Evidence for cross-feeding, metabolic specialization, and niche partitioning in the octocoral holobiont

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Abstract

Background

The role of bacterial symbionts that populate octocorals (Cnidaria, Octocorallia) is still poorly understood. To shed light on their metabolic capacities, we examined 66 high-quality metagenome-assembled genomes (MAGs) spanning 30 prokaryotic species, retrieved from microbial metagenomes of three octocoral species and seawater.

Results

Symbionts of healthy octocorals were affiliated with the taxa *Endozoicomonadaceae*, Candidatus *Thioglobaceae*, *Metamycoplasmataceae*, unclassified *Pseudomonadales*, *Rhodobacteraceae*, unclassified *Alphaproteobacteria* and Ca. *Rhabdochlamydiaceae*. Phylogenomics inference revealed that the *Endozoicomonadaceae* symbionts uncovered here represent two species of a novel genus unique to temperate octocorals, here denoted Ca. *Gorgonimonas eunicellae* and Ca. *Gorgonimonas leptogorgiae*. Their genomes revealed metabolic capacities to thrive under suboxic conditions and high gene copy numbers of serine-threonine protein kinases, type III-secretion system, type IV-pili, and ankyrin-repeat proteins, suggesting excellent capabilities to colonize, aggregate, and persist inside their host. All *Endozoicomonadaceae* MAGs harbored endo-chitinase and chitin-binging protein-encoding genes, enabling these symbionts to hydrolyze the most abundant polysaccharide in the oceans. Other symbionts, including *Metamycoplasmataceae* and Ca. *Thioglobaceae*, may assimilate the smaller chitin-oligosaccharides resulting from chitin breakdown and engage in chitin deacetylation, respectively, providing evidence for substrate cross-feeding and a role for the coral microbiome in overall chitin turnover. We also observed sharp differences in secondary metabolite production capacities between symbiotic lineages. Specific *Proteobacteria* taxa may specialize in chemical defense and guard other symbionts, including *Endozoicomonadaceae*, which lack such capacity.

Conclusion

This is the first study to recover MAGs from dominant symbionts of octocorals, including those of so-far unculturable *Endozoicomonadaceae*, Ca. *Thioglobaceae* and *Mycoplasmoidales* symbionts. We identify a thus-far unanticipated, global role for *Endozoicomonadaceae* symbionts of corals in the processing of chitin, the most abundant natural polysaccharide in the oceans and major component of the natural zoo- and phytoplankton feed of octocorals. We conclude that niche partitioning, metabolic specialization, and adaptation to low oxygen conditions among prokaryotic symbionts likely contribute to the plasticity and adaptability of the octocoral holobiont in changing marine environments. These findings bear implications not only for our understanding of symbiotic relationships in the marine realm abut also for the functioning of benthic ecosystems at large.

Background

Octocorals (Octocorallia, Anthozoa, Cnidaria) are an integral part of benthic marine ecosystems, increasing habitat complexity and biodiversity where they abound [1, 2]. They differ from scleractinian corals (Hexacorallia) by the eight-fold symmetry of their polyps and amount to over 3500 species [3] possessing worldwide distribution, from polar over temperate to tropical regions and from shallow waters to the deep sea [2, 4]. Octocorals suspension-feed on large quantities of debris, phyto- and zooplankton [5] and thus play paramount roles in coastal food chains, helping to regulate primary and secondary production [5, 6]. They also form associations with various microorganisms, including micro-eukaryotes, bacteria, archaea, and viruses [reviewed in 6].

Over the past two decades, heat waves and infectious diseases have led to significant mortalities in Mediterranean and North-East Atlantic octocoral populations [7-11]. These mortality events can alter critical ecosystem processes and result in biodiversity loss in the benthos of temperate zones [8]. In other parts of the world, however, octocorals continued to be thriving while scleractinian corals faced a rapid decline from climate change. Studies in the Red Sea, the Caribbean, and the Pacific and Indian Oceans showed reef community shifts from scleractinian corals towards octocorals, increasing octocoral cover from less than 10% to nearly 50% in some regions [reviewed in 6]. It is yet to be determined why some octocorals resist while others are affected by climate change scenarios - but their associated microbiomes likely play a fundamental role in this response.

The prokaryotic assemblages of corals, and octocorals in particular, are frequently dominated by *Endozoicomonadaceae* and other *Gammaproteobacteria* phylotypes, in addition to members of *Alphaproteobacteria*, *Mollicutes*, *Flavobacteriia, Actinobacteria* and *Spirochaetes* [6, 12-14]. *Endozoicomonadaceae* symbionts can make up to 96% of a coral prokaryotic community and are generally considered indicators of coral health [6, 12, 15]. Despite their ubiquity and abundance in corals worldwide, less than ten coral-associated *Endozoicomonas* strains (comprising five formally described *Endozoicomonas* species) exist in culture or had their genomes sequenced, as shown by a meta-analysis of over 3050 coral bacterial isolates from more than 80 coral species [16]. Moreover, only two cultured *Endozoicomonas* species, *E. euniceicola* and *E. gorgoniicola,* derive from octocorals [17], and no genome sequence of an octocoral-derived *Endozociomonadaceae* symbiont is yet publicly available. Metagenomics studies even showed that cultured *Endozoicomonas* isolates are phylogenetically distant from the dominant *Endozoicomonadaceae* phylotypes populating temperate octocorals [12, 14, 18]. Therefore, we still know little about the role of this ‘core’ bacterial family in octocorals and in the functioning of benthic ecosystems at large.

