shown to play a larger role in supporting the sponge in its ecological function of cycling DOM

compared to low microbial abundance sponges (Rix et al., 2020). Further, the metagenome of *I. ramosa* has been extensively characterised (Engelberts et al., 2020), making it an ideal choice to validate metagenome hypothesised metabolic pathways. Less is known about the microbiota of the closely related *I. microconulosa*, however amplicon data suggest the microbiomes of the two *Ircinia* spp. are highly similar and likely share similar functional potential (O'Brien et al., 2020). Finally, *Phyllospongia* are phototrophic sponges where vertically transmitted Cyanobacteria symbionts potentially contribute up to 50% of the sponge's energy requirements (Abdul Wahab et al., 2021).

## EXPERIMENTAL PROCEDURES

### Sample collection and preservation

Five replicates of three sponge species (*P. foliascens*, *I. ramosa* and *I. microconulosa*) were collected at a depth between 5 and 10 m from Davies Reef ( $-18.82^{\circ}$ ,  $147.65^{\circ}$ ) and Broadhurst Reef ( $-18.97^{\circ}$ ,  $147.72^{\circ}$ ) in the central sector of GBR, Australia, in August 2017. An additional five replicates of *I. ramosa* were collected from the Ribbon Reefs ( $-14.79^{\circ}$ ,  $145.69^{\circ}$ ) in the northern sector of the GBR, Australia, in October 2017, to investigate if microbial community composition displayed a spatial difference and improve the chances of recovering novel symbiont genomes. Since no site effect was observed, data from both locations were used for metagenome binning (see Section 2.6) of *I. ramosa* to increase the number of sample replicates for differential coverage analysis. Sponges were placed in holding tanks with running seawater on the research vessel until sampling and returned to the reef once completed. A small section of each specimen was dissected and rinsed in autoclaved calcium- and magnesium-free artificial seawater (CMFSW; 0.5 M NaCl, 0.01 M KCl, 0.007 M  $\text{Na}_2\text{SO}_4$ , 0.0005 M  $\text{NaHCO}_3$ , Milli-Q water), then cut into small pieces approximately  $0.5\text{ cm}^3$ . Dissected pieces were then added to a 15 mL falcon tube half filled with dimethylsulfoxide-EDTA salt saturated solution (DESS; 0.25 M EDTA, 20% DMSO, NaCl saturated, Milli-Q water, pH 8.0) until the tube was full, reaching a 1:1 DESS: tissue ratio and stored at  $-20^{\circ}\text{C}$  for metagenomic analysis. Three replicates from *P. foliascens* and *I. microconulosa* were additionally preserved for metatranscriptomic analysis using the same approach. Finally, three replicates from *I. ramosa* were collected from Davies Reef in January 2018 for metatranscriptomic analysis as part of an additional study (unpublished). Hence, the following metatranscriptomic laboratory protocols differ for *I. ramosa* compared to *P. foliascens* and *I. microconulosa*.

### Microbial cell enrichment by cell separation for metagenomics

Separation of prokaryotic and eukaryotic cells was performed following the method of Thomas et al. (2010) with minor modifications. Briefly, approximately 2–4 g of sponge sample was transferred to a 50 mL Falcon tube containing 15 mL of autoclaved CMFSW and agitated for 5–10 min at 99 rpm at room temperature using the intelli-mixer RM-2 (Bartelt Instruments Pty Ltd) to remove loosely attached microbes. The washed sample was transferred to  $2 \times 15\text{ mL}$  Falcon tubes half filled with fresh autoclaved CMFSW and homogenised using the Bio-Gen PRO200 Rotary Homogenizer (Pro Scientific Inc) for 10 min. The homogenate was pooled and filter-sterilised collagenase added to a final concentration of  $0.5\text{ mg mL}^{-1}$ . Samples were agitated using the intelli-mixer RM-2 at 99 rpm for 30 min to further break apart the sponge cells. The sponge homogenate was then filtered through a  $100\text{ }\mu\text{m}$  sieve and collected into a new Falcon tube, centrifuged at  $100 \times g$  for 15 min at  $4^{\circ}\text{C}$  and then centrifuged twice at  $300 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The supernatant was filtered twice through an  $8\text{ }\mu\text{m}$  and twice through a  $5\text{ }\mu\text{m}$  filter and then centrifuged for 20 min at  $8800 \times g$  at  $4^{\circ}\text{C}$  to pellet the microbial cells. Two wash steps were performed by adding 10 mL of 10 mM Tris-NaCl, pH 8 to the pellet and then resuspended by vortex for 5 s, followed by centrifugation for 20 min at  $8000 \times g$  at  $4^{\circ}\text{C}$  to re-pellet the cells. Finally, the cell pellet was recovered in 1 mL of 10 mM Tris-NaCl, pH 8 and divided into two  $500\text{ }\mu\text{L}$  aliquots and stored at  $-20^{\circ}\text{C}$ .

### Total DNA extraction for metagenomics

One  $500\text{ }\mu\text{L}$  aliquot of cell separated sponge tissue was used for DNA extraction. Contaminating host DNA was first reduced by the addition of  $1\text{ }\mu\text{L}$  of DNAase 1 (NE bio labs),  $50\text{ }\mu\text{L}$  of DNase buffer and incubated for 30 min at  $37^{\circ}\text{C}$ . DNAase 1 was heat inactivated by adding  $5\text{ }\mu\text{L}$  of 0.5 M EDTA and incubated at  $75^{\circ}\text{C}$  for 10 min and then proceeded directly to DNA extraction using a Phenol:Chloroform:IAA (25:24:1) phase separation extraction protocol (full details can be found in Supporting Information S1).

### Total RNA extraction and microbial mRNA enrichment for metatranscriptomics

For sponge species *I. microconulosa* and *P. foliascens*, approximately 0.25 g of sponge tissue was crushed using liquid nitrogen and a mortar and pestle and total RNA isolated using the RNeasy PowerMicrobiome Kit (Qiagen) following the manufacture's protocol. Total RNA was cleaned using the RNeasy MinElute Cleanup

kit (Qiagen) and quality and quantity of total RNA were checked using the Agilent 2200 TapeStation on the High Sensitivity RNA ScreenTape system. DNA was isolated from approximately 0.05 g of sponge tissue using the DNeasy PowerBiofilm Kit (Qiagen) as per the manufacturer's protocol for the purpose of synthesising rRNA probes for rRNA depletion. Microbial mRNA enrichment was performed following an adapted protocol from Sato et al. (2017) and Stewart et al. (2010) (full details can be found in Supporting Information S1). For *I. ramosa*, methods for RNA extraction and microbial mRNA enrichment are described in Díez-Vives et al. (2017) and Taylor et al. (2022). Briefly, 0.2 g of sponge tissue was homogenised using a mortar and pestle under liquid Nitrogen at  $-80^{\circ}\text{C}$  then processed on a FastPrep24 bead-beater for 30 s at 5.5 m/s with Lysing Matrix E (MPBiomedicals) and 1 mL of TRIzol. RNA was then purified using a PureLink RNA Mini kit (Ambion). Eukaryotic RNA was discarded and prokaryotic mRNA reads retained using the Poly(A)Purist™ MAG Kit (Ambion) and rRNA was removed using the Ribo-Zero rRNA Removal Kit with 50% Bacteria and 50% Plant Leaf probes (Illumina). Prokaryote enriched mRNA was then purified using an ethanol precipitation protocol.

## Library preparation and sequencing

Metagenomic (*P. foliascens*, *I. microconulosa* and *I. ramosa*) and metatranscriptomic (*P. foliascens* and *I. microconulosa*) libraries were prepared using the MGIEasy Universal DNA Library Prep Set (BGI, Shenzhen, China) and sequenced on the MGISEQ-2000 platform under the 100 bp paired-end mode at BGI Australia (Queensland, Australia). An additional technical replicate of sample CS70 (*I. ramosa*, Ribbon Reefs) was prepared for metagenomic sequencing and subsequently used for metagenomic binning, resulting in a total of 21 metagenomic libraries and 6 metatranscriptomic libraries sequenced. Library preparation and sequencing of the *I. ramosa* metatranscriptome was conducted at the Ramaciotti Centre for Genomics, Sydney, Australia using a TruSeq Stranded mRNA kit and a  $2 \times 150$  bp high output sequencing run using the Illumina NextSeq 500 platform.