Genome analyses indicate the provision of vitamins and amino acids, and participation in nutrient cycling and dimethylsulfoniopropionate metabolization as putative roles of bacterial symbionts of scleractinian corals [16, 19, 20]. Comparative genomics of *Alphaproteobacteria*, *Vibrio* and *Aquimarina* isolates from octocorals revealed the presence of various biosynthetic gene clusters (SM-BGCs) coding for polyketide, terpene, and antimicrobial peptide production, suggesting an involvement of octocoral bacteria in chemical defense [16, 21-23]. Our recent metagenomics survey showed that high abundances of eukaryotic-like proteins, exo- and endonucleases, phage lysogenization regulators and micronutrient

acquisition related genes distinguish the prokaryotic communities of healthy from necrotic octocoral tissue, likely contributing to the stability of the symbiotic microbiome [12].

Although our understanding of taxonomic composition, diversity, host-specificity, geographic variability, and seasonal stability of the octocoral microbiome has incresead in the past few years [6, 12, 18, 24-26], the functional contributions and mechanisms of interaction of microbial symbionts within the octocoral holobiont remain largely unknown. Here we examine metagenome-assembled genomes (MAGs) from 17 shotgun-sequenced microbial metagenomes of three octocoral species (healthy and necrotic *Eunicella gazella* tissue, healthy *Eunicella verrucosa* and *Leptogorgia sarmentosa*) and surrounding seawater to connect microbial taxonomy with function in a cultivation-independent fashion and to shed light on the likely roles and mechanisms of interaction of dominant bacterial taxa in octocorals. This study is the first to retrieve and compare multiple draft genomes from uncultured symbionts of octocorals, addressing the hypothesis of microbial niche partitioning in the octocoral holobiont.

Methods

Metagenome samples

The 17 microbial metagenomes used for the binning of MAGs in this study are publicly available (PRJEB13222). These 17 Illumina-sequenced, total community DNA samples were obtained from three octocoral species - healthy (N = 3) and necrotic (N = 3) *Eunicella gazella* tissue (EG15H, EG15N; EG16H, EG16N; EG18H, EG18N), healthy *Eunicella verrucosa* (N = 4; EV01 - EV04) and *Leptogorgia sarmentosa* (N = 3; LS06 - LS08) - and surrounding seawater (N=4; SW01-SW04). Coral sampling, sample processing, DNA extraction and sequencing (on an Illumina HiSeq 2500 device with a sequence depth calibrated at c. 20 million 101- bp reads per sample) were thoroughly described by Keller-Costa et al. [12].

Binning of MAGs

Metagenome reads obtained for each sample were assembled by Keller-Costa et al. [12] using the metawrap v1.0.5 pipeline [27], and encompassed reads quality control with the metawrap galore module, followed by assembly with the meta-SPAdes module 3.13.0 [28]. Eukaryotic contigs were then filtered out of the resulting assemblies using EukRep v.0.6.6 [29], generating individual “prokaryotic-enriched” assembly files per sample [12]. Metagenomic binning was performed in this study on the “prokaryotic-enriched” assemblies using MetaBAT 2 [30], Maxbin 2.0 [31] and CONCOCT [32] within the metawrap binning module. Binning_refiner [33] was used to refine bins and produce a superior bin set within metaWRAP. Genome completeness, contamination and strain heterogeneity scores were calculated using CheckM v1.0.11 [34] with default parameters. Following the approach of Parks et al. [35], an overall quality score was calculated for each MAG (completeness – 5x contamination), and MAGs possessing a quality score above 50% were kept for analysis, resulting in a final dataset of 66 MAGs. We then categorized the 66 MAGs into “High quality MAGs” (when completeness was above 90% and contamination below 5%) and “Medium quality MAGs” (completeness above 50% and contamination below 10%), according to MIMAG guidelines [36]. The Microbial Genome Atlas (MiGA) [37] was used to

obtain genome metrics such as e.g., number of contigs, genome size, GC-content, and N50 values. MiGA (accessed on 25th of November 2021) was also used to identify the closest relative (i.e., genome of a type strain) of each MAG, based on whole-genome average amino acid identity values (AAI%).

Taxonomic assignment and species-level similarity of MAGs

The Genome Taxonomy Database Toolkit (GTDB-Tk) [38] v.1.5.0 (release 06-RS202) was used to perform taxonomy assignment of all MAGs obtained in this study. High taxonomic rank assignments were afterwards manually curated to comply with the List of Prokaryotic names with Standing in Nomenclature (LPSN) [39, 40] as deemed necessary. FastANI [41] was used to compute whole-genome average nucleotide identity (ANI%) values in a pairwise fashion whenever two or more MAGs shared the same taxonomic assignment. MAGs which shared ≥ 95% ANI were considered to belong to the same species [41-43].