## Bioinformatics pipeline for metagenomic analysis

Raw reads were first pre-processed using SOAPnuke v2.1.0 (Chen et al., 2018) to remove adaptor sequences and low-quality reads and each sample was assembled individually using the metaSPAdes option in the SPAdes genome assembler v3.14.0 (Nurk et al., 2017). Cleaned reads from each sample were

mapped back to each assembly of the same host species using CoverM v. 0.5.0 (<https://github.com/wwood/CoverM>), utilising the short-read option within minimap2 alignment programme (Li, 2018). The resulting BAM files were used for differential coverage estimation using the jgi\_summarize\_bam\_contig\_depths script implemented through MetaBAT v2.15 (Kang et al., 2019). Metagenomic binning was performed for each assembly using both MetaBAT v1 (Kang et al., 2015) (with three different parameters: sensitive, specific and superspecific) and MetaBAT v2 executed through MetaBAT v2.15, as well as MaxBin v2.2.7 (Wu et al., 2016) and CONCOCT v1.1.0 (Alneberg et al., 2014), giving a total of six sets of bins per sample. A non-redundant final set of bins was then selected using DAS Tool v1.1.2 (Sieber et al., 2018), by ranking each genome based on a single-copy gene scoring function. Quality of each of the refined set of bins was further scrutinised using CheckM v1.1.2 (Parks et al., 2015) and finally all bins were consolidated and dereplicated at 95 average nucleotide identity (ANI) using CoverM v 0.5.0, retaining only those bins with >50% completion and <10% contamination.

To assign taxonomy to each metagenome assembled genome (MAG) and obtain a phylogenomic tree of MAGs, the Genome Taxonomy Database software toolkit (GTDB-tk) was used by running the 'Classify' workflow. Briefly, this pipeline identifies 120 and 122 bacterial and archaeal marker genes respectively for multiple sequence alignment and phylogenetic inference. Genome classification is achieved by placement of each genome into the GTDB-tk reference tree using pplacer (Matsen et al., 2010). Genome coverage (relative abundance) was calculated in CoverM v0.5.0 using the 'coverm genome' command with default parameters, which mapped the cleaned reads from each sample back to the final set of 95% ANI dereplicated MAGs.

## Bioinformatics pipeline for metatranscriptomic analysis

Raw reads were quality checked using SOAPnuke v2.1.0 to remove adaptor sequences and low-quality reads and further pre-processed to remove rRNA reads using SortMeRNA v2.1 (Kopylova et al., 2012). A custom reference database for each host species was configured by taking the full set of MAGs from each host species and dereplicating each host separately at 95% ANI (as described above), and finally concatenating into a single reference file for each host species. Cleaned metatranscriptomic reads from each host species were aligned to the reference file of the same host species to produce BAM files using CoverM v0.5.0 with the minimap-2 alignment programme (as above). BAM files were sorted by name using samtools v1.11 (Li et al., 2009) and metagenome reference files annotated

using EnrichM v0.6.3 ([github.com/geronimp/enrichM](https://github.com/geronimp/enrichM)) to obtain a gff file. Both the sorted bam files and gff annotation files were then imported into HTSeq v0.9.1 (Anders et al., 2015) to quantify the number of reads aligned to each gene in the annotation file using default parameters.

Following rRNA removal, reads were assembled using Trinity v2.9.1 (Grabherr et al., 2011) to provide an understanding of taxonomic classification of all mRNA reads and therefore assess eukaryotic contamination. Genes were de novo predicted on assembled transcripts using prodigal (v2.6.3) (Hyatt et al., 2010) and gene models with a CDS length <100 bp were excluded from downstream analyses. Protein sequences were then mapped against the National Centre for Biotechnology Information nonredundant (NCBI-nr) database using DIAMOND (v2.0.0) (Buchfink et al., 2015) and taxonomic classification was identified using BASTA (v1.3.2.3) (Kahlke & Ralph, 2019) after filtering hits with a bit score of <60, percent identity <50% and mapped length <50% for queries.

## Metabolic pathways reconstruction

A list of common metabolic pathways found in sponge associated microbes was compiled based on (Engelberts et al., 2020) and refined by manually curating each pathway based on their Kyoto Encyclopedia of Genes and Genomes (KEGG) maps. These included six prokaryote autotrophic carbon fixation pathways (Wood-Ljungdahl pathway, Dicarboxylate/4-hydroxybutyrate cycle, 3-hydroxypropionate bicycle, 3-hydroxypropionate/4-hydroxybutyrate cycle, reductive citric acid cycle and the Calvin-Benson-Bassham cycle), nitrogen metabolism (nitrogen fixation, nitrification, denitrification and dissimilatory nitrate reduction), sulphur metabolism (taurine transport and oxidation, dissimilatory sulphate reduction and thiosulphate oxidation) and B-vitamin biosynthesis. Since ammonia oxidising genes (*amoABC*) are known to contain homologues that are incorrectly annotated (Burgsdorf et al., 2021; Engelberts et al., 2020; Robbins et al., 2021), graftM v 0.14.0 (Boyd et al., 2018) was used to validate KO annotations following the method of Engelberts et al., 2020. Additionally, DOM assimilation was explored through analysis of carbohydrate-active enzyme (CAZyme) expression. Metatranscriptomic datasets were first normalised to reads per kilobase million (RPKM) to give each gene within a sample equal opportunity of being considered expressed. Subsequently, genes with less than 0.1 RPKM mapped to it were not considered expressed to reduce the chance of DNA contamination and spurious mapping influencing pathway expression. Finally, annotation files from both metagenomic and metatranscriptomic datasets were subset to the genes of interest and then combined using a custom R script. The

final dataset consisted of each MAG and their gene annotation, and indicated whether the gene was absent, present but not expressed or expressed, while the CAZyme dataset included quantitative results on all CAZymes that were expressed within a sample and log transformed for visualisation. Pathways are reported as expressed if >70% of genes within the pathway were expressed and included key enzymes as determined by Engelberts et al., 2020.

## RESULTS

### Metagenomic binning and metatranscriptomic mapping results

A total of 1290 MAGs, including 1195 medium-high quality MAGs (>50% completion <10% contamination), were retrieved across the three species of sponges. Following dereplication, the total number was reduced to 781 medium-high quality MAGs at 99% ANI and 415 medium-high quality MAGs at 95% ANI (Table S2), representing clonal and species boundaries respectively (Jain et al., 2018). Mapping metagenomic reads from each sample to the 415 MAGs (95 ANI) showed that between 75.62% and 91.85% of reads successfully aligned to our final set of MAGs, indicating a good representation of the total microbial community in each sample (Table S1). This was highest for *I. microconulosa*, with  $88.97 \pm 0.88\%$  (mean  $\pm$  standard error) of reads mapping to the metagenome and lowest for *I. ramosa*, with  $82.39 \pm 2.17\%$ , while  $85.96 \pm 0.36\%$  of reads from *P. foliascens* mapped to the metagenome.

Following rRNA removal, metatranscriptomic reads were reduced to 201–296 million reads per sample for *P. foliascens* and *I. microconulosa*, and 18–22 million for *I. ramosa*. Mapping the remaining mRNA reads to the corresponding metagenomic reference files revealed  $81 \pm 1.6\%$  of reads did not align to the *P. foliascens* metagenome,  $85 \pm 3.3\%$  of reads did not align to the *I. microconulosa* metagenome and  $78 \pm 1.2\%$  did not align to the *I. ramosa* metagenome. Low mapping results are likely due, in part, to eukaryotic mRNA contamination, based on taxonomic classification of assembled metatranscriptomic contigs (Table S3). Additionally, the potential for errors in identifying coding sequences in the metagenome may further reduce mapping success (Hao et al., 2020). Of the mRNA reads that successfully aligned, a further  $3.0 \pm 0.2\%$ ,  $4.3 \pm 1.7\%$  and  $12 \pm 0.2\%$  of reads in *P. foliascens*, *I. microconulosa* and *I. ramosa* respectively were ambiguously aligned (i.e., mapped to multiple genes) and were therefore excluded. All subsequent analyses are based on the mRNA reads that were successfully aligned to the metagenomes.

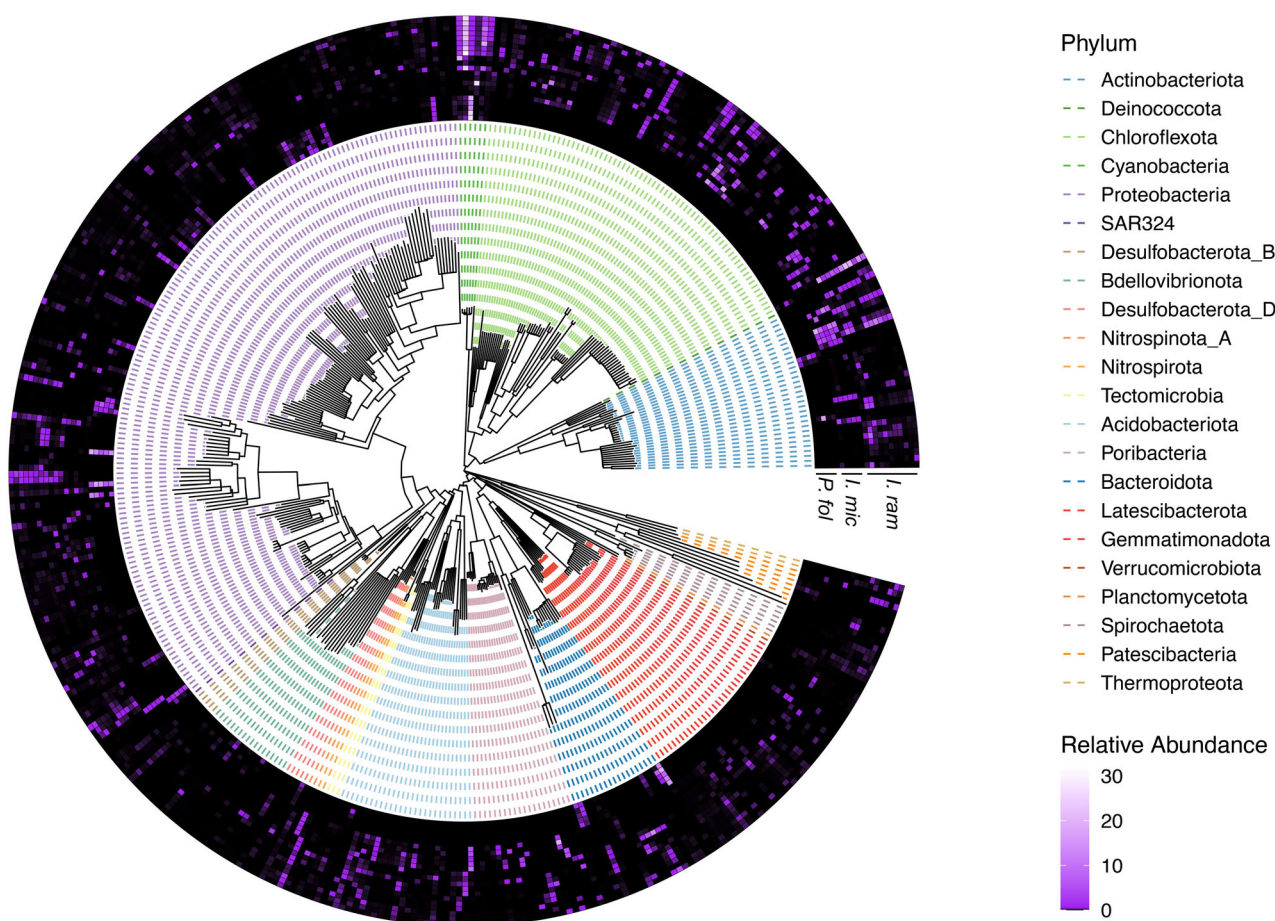
## MAG abundance is not proportional to metatranscriptomic activity

The 415 MAGs across all three sponge species represented 21 bacterial phyla and one archaeal phylum classified as Thermoproteota (formerly Thaumarchaeota) (Figure 1). Gene expression was detected in all phyla, however analysis of the successfully aligned metatranscriptomic reads showed that taxon-specific gene expression was disproportionate to the relative abundance of microbial genomes (Figure 2). For example, Cyanobacteria was the most abundant phylum in the *I. ramosa* metagenome representing  $29 \pm 10.7\%$  of the microbiota, with one MAG from the genus *Synechococcus* showing a relative abundance of  $19 \pm 2.9\%$ . In contrast, Cyanobacteria gene expression encompassed  $14 \pm 1.9\%$  of the metatranscriptomic reads, while  $62 \pm 5.1\%$  of the metatranscriptomic reads aligned to Poribacteria MAGs. Similarly, the most abundant phylum in the *I. microconulosa* metagenome was the Chloroflexota ( $34 \pm 1.2\%$ ), yet  $13 \pm 2.2\%$  of the metatranscriptomic reads aligned to this phylum, while  $22 \pm 10.4\%$  and  $21 \pm 0.6\%$  of reads aligned to Poribacteria and Proteobacteria MAGs respectively. Within the

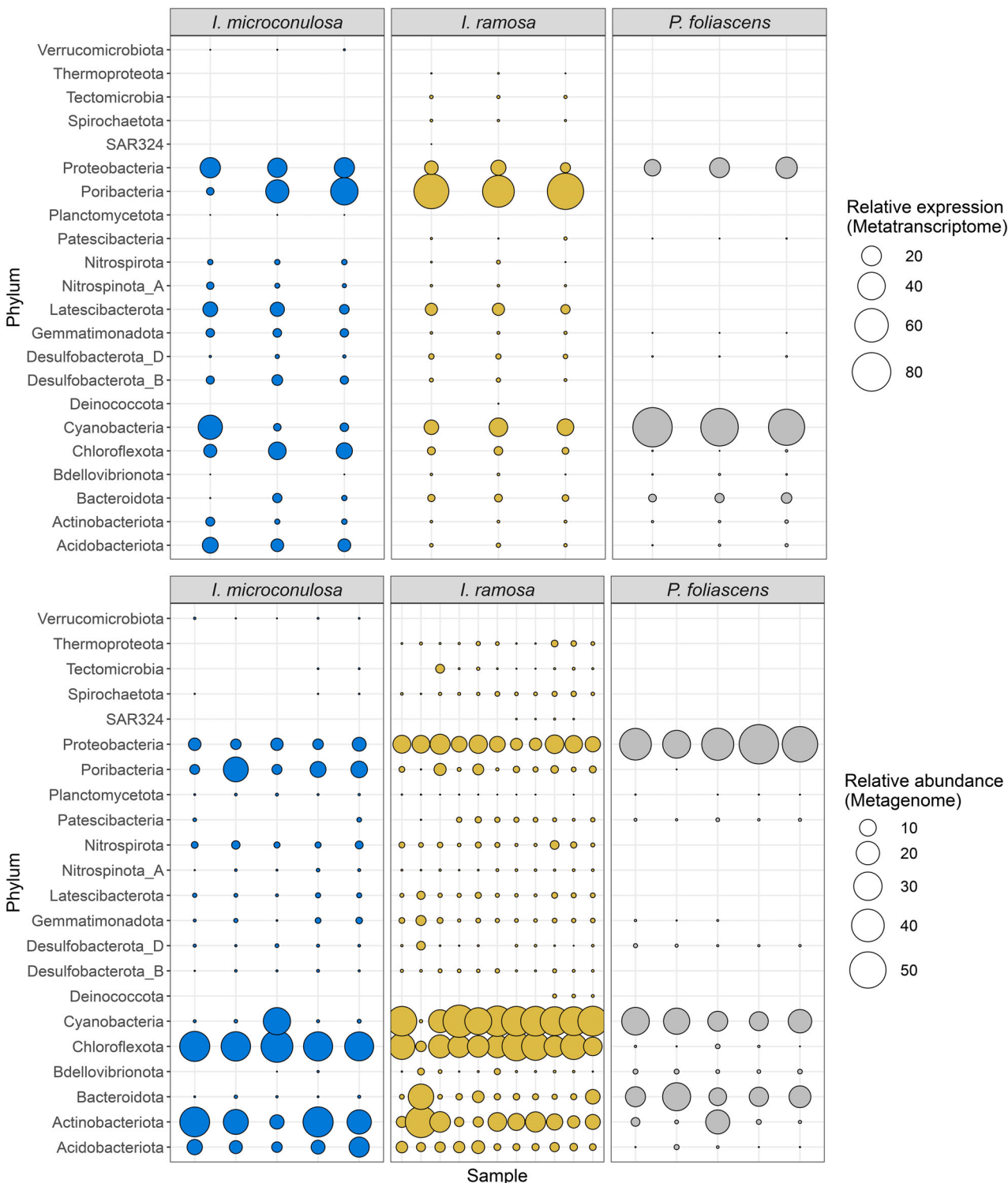
*P. foliascens* metagenome, Proteobacteria showed the highest relative abundance ( $42 \pm 5.1\%$ ) whereas  $20 \pm 2.9\%$  of metatranscriptomic reads aligned to this phylum. However, the most active phylum was Cyanobacteria, where  $76 \pm 3.9\%$  of metatranscriptomic reads aligned to MAGs within this phylum. While species replicates overall had a consistent level of gene expression based on microbial taxonomy, one *I. microconulosa* replicate showed a lower expression of Poribacteria and a higher expression of Cyanobacteria compared to the other two replicates (Figure 2).