Phylogenomics of the *Endozoicomonadaceae* and Ca. *Thioglabaceae* families

Owing to their high frequency across healthy octocoral samples and presumed taxonomic distinctiveness and novelty, we thoroughly explored the phylogenomic relatedness of the 11 *Endozociomonadaceae* and six Ca. *Thioglobaceae* MAGs of this study with their closest relatives. Two phylogenomic trees were created, one for each family. Details on the genomes used for tree construction are provided in Additional File 1. Both trees were constructed with the SpeciesTreeBuilder v.01.0 application of the DOE Systems Biology Knowledgebase (KBase) [44] using the function "Insert Set of Genomes into Species Tree", after annotating all isolate genomes, MAGs and SAGs with Prokka [45]. SpeciesTreeBuilder uses the FastTree2 algorithm [46] to infer Maximum-Likelihood (ML) phylogenies for large alignments. Alignments were based on a set of 49 core genes defined by COG families. Graphical visualization and editing of the trees was made in iTOL v4 (Interactive Tree Of Life) [47].

Functional annotation of MAGs

Functional annotation of MAGs encompassed the generation of Clusters of Orthologous Groups of proteins (COG) profiles, metabolic pathway reconstruction, and genome mining for secondary metabolite biosynthetic gene clusters (SM-BGCs). COG annotation was performed for all MAGs using our in-house, automated genome annotation pipeline MeLanGE as documented on GitHub (https://sandragodinhosilva.github.io/MeLanGE). Briefly, all MAGs (contig fasta files) were first annotated with Prokka v1.14.6 [45] to obtain GenBank (gbk) format and amino acid fasta files. Thereafter, proteins were queried against the COGs database implemented within NCBI's Conserved Domain Database (CDD) through Reversed Position Specific Blast (RPS-BLAST) from the BLAST+ suite (v2.9.0) and the best hit per ORF, above the cut-off of E 1e-5, was selected. MAGs were further annotated with the RAST server version 2.0 [48, 49] using the RASTtk annotation scheme and the “Build metabolic models” option with default settings. Using the ‘KEGG Metabolic Analysis’ tool within RAST, KEGG metabolic maps were constructed for selected pathways and MAGs. Complete chitinase coding sequences (CDS) present on our MAGs were retrieved from RAST and characterized as described in

Additional File 1. Identification of SM-BGCs across all MAGs was performed using antiSMASH v5.0 [50] with default parameters and extra features “All on”. The degree of novelty of SM-BGCs was assessed through matches with the Minimum Information about a Biosynthetic Gene cluster (MIBiG) database [51, 52].

Data analyses and statistics

Multivariate analysis of COG-based functional profiles was carried out on Hellinger-transformed data (i.e., square root of the relative abundance of each COG entry on a MAG). Euclidean distances were then calculated from COG abundance distributions across MAGs and a principal components analysis (PCA) was performed using PAST v3.25 [53]. One-way permutational analysis of variance (PERMANOVA) was used to test for overall differences in functional profiles among MAGs belonging to different orders. To determine COG functions that contributed most to the dissimilarity between MAGs at the order level, similarity percentage analysis (SIMPER) was performed in PAST v 3.25 [84]. The top ten most differentiating COG functions were then plotted as vectors on the PCA diagram to explore relationships between gene functions and symbiont taxonomy. Moreover, Welch's unequal variances *t*-tests (one-sided) were conducted within STAMP v2.0.953 [54] to identify COG entries that were significantly enriched on MAGs affiliated with the *Endozoicomonadaceae* (*N*=11) and Ca. *Thioglobaceae* (*N*=6) families. Multiple test correction was performed with the Benjamini-Hochberg method to decrease false discovery rates, and COG entries representing 5-fold (Ca. *Thioglobaceae* MAGs) and 10-fold (*Endozoicomonadaceae* MAGs) enrichments were selected for further analysis.

Results

Dataset overview

The final dataset analysed in this study comprised 66 MAGs, 65 of which derived from *Bacteria* and one from *Archaea* (Table S1, Additional File 2). Twenty-five MAGs were obtained from healthy octocoral samples (all species together), 14 MAGs from necrotic *E. gazella* tissue and 27 MAGs, including the archaeal one, from seawater. Of the 66 MAGs, 30 and 36 were of high and medium quality, respectively (Table S2, Additional File 2). Average genome size and GC-content ranged from only 0.63 Mb and 22.3% in *Metamycoplasmataceae* to 4.2 Mb and 59.6% in Ca. *Inquilinaceae* (*Alphaproteobacteria*), both symbionts of healthy octocorals (Table S1, Additional File 2).