## Carbohydrate-active enzymes are expressed by diverse members of the sponge microbiome

All three sponge microbiomes exhibited widespread expression of CAZymes including multiple carbohydrate esterases (CE) and glycosyl hydrolases (GH) (Figure 3). Notably, expression of CE family 11 was common among Cyanobacteria, Gammaproteobacteria and Alphaproteobacteria lineages in all three sponge metatranscriptomes, along with Nitrospirota in the two *Ircinia*



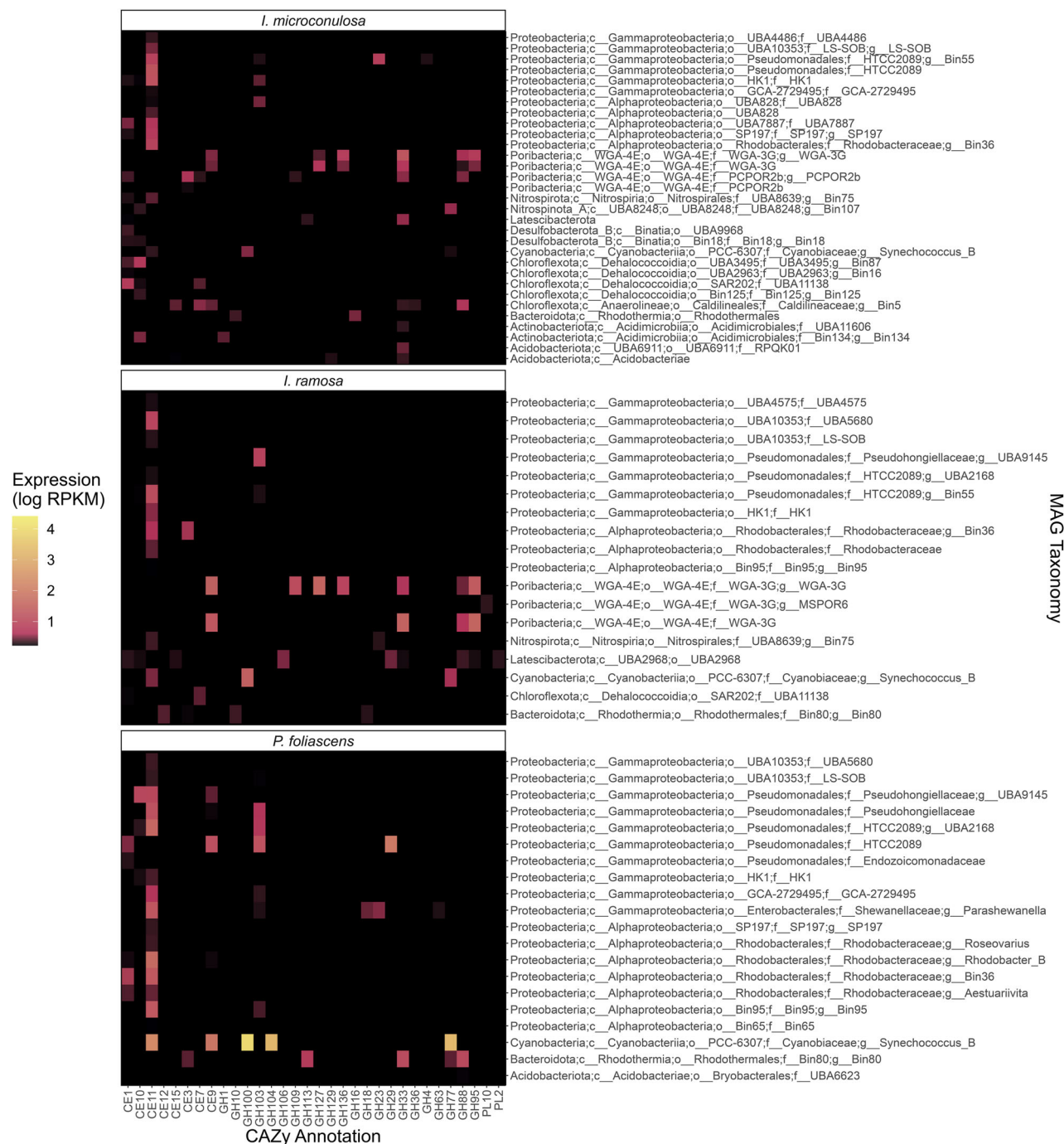
**FIGURE 1** Phylogeny of 415 metagenome assembled genomes (MAGs) isolated from *Ircinia ramosa*, *Ircinia microconulosa* and *Phyllospongia foliascens*. Tree clades are coloured by microbial phylum while the outer heatmap indicates the relative abundance of each MAG in each sponge sample.



**FIGURE 2** Relative expression of metatranscriptomic reads (top panel) compared against the relative abundance of metagenomic reads (bottom panel). All reads (metatranscriptomic and metagenomic) are mapped against the same set of metagenome assembled genomes (MAGs). The y-axis displays the taxonomic assignment of each MAG grouped by microbial phylum while the x-axis shows each metagenome and metatranscriptome sample. Each plot is faceted by the sponge host.

species. Similarly, CE 1 and 10 were expressed in multiple Alpha- and Gammaproteobacteria in *P. foliascens* as well as Chloroflexota in *I. microconulosa*. Multiple GHs were expressed in Poribacteria symbionts from both

*Ircinia* species, including GH33, GH88, GH95 and GH127. Within the *P. foliascens* metatranscriptomes, GH33, GH88 and GH95 were expressed by Bacteroidota symbionts, however no expression of GH127 was



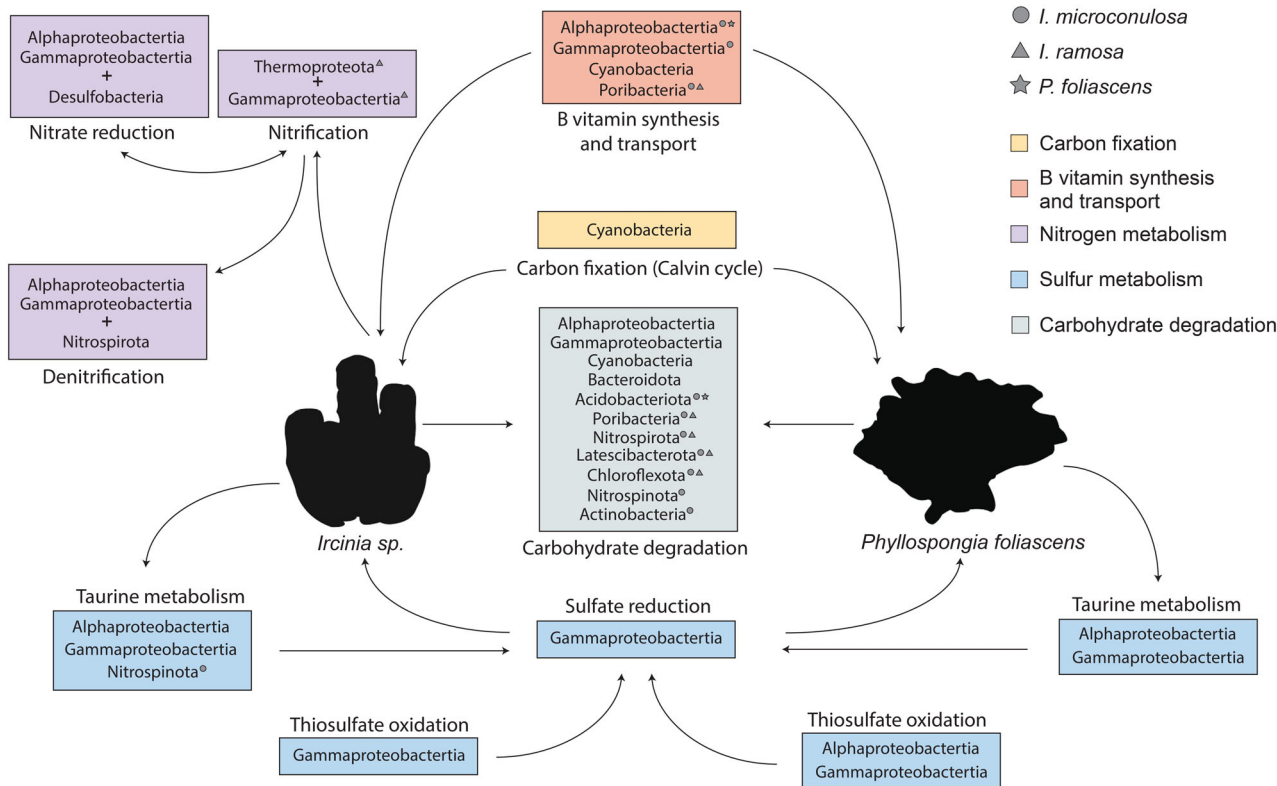
**FIGURE 3** Expression levels of CAZymes found across all three sponge metatranscriptomes. CAZymes are filtered to remove those with expression levels  $<0.25$  reads per kilobase million (RPKM) and metagenome assembled genomes (MAGs) with no CAZyme expression are removed. MAGs are grouped to the highest level classified and the resulting taxonomy is displayed on the y-axis, while each CAZyme that was expressed in at least one sponge is displayed on the x-axis. Transcript abundance is displayed in RPKM and log transformed to visualise expression levels more clearly.

observed. Similarly, Cyanobacteria symbionts from *P. foliascens* showed relatively high expression of multiple GHs, including GH77, GH100 and GH104. Finally, expression of polysaccharide lyases were less common among the three sponge microbiomes, which was only observed in Latescibacterota and Poribacteria symbionts associated with *I. ramosa*.

## Nutrient cycling in the sponge microbiome

All three sponge microbiomes had the metabolic potential for carbon fixation through the Calvin cycle and complete expression of this pathway was observed in *Synechococcus* for all sponges (Figure 4; Figure S1). Similarly, most of the genes necessary for carbon





**FIGURE 4** Overview of gene expression for metabolic pathways (>70% complete) and carbohydrate active enzymes found within the microbiomes of *Ircinia microconulosa*, *Ircinia ramosa* (grouped as *Ircinia sp.*) and *Phyllospongia foliascens*. Arrows indicate proposed transfer of nutrients within the holobiont. A '+' symbol is used to indicate where microbes from multiple taxonomic classifications are needed to complete a pathway. Bacteria expressing B-vitamin synthesis pathways are only included if expression of transporter genes were observed. Legend symbol indicates which sponge species gene expression was observed in where pathways are grouped for multiple sponges (no symbol indicates all sponges).

fixation through the reductive citric acid (rTCA) cycle were present in the metagenomes of both *Ircinia* species (Figure S2). In *I. microconulosa*, rTCA transcripts mapped to Nitrospirales MAGs (phylum Nitrospirota), however the genes necessary to synthesise 2-oxoglutarate (*korABCD*) were not found. In the case of *I. ramosa*, expression of Nitrospirota symbionts was incomplete for the rTCA cycle, where no transcripts were found for the key enzyme ATP-citrate lyase (*acIAB*). Near complete pathways for carbon fixation through the 3-hydroxypropionate (3-HP) bicycle were found in the metagenomes of all three sponges, however genes for the reduction of malonyl-CoA to propionyl-CoA (*mcr* and *pcc*) were absent from all symbionts, representing a key step missing in this pathway. Thermoproteota MAGs were only recovered from *I. ramosa* and although most key enzymes necessary for the HP-HB pathway were detected, such as methylmalonyl-CoA mutase (*mcmA12*) and 4-hydroxybutyryl-CoA dehydratase (*abfD*), no transcripts for these enzymes were found (Figure S3).

Genes for nitrogen fixation were absent from the microbiota of all three sponges, however both *Ircinia* species possessed microbes with the genomic potential for dissimilatory nitrate reduction, denitrification, and

nitrification. The first step in denitrification and nitrate reduction, the reduction of nitrate to nitrite by nitrate reductase (*narGHI*), was expressed in Gammaproteobacteria (family *HK1*) and Alphaproteobacteria (family *Rhodobacteraceae*) symbionts within the microbiota of *I. microconulosa* and *I. ramosa* respectively (Figure 4; Figure S4). Similarly, *narGH* was expressed by Nitrospirales in both *Ircinia* species, and may represent either nitrate reduction or nitrite oxidation (nitrification pathway), however given the expression of nitrate reductase (*nirK*) was also observed, this would suggest the Nitrospirales were involved in denitrification at the time of sampling. Alphaproteobacteria and Gammaproteobacteria MAGs additionally contained genes for the remaining steps of denitrification (*nirKIS*, *norBC*, *nosZ*), and although gene expression was incomplete, the last step in denitrification, the reduction of nitrous oxide to nitrogen (*nosZ*), was expressed by MAGs classified as Alphaproteobacteria in the *I. microconulosa* microbiome. Finally, Desulfobacterota showed either full or partial expression of nitrite reduction to ammonia (nitrate reductase, *nirBD*) in *I. microconulosa* and *I. ramosa* respectively, thereby completing the nitrate reduction pathway.

For nitrification, we identified complete expression of ammonia monooxygenase (*amoABC*) in *Nitrosopumilaceae* (Thermoproteota), confirming their role in nitrification (Figure S4). Additionally, all three genes for ammonia oxidation (*amoABC*) were found and partially expressed in Desulfobacterota in both *Ircinia* species (Figure S4). However, further characterisation of these genes using GraftM showed these were more closely related to Actinobacteria/Deltaproteobacteria homologues of *amoA* that are not involved in nitrification. Genes for the remaining steps in nitrification, oxidation of hydroxylamine to nitrate (*Hao*) and nitrite to nitrate (*narGH*), were expressed in the Gammaproteobacteria family *HK1* and Nitrospirales respectively in both *Ircinia* species, however, as mentioned above, it is likely the Nitrospirales were involved in denitrification rather than nitrification. In contrast, nitrogen metabolism was largely absent in the *P. foliascens* microbiota, where pathways for nitrate reduction, denitrification and nitrification were incomplete (Figure S4). Nonetheless, genomic potential was observed for denitrification, where genes for nitrate reductase were retrieved (*narGHI*) in Rhodospirillales (Alphaproteobacteria) and expressed (*napAB*) in Gammaproteobacteria (family *UBA10353*).

Since nitrification and denitrification/nitrate reduction represent aerobic and anaerobic processes respectively, and both these pathways were expressed in *I. ramosa*, we further analysed the relative expression within an individual to determine if transcript abundances were higher for aerobic or anaerobic processes, or if these were occurring in different individuals. All three individuals showed expression of denitrification/nitrate reduction genes (nitrate reductase, *narGHI* and nitrite reductase, *nirDK*), while two of the three individuals showed expression of nitrification genes (ammonia monooxygenase, *amoABC*) (Figure 5). However, in both individuals expressing nitrification genes, the relative expression of genes for aerobic metabolism (2.04 and 4.91 RPKM) was far lower compared to the expression of genes for anaerobic metabolism (27.17 and 43.47 RPKM). In particular, nitrite reductase (*nirD*) within the dissimilatory nitrate reduction pathway was highly expressed in Poribacteria MAGs in all three sponges (20.0, 27.19 and 21.42 RPKM), suggesting nitrite reduction was the dominant process at the time of sampling (Figure 5).

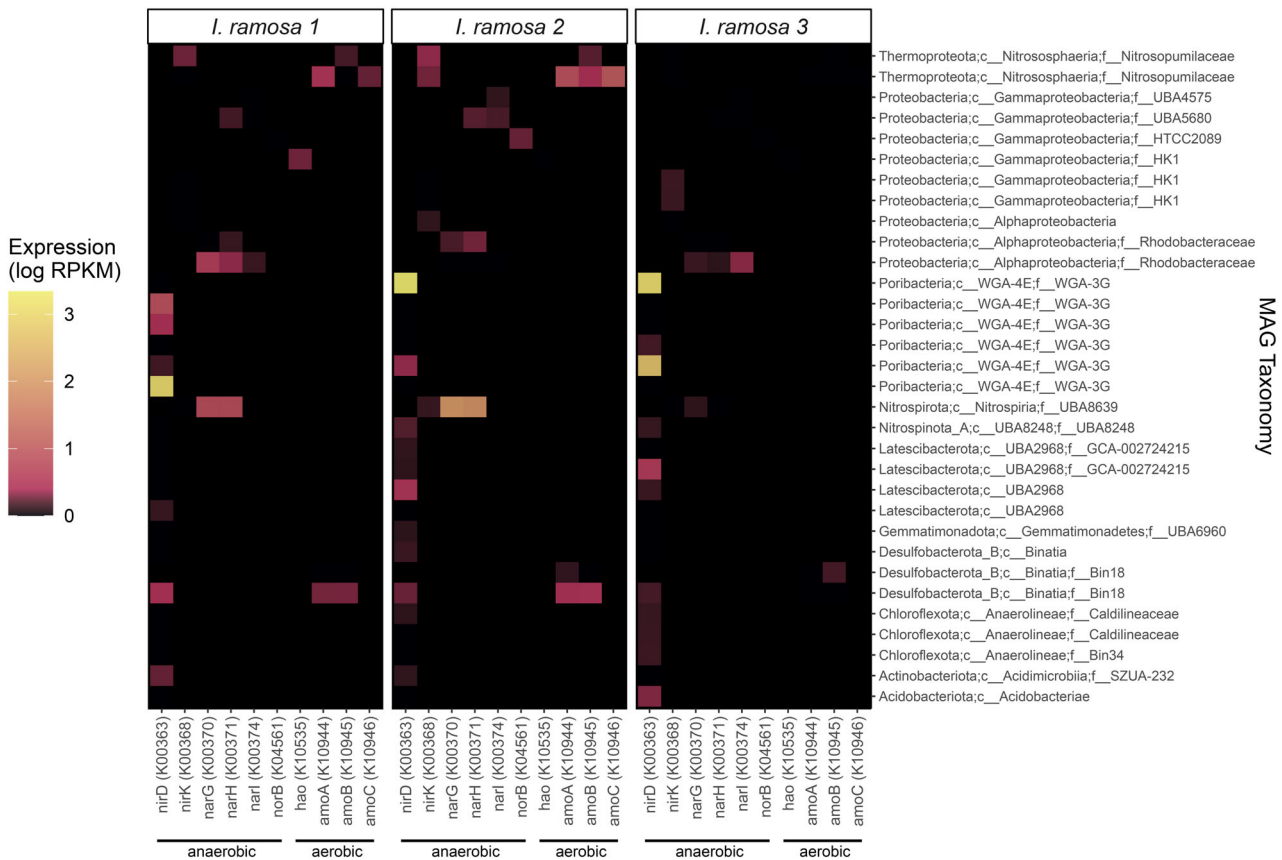
Genes for sulphur metabolism were present in all three sponge metagenomes, which included sulphate reduction, thiosulphate oxidation and taurine transport and oxidation. In all sponges, gene expression was observed for taurine transport and its metabolism via the ABC transport system (*tauABC*) and taurine dioxygenase (*tauD*) in Gammaproteobacteria MAGs, including the family *HK1*. Additionally, Alphaproteobacteria and Nitrospinota MAGs also expressed these genes in *I. microconulosa*, however expression was incomplete

for Alphaproteobacteria MAGs in the remaining two sponges (Figure 4; Figure S5). Similarly, Gammaproteobacteria MAGs (including *HK1*) showed complete expression for sulphate reduction (*sat*, *aprAB*, *dsrAB*) in both *P. foliascens* and *I. microconulosa*, while all genes except sulphate adenylyltransferase (*sat*) were expressed in Gammaproteobacteria MAGs from *I. ramosa*. Gammaproteobacteria MAGs (including *HK1*) also expressed the necessary genes for thiosulphate oxidation, however one gene was missing from the SOX complex (*soxC*) in MAGs from *P. foliascens*, while those from both *Ircinia* species were found but not expressed. In *P. foliascens*, *Rhodobacteraceae* expressed the full pathway for thiosulphate oxidation, however these genes were found but not expressed in *Rhodobacteraceae* MAGs from *I. microconulosa* and partially expressed in *I. ramosa*. Taken together, all sponges had metatranscriptomic profiles that were consistent in their ability to metabolise sulphur, particularly the Gammaproteobacteria family *HK1*, which showed complete or near complete expression for all sulphur transformations in all three sponges (Figure S5).