Taxonomic affiliation of MAGs

The 66 MAGs belonged to six phyla, seven classes, 15 orders, 16 families, and at least 30 species as defined by 95% ANI thresholds (Figure 1a-c; Table S1, Additional File 2). The 21 *Gammaproteobacteria* MAGs retrieved from octocorals all represented so-far uncultured and unclassified lineages, with 11 MAGs affiliating with the *Endozoicomonadaceae* family (Figure 1b). Of these, nine derived from the microbiomes of healthy octocoral tissue and two from necrotic tissue. Six octocoral-derived MAGs affiliated with the family Ca. *Thioglobaceae*, five of them obtained from the microbiomes of healthy

tissue. Moreover, two *Gammaproteobacteria* MAGs from healthy *L. sarmentosa* were affiliated with candidate taxon DT-91 of the order *Pseudomonadales*,while two MAGs from necrotic *E. gazella* were identified as unclassified *Cardiobacterales*. Contrarily, the three *Gammaproteobacteria* MAGs found in seawater belonged to the genus *Luminiphilus* (*Halieaceae*, *Cellvibrionales*). Notably, four MAGs from healthy octocoral tissue were affiliated with intracellular bacterial symbionts [55] of the families *Metamycoplasmataceae* and Ca. *Rhabdochlamydiaceae*. The five *Alphaproteobacteria* MAGs from healthy octocoral tissue were either unclassified at order level (*N*=2) or belonged to the candidate family *Inquilinaceae* and the *Rhodobacteraceae* genera *Ruegeria* and *Yoonia* (Figure 1c). Among the MAGs obtained from necrotic *E. gazella* tissue was also one affiliating with the genus *Aquimarina* (*Bacteroidetes*), a taxon frequently cultured from octocorals [14, 22], as well as three MAGs affiliating with the *Rhizobiaceae* family, one of them identified as *Lentilitoribacter*. Overall, little overlap was observed at species level between MAGs reconstructed from healthy octocoral tissue versus necrotic tissue versus seawater. Of 11 bacterial species recovered from healthy octocorals, only two species were also recovered from necrotic samples. However, their average genome coverage, a proxy for relative abundance, was much lower in necrotic than healthy tissue (Figure 1c).

Phylogenomics of *Endozoicomonadaceae* and Ca. *Thioglobaceae* MAGs

Phylogenomics analysis of the *Endozoicomonadaceae* family comprised 29 publicly available type genomes, MAGs, and SAGs plus the 11 octocoral-derived MAGs retrieved in this study (Figure 2). The latter formed their own, well supported and deeply branching clade, separate from the genomes of all described genera with cultured representatives (i.e., *Kistimonas*, *Parendozoicomonas* and *Endozoicomonas*). This clade comprised two subclusters, each representing a novel species, sharing ~89.8% ANI between them. Subcluster I contained seven MAGs, all obtained from *Eunicella* spp. Subcluster II comprised four MAGs, three from *L. sarmentosa* and one from *E. gazella*. The closest type strain genome to our MAGs, was *Endozoicomonas atrinae* GCA_001647025T, which shared only 52-53% AAI with our MAGs (Table S1, Additional File 2), well below the 65% threshold considered by MiGA for same-genus classification. This indicates that our 11 *Endozoicomonadaceae* MAGs represent two distinct species, forming a novel yet uncultured genus unique to temperate octocorals. We propose the names Candidatus *Gorgonimonas eunicellae* (corresponding to subcluster I) andCa*. Gorgonimonas leptogorgiae* (corresponding to subcluster II) for the two species.

Phylogenomics inference of the Ca. *Thioglobacaceae* family **showed that our** six octocoral-derived *Thioglobaceae* MAGs formed two separate clusters, representing distinct, novel species as judged by ANI values way below 80% compared with the remainder genomes of the family **(Figure S1,** Additional File 1**). The first clade comprised two *E. gazella*-derived MAGs which** formed a subcluster within other Ca. *Thioglobaceae* clusters of the genera *Thioglobus* and *Thiomultimodus*. The second clade was composed of the otherfour *Thioglobaceae* MAGs, derived from healthy *L. sarmentosa* and *E. gazella* tissue, which formed a well-supported, deeply branching phylogenomic node on their own, sharing only 46% AAI with genomes of their closest type strains, namely *Sulfurivirga caldicuralii* GCA_900141795T and *Thiohalobacter thiocyanaticus* GCA_003932505T (Table S1, Additional File 2). This indicates that the six

octocoral-derived Ca. *Thioglobaceae* MAGs not only represent two distinct species but most likely two distinct, novel genera, here proposed Ca. *Thiocorallibacter gorgonii* and Ca. *Microaerophilica antagonistica* (Figure S1, Additional File 1), which so far lack cultured representatives.

Functional profiling of MAGs from octocoral and seawater microbiomes

Multivariate analysis based on COG functional profiles showed that the MAGs grouped primarily according to their (order level) taxonomic affiliations (PERMANOVA, $F = 9.869$, $P = 0.0001$) (Figure 3). The 11 *Endozoicomonadaceae* MAGsformed a very tight cluster, much distant from all other MAGs. Such separate clustering was mostly determined by the high copy number of ankyrin repeat motifs (COG0666) and serine/threonine protein kinase-encoding genes (COG0515) on the *Endozoicomonadaceae* MAGs. SIMPER analysis showed that these two COGs were indeed the functions that contributed most to the dissimilarity between all MAGs at order level (Tables S3, S4, Additional File 2).