## Biosynthesis of B vitamins

The metagenomes of the three sponge species contained the necessary genes for all B vitamin synthesis including the bioactive forms for thiamine (B<sub>1</sub>), riboflavin (B<sub>2</sub>), pantothenate (B<sub>5</sub>), pyridoxine (B<sub>6</sub>), biotin (B<sub>7</sub>) and cobalamin (B<sub>12</sub>) as well as thiamine and biotin transporters. For thiamine biosynthesis, expression of thiamine phosphate pyrophosphorylase (*thiE*), necessary for the synthesis of thiamine phosphate, was widespread encompassing 11 phyla across all sponge metatranscriptomes (Figure 4; Figure S6). Similarly, the conversion of thiamine phosphate to thiamine diphosphate was widespread across all sponges, either directly through thiamine monophosphate kinase (*thiL*), or first converting to thiamine and subsequently thiamine diphosphate through thiamine pyrophosphokinase (*thiN*). Interestingly, it was only the Alphaproteobacteria and Gammaproteobacteria symbionts that showed full expression of thiamine transport genes, suggesting the majority of those involved in synthesising thiamine may not necessarily transport it. Specifically, *Rhodobacteraceae* and the Alphaproteobacteria family *SP197* expressed both thiamine synthesis and transporter genes, indicating they may be capable of transporting synthesised thiamine to the host.

Expression of the partial biosynthetic pathways for riboflavin were widespread in all sponge metatranscriptomes. In particular, the synthesis of riboflavin through riboflavin synthase (*ribE*), and its conversion to the cofactor flavin adenine dinucleotide (FAD) by riboflavin kinase (*ribF*), were expressed among the majority of



**FIGURE 5** Expression levels of nitrogen metabolism genes in three *Ircinia ramosa* metatranscriptomes. Only metagenome assembled genomes that expressed nitrogen metabolism genes are shown and the taxonomy is displayed on the y-axis (shortened to Phylum; Class; Family). The x-axis shows which genes were expressed and are grouped by aerobic and anaerobic processes and each sponge sample is displayed in a separate panel. Expression levels show aerobic and anaerobic processes are occurring in the same individual with higher levels of anaerobic expression.

microbial phyla (Figure S7). Similarly, expression for partial biosynthetic pathways for pantothenate was widespread across all sponges metatranscriptomes (Figure S8), particularly through the synthesis of pantoate (*ilvE*, *panBE*) and its conversion to pantothenate (*panC*). Pseudomonadales (Gammaproteobacteria) within the *P. foliascens* microbiome expressed the necessary genes for pyridoxine synthesis using D-Erythrose-4-phosphate (Figure 4; Figure S9), while partial expression of this pathway (*pdxAJH*) was observed in Bacteroidota, Alphaproteobacteria and Gammaproteobacteria symbionts in all three sponges (Figure S9). Importantly, pyridoxine can also be synthesised using pyridoxal 5'-phosphate synthase (*pdxTS*), which was expressed or partially expressed in Acidobacteriota, Chloroflexota and Actinobacteria symbionts from all sponges, as well as Latescibacterota symbionts in *I. ramosa*.

Genes for biotin synthesis (*bioFADE*) and its transporter (*bioY*, *ecfAA7*) were expressed by Cyanobacteria in all three sponge metatranscriptomes, suggesting these symbionts may secrete biotin (Figure 4; Figure S10). Similarly, Poribacteria symbionts from the *Ircinia* species

expressed both biotin synthesis and transporter genes. Interestingly, in all three sponge metatranscriptomes *Rhodobacteraceae* expressed genes for a second biotin transporter (*bioYNM*), yet no evidence of biotin synthesis was observed, suggesting this group may utilise biotin synthesised by other members of the microbiome. Likewise, the Gammaproteobacteria family *HK1* also expressed genes for biotin transport, however MAGs retrieved from the *Ircinia* species also encoded the genes for biotin synthesis, although these were not expressed. Additionally, Gammaproteobacteria and Alphaproteobacteria MAGs in *P. foliascens*, and Desulfobacterota MAGs in the *Ircinia* species, expressed the necessary genes for biotin synthesis, however no evidence of biotin transport was observed (Figure S10).

Cobalamin metabolism from cobyrinate a,c-diamide (*pduO*, *cbiB*, *cobAQC1P*) was detected in the metatranscriptomes of all sponge species (Figure 4; Figure S11), performed by Alphaproteobacteria, Gammaproteobacteria and Cyanobacteria symbionts. The synthesis of cobyrinate a,c-diamide in the cobalamin pathway may occur via both the precorrin (aerobic) pathway and co-precorrin (anaerobic) pathways, and

all sponges showed expression of the precorrin pathway in *Rhodobacteraceae* MAGs, although expression was incomplete for *I. ramosa* symbionts, as well as *HK1* in *P. foliascens* and *I. microconulosa*. Additionally, Cyanobacteria symbionts showed partial expression of this pathway in all three sponges. Overall, expression of vitamin synthesis was widespread in these coral reef sponge microbiomes, with high similarities between the two *Ircinia* species.

## DISCUSSION

Coral reefs generally exist in oligotrophic environments and therefore reef fauna must obtain and recycle nutrients efficiently to contribute to the high productivity of these ecosystems (De Goeij et al., 2013; Rix et al., 2020). While genomic research on the sponge microbiome has provided hypotheses on the genes and pathways that may allow sponges to contribute to these processes (Engelberts et al., 2020; Kamke et al., 2013; Robbins et al., 2021), little validation has been performed to establish which of these hypotheses are occurring. Our genome-centric metatranscriptomic analysis revealed that genes for carbohydrate degradation and pathways for nutrient metabolism and potential provision to the host are expressed within the microbiome of coral reef sponges. Although metagenomic data could predict microbial activity in terms of the genes and pathways utilised by symbionts, the disparity between microbial abundance and microbial activity suggests metagenomics alone can be misleading in the relative importance of different microbial taxa. These results highlight the need for obtaining both metagenomes and metatranscriptomes (or additional 'omic' datasets) from reef species to better understand benthic-pelagic nutrient cycling and subsequent reef productivity.

### Poribacteria, Bacteroidota and Cyanobacteria have central roles in the degradation of carbohydrates

A major ecological role of sponges is the breakdown and assimilation of DOM, performed in part by the sponge microbiome (Campana et al., 2021, 2022; Rix et al., 2020). The microbial taxa responsible for carbohydrate degradation are thought to be widespread, with symbionts from the phyla Poribacteria, Latescibacteria, Spirochaetota, Chloroflexota and Acidobacteriota all possessing an enrichment in GH and CE across seven sponge species (Robbins et al., 2021). Similarly, a coupling of DNA-stable isotope probing with 16S rRNA gene sequencing revealed that DOM degradation in the *Plakortis angulospiculatus* microbiome was largely attributed to symbionts belonging to the phyla PAUC34F

(Latescibacteria in GTDB), Proteobacteria, Poribacteria Nitrospirata and Chloroflexi (Campana et al., 2021). Here, we observed that carbohydrate degradation genes are expressed across 10 microbial phyla in the three sponge species investigated. In particular, CE families 1 and 11 were widely expressed across the three sponge microbiomes. These enzymes catalyse the hydrolysis of ester bonds between carbohydrates and functional groups, thereby making them more accessible to further degradation by GHs which act on glycosidic bonds (Nakamura et al., 2017). Across the three sponge metatranscriptomes, relatively high expression was observed for GH33, GH88, GH95 and GH127, responsible for the breakdown of sialic acids, glycosaminoglycans, fucose and arabinose respectively. Further, the genes encoding these enzymes are enriched in the microbiome of some coral reef demosponges (Robbins et al., 2021), which together with their expression patterns observed in this study, support their involvement in breaking down DOM released from marine algae and coral mucus (Hadaidi et al., 2019; Øverland et al., 2019; Rix et al., 2016). Within the *Ircinia* species, Poribacteria were largely responsible for the expression of these GHs, while Bacteroidota and Cyanobacteria were responsible for most of the expression of GHs in the *P. foliascens* microbiome. This supports previous findings that Poribacteria may be central to carbohydrate degradation given the high number of GHs encoded in their genome (Kamke et al., 2013), however when Poribacteria are not present, this role may be fulfilled by other members of the microbiome, such as Bacteroidota. Overall, these results suggest a cooperation of carbohydrate degradation between CEs and GHs, with widespread cleavage of ester bonds (CE), while a select few lineages are responsible for the breakdown of glycosidic bonds in specific carbohydrates (GH).

### Autotrophic carbon fixation in sponge symbionts

Expression of complete autotrophic carbon fixation was observed in the phototrophic cyanobacterium, *Synechococcus*, contributing to evidence that microbial carbon fixation provides a carbon source for the host and other members of the microbiome (Burgsdorf et al., 2021; Freeman et al., 2013). However, microbially fixed carbon is not universally transferred to the host and the presence of photosymbionts may serve other functions such as protection from solar radiation (Britstein et al., 2020; Burgsdorf et al., 2021). Interestingly, each sponge was dominated by a distinct *Synechococcus* population, where genome dereplication indicated that dominant MAGs from different host species shared less than 95% ANI. The presence of *Synechococcus* in sponges has been documented

extensively, including in high abundance within the microbial community of *Stylissa carteri* collected from the Red Sea, which showed high expression of Cyanobacteria photosystems and carbon fixation genes (Moitinho-Silva et al., 2014). Similarly, genomic analysis of the abundant symbiont *Candidatus Synechococcus spongarium* within the microbiota of *P. foliascens* collected from the Red Sea suggests that its genome had undergone streamlining and contained the necessary genes for carbon fixation through the Calvin cycle (Gao et al., 2014). Our *Synechococcus* genomes were closely related to the genome of *Ca. Synechococcus spongarium* (based on GTDB taxonomic assignment), indicating this *Synechococcus-Phyllospongia* symbiosis has been maintained across the vast geographic range the sponge is found. This may reflect the importance of *Synechococcus* as a sponge symbiont and its ability to contribute to the sponge carbon budget.

Additional pathways for autotrophic carbon fixation were near complete in our sponge microbiomes, highlighting the potential for alternative sources of fixed carbon. Previously, Nitrospirata from *I. ramosa* had been documented as possessing the necessary genes for the rTCA cycle (Engelberts et al., 2020), while expression of this pathway was observed in Nitrospirata from *Cymbastela concentrica* (Moitinho-Silva et al., 2017) and translocation observed using <sup>14</sup>C-labelling experiments in *Petrosia ficiformis* and *Theonella swinhoei* (Burgsdorf et al., 2021). Here, the microbiomes of both *Ircinia* species contained most of the genes required for the rTCA cycle and Nitrospirata symbionts had near complete expression of this pathway. Similarly, the 3-hydroxypropionate/4-hydroxybutyrate (HP-HB) cycle has been identified in Thermoproteota symbionts (formerly Thaumarchaeota) from *I. ramosa*, *C. concentrica*, *P. ficiformis* and *T. swinhoei*, using techniques such as metagenomics, metatranscriptomics and <sup>14</sup>C-labelling experiments (Burgsdorf et al., 2021; Engelberts et al., 2020; Moitinho-Silva et al., 2017). Thermoproteota MAGs retrieved here contained the near complete pathway for the HP-HB cycle, however no expression of these genes was observed. Given that carbon fixation through the HP-HB cycle is known to occur in Thermoproteota (Berg et al., 2010), it is likely transcripts here were not recovered rather than this pathway not being functional. Taken together, these results suggest carbon is fixed by a variety of symbionts using both light and dark fixation pathways.

## Anaerobic nitrogen metabolism is more dominant than aerobic in the sponge metatranscriptome

Within an oligotrophic environment, nitrogen is of particular interest as it is a limiting element for primary productivity in the ocean (Falkowski, 1997; Tyrrell, 1999). Given no sponge-associated microbes had the capability

of nitrogen fixation in this study, assimilation of inorganic nitrogen may provide an important source of nitrogen for the sponge microbiome. The metatranscriptome of both *Ircinia* species revealed evidence for denitrification and dissimilatory nitrate reduction, while *I. ramosa* also expressed genes for nitrification. Previously, *Nitrosopumiliaceae* has been suggested as a key sponge symbiont of *I. ramosa* given it was the only microbe containing ammonia oxidising genes (Engelberts et al., 2020). Our analysis confirms that *Nitrosopumiliaceae* MAGs from *I. ramosa* express ammonia oxidising genes and appear to play a key role in nitrification within the *I. ramosa* microbiome. While the Desulfobacterota also expressed KOs of ammonia oxidising genes, further investigation revealed these genes were more closely related to homologues of *amoA*, which have been attributed to the oxidation of methane or hydrocarbons based on gene sequence similarity (Burgsdorf et al., 2021; Robbins et al., 2021). Similarly, the Gammaproteobacteria family *HK1* expressed the next step in nitrification, hydroxylamine oxidation (*hao*) to nitrite, however it did not contain the additional ammonia oxidising genes and this has previously been attributed to the detoxification of hydroxylamine rather than nitrification (Engelberts et al., 2020). Finally, Nitrospirata can be involved in either nitrification (nitrite oxidation) or denitrification (nitrate reduction), depending on the presence of suitable electron donors (Koch et al., 2019). Given this group also expressed nitrite reductase (*nirk*) within the denitrification pathway, these findings suggest that at the time of sampling, Nitrospirata were contributing to denitrification in the sponge microbiome. Hence, our results imply nitrification is not a dominant process of the sponge microbiome and is reliant upon the presence of the ammonia oxidising archaea (Thermoproteota).

Denitrification and dissimilatory nitrate reduction both begin with the reduction of nitrate to nitrite and here we find the expression of this process in all three sponge metatranscriptomes. Moreover, the remainder of these pathways were found in both *Ircinia* species and showed near complete expression. While it may sound counterintuitive to observe expression of anaerobic (denitrification) and aerobic (nitrification) processes within the same sample, the higher expression levels of nitrate reduction within *I. ramosa* suggest denitrification was the dominant process at the time of sampling. Suitable niches for anaerobic metabolism are known to occur in the inner mesohyl of the sponge, which can experience microenvironments of anoxia through increased cellular respiration and reduced pumping rates (Lavy et al., 2016; Zhang et al., 2019). This may have been further exacerbated here during sampling, where the sponge is removed from the seawater causing inhibition of pumping. Moreover, both nitrification and denitrification have been observed in the same individual sponge using  $\text{NH}_4^+$  and <sup>15</sup>N incubation experiments (Hoffmann et al., 2009; Schläppy

et al., 2010), providing evidence of these processes occurring simultaneously. Finally, denitrification is known to occur aerobically under certain conditions, where some denitrifying bacteria (facultative anaerobes) may switch to oxygen instead of nitrate as the electron acceptor, allowing simultaneous denitrification and nitrification to occur (Seifi & Fazelipour, 2012). These studies suggest that nitrification and denitrification can be coupled within the sponge microbiome reflecting the expression of both these pathways in the *I. ramosa* metatranscriptome.

Although denitrification is known to occur in the sponge microbiome (Rooks et al., 2020; Schläppy et al., 2010), genes for the conversion of nitrous oxide to nitrogen (nitrous oxide reductase, *nosZ*), are often missing in sponge metagenomic data (Engelberts et al., 2020). Here we observed the expression of nitrous oxide reduction in the Alphaproteobacteria family *UBA828* associated with *I. microconulosa*, while additional Alphaproteobacteria MAGs contained this gene in all three sponges. Alphaproteobacteria also showed expression of the first steps of denitrification, both nitrate and nitrite reduction, suggesting this lineage may have an important role for denitrification within the sponge microbiome. Similar to denitrification, dissimilatory nitrate reduction is an anaerobic process, which involves the reduction of nitrate and nitrite resulting in ammonia. Our analysis showed that both Gammaproteobacteria and Desulfobacterota expressed nitrite reduction to ammonia, highlighting further capabilities of nitrogen cycling in the *Ircinia* species via anaerobic processes. Within the *P. foliascens* microbiota, genes for nitrogen metabolism were far less common, and only the expression of nitrate and (*napAB*) and nitrite (*nirBD*) reduction was observed. Hence in contrast to the *Ircinia* species, the microbiome of *P. foliascens* displayed a limited capacity for cycling nitrogen, which was restricted to dissimilatory nitrate reduction. Overall, all three sponges showed expression of anaerobic nitrogen pathways, suggesting these are the dominant processes for nitrogen metabolism within the sponge microbiome.