The six Ca. *Thioglobaceae* MAGs formed two, well-separated clusters in the ordination space, one comprising the two Ca. *Thiocorallibacter gorgonii* MAGs and another one with the four Ca. *Microaerophilica antagonistica* MAGs, congruent with our phylogenomic assessment. The positioning of *Alphaproteobacteria* MAGs in the PCA diagram was influenced, amongst others, by the presence and abundance of genes encoding for LysR family transcriptional regulator (COG0583), ABC sugar transport system (COG3839), Acyl-CoA and NAD(P)-dependent alcohol dehydrogenases (COG1960 and COG1028) and drug metabolite transporters (COG0697).

Functional features enriched in *Endozoicomonadaceae* symbionts of octocorals

The 11 *Endozoicomonadadeae* MAGs were drastically enriched in COGs related to eukaryotic-like proteins (Figure 4), mainly ankyrin repeats ($q < 0.0001$; Welch's test)and, to a lesser extent, WD40 repeats and tetratricopeptide repeats (Table S5, Additional File 2, Figure S2, Additional File 1). These MAGs also displayed high abundance of COG entries related to thetype III secretion system ($q < 0.0001$; Welch's test), serine/threonine protein kinases, serine protease inhibitors ($q < 0.0001$; Welch's test) and the membrane-anchored periplasmic protein YejM.Other typical features of all 11 *Endozoicomonadaceae* MAGs were several COG entries associated with Type IV-pilus (Tfp) production (Figures 4 and 5), and the consistent presence of COG3206 encoding a protein involved in exopolysaccharide (EPS) biosynthesis, which was not observed to such extent in any of the other 55 MAGs investigated here. The two *Endozoicomonadaceae* species identified in this study were distinguished by a consistent presence of serine/threonine phosphatase encoding genes on the four MAGs of Ca. *Gorgonimonas leptogorgiae* which were absent on the seven MAGs of Ca. *G. eunicellae.*

The *Endozoicomonadaceae* MAGs show capacity for pyruvate metabolism and to convert acetyl-CoA to acetate via acetyl-phosphate, through a phosphate acetyl transferase (EC 2.3.1.8) and an acetate kinase (EC 2.7.2.1), respectively, a process able to generate ATP independently from aerobic conditions (Figure S3, Additional File 1). The consistent presence of pyruvate-formate lyase activating enzyme encoding genes (COG1189) on the 11 *Endozoicomonadaceae* genomes (Figure S2, Additional File 1, Welsh's-test q

< 0.0001) further suggests that these symbionts may supply the citric acid cycle with acetyl-CoA from pyruvate during anaerobic glycolysis. Evidence for adaptation of *Endozoicomonadaceae* symbionts to suboxic conditions could also be found through the consistent presence of feoA/B genes (COG1918, COG0370) encoding for ferrous iron (Fe^{2+}) uptake systems, distinguishing this taxon from the other 55 MAGs of this study (Welch's t-test, q < 0.0001). In this regard, *Endozoicomonadaceae* MAGs were also distinguished by the presence of rubredoxin encoding genes (Figures 4-6;Figure S2, Additional File 1), a class of iron-containing proteins which play an important role in superoxide reduction that can be found in several anaerobic and sulphate-reducing bacteria.

All *Endozoicomonadaceae* MAGs possessed a gene encoding an endo-chitinase (COG3469) involved in the extracellular breakdown of chitin polymers (Figures 4-6). We found a high degree of novelty within these 11 endo-chitinase encoding genes, as they clustered into two distinct groups and possessed less than 50% amino acid sequence similarity to publicly available endo-chitinases (Blastp search). Remarkably, these groups mirror the phylogenomic relatedness of the *Endozoicomonadaceae* MAGs, representing two endo-chitinase gene clades, each from Ca. species *Gorgonimonas leptogorgiae* and *G. eunicellae* (Figure S4, Additional File 1). Protein family (Pfam) analysis confirmed the presence of a GH18 domain with an active site on all 11 genes. These genes were all complete (start and stop codon present) and carried a signal peptide sequence, indicating the protein can be excreted from the cell (Table S6A, Additional File 2). We also screened all publicly availablegenomes from cultured and uncultured *Endozoicomonadaceae* representatives and detected endo-chitinases on 32 out of 42 *Endozoicomonadaceae* genomes (Table S6B, Additional File 2).

Metabolic inference of Ca. *Thioglobaceae* symbionts of octocorals

The Ca. *Thioglobaceae* MAGs were significantly enriched in CRISPR/Cas system-associated endoribonuclease Cas2 (COG1343) (Figure S5, Additional File 1). Several other CRISPR/Cas protein-encoding genes were found on all Ca. *Thioglobaceae*, all *Metamycoplasmataceae*, and many *Endozoicomonadaceae* MAGs (Figure 4; Table S5, Additional File 2). Ca. *Thioglobaceae* MAGs were further significantly enriched in Na+-translocating ferredoxin:NAD+ oxidoreductase (Rnf complex) encoding genes (Figure S5, Additional File 1), which were also present in great abundance in nine of the 11 *Endozoicomonadaceae* MAGs. An assimilatory sulfite (SO_3^{2}) reductase (EC 1.8.1.2) was also found on the Ca. *Thioglobaceae* MAGs, pointing towards a role in sulfur cycling in the octocoral holobiont. The four Ca. *Microaerophilica antagonistica* MAGs encoded for versatile taurine utilization pathways and its metabolization to aminoacetaldehyde and sulfite, via taurine deoxygenase (TauD; EC 1.14.11.17), or to sulfoacetaldehyde via taurine-pyruvate-aminotransferase (EC 2.6.1.77) (Figure S6, Additional File 1).

All Ca. *Thioglobaceae* MAGs showed an extensive genetic repertoire for ammonium assimilation and transformation of inorganic nitrogen into amino acids, possessing genes coding for glutamine and asparagine synthetase (EC 6.3.1.2; EC 6.3.5.4), L-asparaginase (EC 3.5.1.1), glutamine amidotransferase (EC 6.3.1.5), aminoethyltransferase (EC 2.1.2.10), and glutamate synthase (EC 1.4.1.13; EC 1.4.7.1). The

latter was significantly enriched in this symbiotic family compared to the other 60 MAGs obtained in this study (One-sided Welch's-test, p>0.001, Figure S5 Additional File 1). Finally, Ca. *Thiocorallibacter gorgonii* MAG EG15H_bin1 stood out as the only MAG harboring ribulose 1,5-bisphosphate carboxylase - RuBisCo (EC 4.1.1.39, COG4451, COG1850) and several other genes involved in the reductive dicarboxylate cycle (Figure S7, Additional File 1), suggesting a chemoautotrophic lifestyle and "dark carbon fixation" ability of this octocoral symbiont.

Secondary metabolite biosynthetic capacities of octocoral symbionts

Genome mining with antiSMASH revealed that 46 MAGs harboured between one and 16 (Ca. *Inquilinaceae* EV04_Bin1) SM-BGCs, while 20 MAGs lacked SM-BGCs, among them all *Endozoicomonadaceae, Metamycoplasmataceae*, Ca. *Rhabdochlamydiaceae* and unclassified *Aphaproteobacteria* MAGs from octocorals (Figure 7a; Table S7, Additional File 2). Notably, the two Ca. *Thioglobaceae* species presented distinct secondary metabolite coding potential. While Ca. *Thiocorallibacter gorgonii* MAGs harboured one or two arylpolyene cluster(s) (which may function as antioxidants), *Ca. Microaerophilica antagonistica* MAGs harbored one T3PKS and one bacteriocin/RiPP SM-BGC each, which may indicate antagonistic potential. The two *Pseudomonadales* MAGs from healthy *L. sarmentosa* samples showed rich SM-BGC profiles, with 7-8 NRPS and 2-3 bacteriocin/RiPP/proteusin clusters, plus a siderophore SM-BGC (Figures 6 and 7a).

Only 24 out of 163 SM-BGCs detected across all MAGs showed some homology with SM-BGCs encoding known compounds present in the MIBiG database, with 14 SM-BGCs sharing a similarity of 60-100% (Figure 7b; Table S8, Additional File 2). One of the NRPS clusters of *Pseudomonadales* MAG LS06H_Bin2 showed 100% similarity to the NRPS cluster of the antimicrobial peptide bicornutin A [56] (Figure 7c, d). Another NRPS cluster with 100% similarity to a SM-BGC of a known compound, namely the antibiotic and cyclic depsipeptide isocalide A [57], was identified on the Ca. *Inquilinaceae* MAG from healthy *E. verrucosa,* which was also the MAG with the richest SM-BGC profile across the entire dataset.

Discussion

This is the first functional genomics study of uncultivated symbionts of octocorals. Notably, MAGs of dominant *Endozoicomonadaceae* symbionts were retrieved from all octocoral species and from nine out of 10 healthy coral specimens analysed, strengthening the current understanding of these symbionts as indicators of coral health. Extensive amino acid and b-vitamin biosynthesis capacities were common traits of the dominant *Gammaproteobacteria* fraction (*Endozoicomonadaceae,* Ca. *Thioglobaceae* and *Pseudomonadales* MAGs) characteristic of healthy octocorals (summarized in Figure 6). This outcome corroborates recent research on scleractinian coral symbionts, which suggested amino acid and vitamin biosynthesis among the key traits for holobiont functioning [19]. Our data suggest that cross-feeding and niche partitioning, particularly of chitin and its breakdown products, b-vitamins, and amino acids, might be important mechanisms to promote co-existence and energy conservation among symbionts of azooxanthellate octocorals. Niche partitioning is further proposed by sharp differences in secondary

metabolite biosynthesis potential between symbiotic lineages, whereby *Endozoicomonadaceae*, *Mycoplasmoidales* and *Chlamydiales* symbionts did not possess a single SM-BGC, indicating they might not engage in chemical defense. Instead, *Pseudomonadales* and specific *Alphaproteobacteria* symbionts of healthy octocorals are likely to fulfill this task. They possessed the richest secondary metabolism across all 66 MAGs examined, including the genomic blueprint for the biosynthesis of antibiotics such as isocalide A [57]. Below we highlight key genomic features and putative mechanisms of interaction among octocoral symbionts, focusing on the *Endozoicomonadaceae-Thioglobaceae-Metamycoplasmataceae* triad.