### Multiple transformations of sulphur by the Gammaproteobacteria family HK1 in all sponge metatranscriptomes

Sulphur is an essential nutrient required for microbial synthesis of certain amino acids (cysteine, cystine and methionine), vitamins (thiamine and biotin) and enzymes (Soda, 1987). One mechanism that sponge-associated microbes use to obtain sulphur is through sponge derived taurine, where sponge symbionts are often enriched in taurine dioxygenases and many of which also possess taurine transporters (Robbins et al., 2021; Moeller et al., 2023). Here, all

three sponge microbiomes contained the necessary genes to transport taurine across the cell membrane as well as taurine dioxygenase to degrade taurine to sulphite. The expression profiles of all sponges showed these genes were mostly active in Alphaproteobacteria and Gammaproteobacteria MAGs (including the family *HK1*), which is consistent with the metaproteome of the sponge *lanthella basta* (Engelberts et al., 2023). Hence, the degradation of host derived taurine by diverse bacteria potentially provides the sponge holobiont with a reliable source of sulphite, which can be converted to either sulphide or sulphate within the dissimilatory sulphate reduction and oxidation pathway. Genes for the dissimilatory sulphate reduction and oxidation pathway were expressed by the *HK1* family in all three sponges, as well as additional Gammaproteobacteria MAGs in *P. foliascens* and *I. ramosa*. Moreover, multiple Alpha- and Gammaproteobacteria MAGs expressed genes for the reduction of sulphate to sulphite (*aprAB*, *Sat*) supporting the idea that sulphate is an important energy source for sponge symbionts (Zhang et al., 2019). This may be acquired from sulphate produced through thiosulphate oxidation via the SOX complex, which has been identified in sponge metagenomes and is of interest as thiosulphate may be produced by incomplete oxidation of sulphides (Engelberts et al., 2020). Our metatranscriptomes showed complete and near complete expression of the SOX complex in multiple Alpha- and Gammaproteobacteria, including *Rhodobacteraceae* from *P. foliascens* and *HK1* in all three sponge species. These results show active sulphur cycling in the sponge microbiome and verify previous metagenomic conclusions of widespread potential for sulphur metabolism. Further, we show that the Gammaproteobacteria family *HK1* is instrumental in sulphur cycling, where expression was observed for taurine transport and oxidation, thiosulphate oxidation and the sulphate reduction and oxidation pathway in all three sponges.

### Potential for supplementation of B-vitamins by sponge symbionts

Since animals cannot synthesise their own B-vitamins they must acquire these essential cofactors from their diet or from microbial symbionts. Although sponges may obtain B-vitamins from filter feeding, previous metagenomic studies have identified widespread potential for microbial biosynthesis and provisioning suggesting supplementation of B-vitamins (Engelberts et al., 2020; Fiore et al., 2015; Thomas et al., 2010). Our analysis shows all three sponge microbiomes contain the necessary genes for biosynthesis of six essential B-vitamins; thiamine (B<sub>1</sub>), riboflavin (B<sub>2</sub>), pantothenate (B<sub>5</sub>), pyridoxine (B<sub>6</sub>), biotin (B<sub>7</sub>) and cobalamin (B<sub>12</sub>), with expression of these pathways

detected across diverse microbial lineages. Of particular interest were the families *HK1* and *Rhodobacteraceae*, which expressed thiamine synthesis and transporter genes (*thiBPQ*), as well as *Synechococcus* and Poribacteria which expressed biotin synthesis and transporter (*ecfAAT*) genes, suggesting these bacteria might provision thiamine and biotin to the host respectively (Zhang, 2013). However, most symbionts synthesising biotin and thiamine did not additionally express genes for transport, and therefore likely use it for their own cellular and metabolic function and may not necessarily transport it. Further, functional studies of the transporters investigated here (*thiBPQ*, *ecfAAT*, *bioNMY*) indicate these transporters are involved in vitamin uptake rather than export (Hebbeln et al., 2007; Jaehme & Slotboom, 2015; Webb et al., 1998). Hence, the ability of a symbiont to synthesise B-vitamins should not be assumed to be a benefit to the host. There are other avenues for vitamin transport that may not have been identified, as recent reports have shown cobalamin can be transported into the mitochondria via alternative ABC transporters (McDonald et al., 2017). Therefore, future studies would benefit from tracking biosynthesis of B-vitamins by sponge symbionts to provide further evidence on whether they are translocated to the host.

## Conclusions and future studies

Our genome-centric metatranscriptomic results confirm previously hypothesised microbial functions and pathways are expressed within the sponge microbiome, and although the metatranscriptomic mapping rate was low, this was consistent with previous studies using environmental metagenomes (Hao et al., 2020). Importantly, in some cases, incomplete expression of pathways or multi-enzyme complexes was observed despite the full suite of genes being assembled and this may be the result of low microbial mRNA recovery. Hence further work to optimise the subtraction of rRNA and eukaryotic mRNA within metatranscriptomic laboratory protocols would be highly beneficial to understanding the meta-metabolism within a host microbiome such as a sponge. Further, our metatranscriptomes represent a snapshot in time, where different conditions would likely lead to different pathways and enzymes being expressed. Thus, we can only confirm positive results of gene expression, while genes that showed no expression cannot be interpreted as never being expressed. In other cases, missing genes within metagenomes resulted in incomplete pathways. It is possible these genes were not assembled since the minimum requirement for MAGs was greater than 50% complete with less than 10% contamination. However, it could also represent metabolic collaboration with the host and therefore combining host genomes

and transcriptomes with microbial data may provide a unique perspective on how microbes interact with their host.

The microbiome is integral to the provision and efficient recycling of nutrients to the host, facilitating the success of sponges within reef ecosystems. Here we provide metatranscriptomic validation of enzymes and metabolic pathways involved in carbohydrate degradation, carbon fixation, nitrogen and sulphur metabolism and biosynthesis of B-vitamins for three coral reef sponge species (*I. microconulosa*, *I. ramosa* and *P. foliascens*). We show that carbohydrate degradation is likely coordinated through widespread expression of CEs coupled with GHs expressed by specific lineages. *Synechococcus* appears to be an important symbiont in all three sponges, where full expression of carbon fixation through the Calvin cycle was detected as well as biosynthesis and transport of B-vitamins. Similarly, the Gammaproteobacteria family *HK1* was also responsible for multiple functions, where it was involved in nitrification, sulphur metabolism and expressed both thiamine biosynthesis and transport. We demonstrate that within the microbiome of *I. ramosa* both nitrification and denitrification genes were expressed, suggesting a coupling of aerobic and anaerobic nitrogen metabolism, potentially across oxygen gradients within the sponge mesohyl. However, overall anaerobic nitrogen pathways were the dominant nitrogen processes in our sponge metatranscriptomes. Our work demonstrates the activity of microbial communities within coral reef sponges and highlights microbes with key functions of limited redundancy, while further clarifying the role of the microbiota in providing nutrients in a nutrient poor ocean.

## AUTHOR CONTRIBUTIONS

**Paul A. O'Brien:** Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (equal); methodology (equal); project administration (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Shangjin Tan:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); resources (supporting); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Pedro R. Frade:** Conceptualization (supporting); investigation (equal); methodology (equal); supervision (supporting); validation (equal); writing – review and editing (supporting). **Steven J. Robbins:** Formal analysis (supporting); methodology (supporting); supervision (supporting); validation (supporting); writing – review and editing (supporting). **J. Pamela Engelberts:** Methodology (supporting); visualization (supporting); writing – review and editing (supporting). **Sara C. Bell:** Investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Inka Vanwongerghem:** Methodology (supporting); resources (supporting); writing – review and

editing (supporting). **David J. Miller:** Conceptualization (supporting); supervision (supporting); writing – review and editing (supporting). **Nicole S. Webster:** Conceptualization (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Guojie Zhang:** Conceptualization (supporting); project administration (equal); resources (lead); writing – review and editing (supporting). **David G. Bourne:** Conceptualization (equal); funding acquisition (lead); methodology (supporting); project administration (lead); resources (equal); supervision (lead); validation (equal); writing – review and editing (equal).

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.


## DATA AVAILABILITY STATEMENT

Metatranscriptomic data for *Ircinia ramosa* can be found under the accession numbers SAMN34506096, SAMN34506103 and SAMN34506110 within the BioProject PRJNA965831. All remaining metagenomic and metatranscriptomic data are available at the CNGB sequence archive (CNSA) (Guo et al., 2020) of China National GeneBank DataBase (CNGBdb, <https://db.cngb.org/>) (Chen et al., 2020) under the project CNP0003428. Assemblies of metagenomes and metatranscriptomes are available under the accession numbers CNA0052221-CNA0052246, MAGs under CNA0052247-CNA0052661 and raw data under CNR0818197-CNR0818403. All code used for the analysis is available at [https://github.com/paobrien/sponge\\_metatranscriptome](https://github.com/paobrien/sponge_metatranscriptome).

## ORCID

Paul A. O'Brien  <https://orcid.org/0000-0003-3932-1461>


Shangjin Tan  <https://orcid.org/0000-0002-5688-9334>

Pedro R. Frade  <https://orcid.org/0000-0002-4010-255X>

J. Pamela Engelberts  <https://orcid.org/0000-0002-1185-3741>

Nicole S. Webster  <https://orcid.org/0000-0002-4753-5278>

Guojie Zhang  <https://orcid.org/0000-0001-6860-1521>

David G. Bourne  <https://orcid.org/0000-0002-1492-8710>

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## SUPPORTING INFORMATION

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