Discovery ofnovel *Endozoicomonadaceae* symbionts highly adapted to life in octocorals

This study uncovered two species of a novel *Endozoicomonadaceae* genus unique to temperate octocorals, here denoted Ca. *Gorgonimonas eunicellae* and Ca. *Gorgonimonas leptogorgiae.* These *Gorgonimonas* MAGs showed many traits that facilitate colonization of and persistence inside the host animal, including high abundances of ankyrin repeats. Studies on marine sponges showed that bacteria expressing ankyrin genes avoid phagocytosis by sponge amoebocytes, thus residing inside the sponge by evading its immune system [58]. It is conceivable that a similar mechanism exists in coral-associated bacteria. Some bacteriophages also carry genes for ankyrin biosynthesis and possibly even transfer this information across different microbial symbionts [59]. Our earlier research demonstrated that ankyrin repeats are enriched in the microbial metagenomes of healthy octocorals [12] and this study now allows the attribution of this trait to specific *Endozoicomonadaceae* symbionts. Although a high copy number of ankyrin repeats was recently found among few, cultured *Endozoicomonas* strains [16], our study is the first to show the enrichment of this trait in dominant, deeply branching and uncultivated *Endozoicomonadaceae* lineages within the octocoral microbiome. *Gorgonimonas* MAGs further possess a high abundance of genes related to thetype III secretion system, serine/threonine protein kinases, serine protease inhibitors and the protein YejM.These features enable colonization and interference with host defense mechanisms amog bacterial pathogens [60-62]. There is, however, growing evidence that beneficial bacteria use similar mechanisms to suppress immune responses and facilitate host colonization [63]. Indeed, parasitic and mutualistic symbioses often share similar evolutionary histories. Thus, host-microbe interactions may rather represent a flexible gradient, known as the ‘parasite-mutualist continuum’, than a static binary system, and can shift and evolve depending on ecological context [64]. To accurately discern the beneficial-to-parasitic behavior of coral symbionts in a context-dependent manner, future studies need to combine multi-omics analyses with controlled *in-vivo* mesocosm experiments.

All *Gorgonimonas* MAGs showed great capacities to produce tfp pili, bacterial surface appendages required for adherence to solid surfaces or cells, including host cells [65]. Tfp pili are also involved in bacterial aggregation (agglutination) and flagellum-independent movement on solid surfaces via gliding or twitching motility, coupled with chemotaxis [65]. CARD-FISH analysis demonstrated that *Endozoicomonas* bacteria indeed aggregate in dense, cyst-like structures inside the coral endoderm [66].

The here identified tfp pili likely provide the mechanisms for *Endozoicomonadaceae* symbionts to form such aggregations and attach to and move inside their host.

Ca. *Thioglobaceae* genomes code for a sophisticated antiviral defense system and chemoautotrophic metabolism

The Ca. *Thioglobaceae* MAGs were significantly enriched in CRISPR/Cas protein-associated genes, also found in all *Metamycoplasmataceae* (DT-68) and many *Endozoicomonadaceae* MAGs. These proteins are prokaryotic defense mechanisms against bacterial viruses and foreign DNA known to be enriched in the microbiomes of marine sponges [59, 67] and octocorals [12]. A recent study of the *Thioglobaceae* pan-genome showed that CRISPR/Cas systems are relatively enriched in mussel, sponge and scleractinian coral symbionts compared to free-living *Thioglobaceae* lineages [68]. Our Ca. *Thioglobaceae* and *Endozoicomonadaceae* MAGs exhibited intra-specific variability in CRISPR/Cas gene numbers, leading to a flexible pan-immune system within host-associated populations, as suggested elsewhere [69].

We found one Ca. *Thioglobaceae* MAG to harbour RuBisCo and others genes for light-independent carbon fixation and chemoautotrophic lifestyle - as known for some *Thioglobaceae s*ymbionts from deep-sea sponges and hydrothermal vent bivalves [70]. It could represent an alternative carbon fixation route in azooxanthellate octocorals that lack photosynthetic *Symbiodinaceae* and other algae symbionts. It also indicates that the ecological range of chemoautotrophic, symbiotic *Thioglobaceae* extends beyond hydrothermal vent and deep-sea ecosystems well into mesophotic zones and above.

Beyond their putative role in carbon supply, most Ca. *Thioglobaceae* MAGs possessed the genetic repertoire for ammonium assimilation, sulfur cycling and taurine processing within the octocoral holobiont. Taurine is a sulfur-containing amino acid widely found in animal tissue and several studies suggested a role of sponge and coral symbionts in sulfur cycling via taurine metabolism [16, 19, 67, 71]. However, as observed in this study, taurine utilization is a widespread trait among marine host-associated and free-living *Alpha-* and *Gammaproteobacteria* and not restricted to obligate symbiotic clades.

Endozoicomonadaceae, Ca. *Thioglobaceae* and *Metamycoplasmataceae* symbionts may adopt a facultative anaerobic lifestyle

We found genome-based evidence for *Endozoicomonadaceae,* Ca. *Thioglobaceae* and *Metamycoplasmataceae* symbionts to possess a facultative anaerobic metabolism and the ability to thrive in low oxygen conditions. *Endozoicomonadaceae* symbionts may generate energy without oxygen via the pyruvate metabolism and with the help of acetate kinases and pyruvate formate-lyases [72, 73]. All Ca. *Thioglobaceae* and most *Endozoicomonadaceae* MAGs also harbored Na+-translocating ferredoxin:NAD+ oxidoreductase (Rnf complex) encoding genes. The Rnf complex is involved in anaerobic respiration and alternative routes of energy generation by translocating sodium across the cell membrane, a common feature of anaerobic (acetate-forming) acetogens (e.g., *Acetobacterium woodii)* [74]. Recent studies demonstrated that the Rnf complex is not limited to acetogens and instead can be

found in several Gram-negative, facultative anaerobic bacteria [75-77]. The *Metamycoplasmataceae* (DT-68) and some Ca. *Thioglobaceae* MAGs further possessed lactate dehydrogenase encoding genes to ferment pyruvate into lactate and thus recycle NADH + H^+, for glycolysis to continue under anaerobic conditions. Facultative anaerobe symbionts and anaerobic metabolism seem to play a crucial role in nutrient cycling in marine sponges [78]. Few cultured *Endozoicomonas* strains from octocorals are classified as facultative anaerobes, such as type strains *Endozoicomonas euniceicola* and *E. gorgoniicola* [17], indicating that octocoral holobionts might benefit from the facultative anaerobic lifestyles of their symbionts. The consistent presence of genes coding for ferrous iron (Fe^{2+}) uptake systems on all *Endozoicomonadaceae* MAGs provides further support for this hypothesis, since Fe^{2+} is usually available in anaerobic environments while ferric iron, (Fe^{3+}) is dominant under oxygenated conditions, and organisms frequently exposed to oxygen limitation in their natural habitat rely on ferrous iron uptake mechanisms [79]. The 11 *Endozoicomonadaceae* MAGs were also enriched in ferritin encoding genes for iron storage, likely supporting growth of these symbionts during iron starvation [80], a situation they may frequently encounter in a host animal that is craving for iron itself. Together, such metabolic capacities could enable these symbionts not to drain their obligate aerobic host from oxygen but to provide it with iron whenever environmental oxygen is limited. Another distinguishing feature of the *Endozoicomonadaceae* symbionts of this study was the presence of rubredoxins, a class of iron-containing proteins found in several anaerobic and sulfate-reducing bacteria. Rubredoxins act as electron carriers in many biochemical processes and can play a crucial role in reducing reactive oxygen species [81-83]. Both, effective oxidative stress response and iron-sequestration mechanisms, were recently proposed as key beneficial traits of coral probiotics [84].

Chitin breakdown and cross-feeding capacities in the octocoral holobiont revealed

All *Endozoicomonadaceae* MAGs harbored endo-chitinase and chitin-binding protein-coding genes involved in the extracellular breakdown of chitin polymers, the most abundant polysaccharide in the ocean. Genes for exo-chitinases, the enzymes that cleave the smaller chito-oligomer products into mono-sugars, were further present in *Metamycoplamataceae*, Ca. *Rhabdochlamydiaceae* and *Pseudomonadalaes*, while polysaccharide/chitin deacetylase encoding genes, which lead to the production of chitosan, were found in Ca. *Thioglobaceae* and several *Alphaproteobacteria* symbionts of octocorals. This points towards a likely role of octocoral symbionts in chitin processing and carbon and nitrogen turnover in their host and indicates possible mechanisms of substrate cross-feeding [85] between symbiotic partners. Cross-feeding cascades among microbes may promote the evolution of small genomes as here observed, for example, in *Metamycoplasmataceae* (0.63 Mb) and Ca. *Rhabdochlamydiaceae* (1 Mb) symbionts. It may also reduce competition and enable co- existence of multiple host-associated taxa through functional specialization. Exo-chitinase (EC 3.2.1.52) activity was measured earlier in crude extracts of the octocoral *Gorgonia ventalina* [86]. Moreover, chitinolytic activity was reported in seven scleractinian coral species and chitinase-like genes were identified in the genome of *Acropora digitifera* [87]. Raimundo and colleagues reported that the abundance of endo-chitinase and chitin-binding-protein encoding genes in healthy octocoral tissue levels up with those from surrounding

environments [88]. All this evidence shows that chitinases are widely distributed among corals, many of which feed on chitin-rich phyto- and zooplankton [5]. These enzymes may also play a role in the animals' defense against fungal infections [6] whereby chitinases from bacterial symbionts may further strengthen the coral immune system.

Conclusion

This study examines the first microbial MAGs ever retrieved from octocorals, revealing that uncultured *Endozoicomonadaceae*, Ca. *Thioglobaceae* and *Mycoplasmoidales* symbionts are seemingly well adapted to life in low oxygen conditions. Moreover, we identified a thus-far unanticipated, global role for *Endozoicomonadaceae* symbionts of corals in chitin processing and C and N cycling across benthic ecosystems. Other symbionts, such as Ca. *Thioglobaceae* were well equipped to control bacteriophage attacks and to adopt a chemoautotrophic lifestyle. We conclude that the prokaryotic symbiome of octocorals participates in carbon (particularly chitin), nitrogen and sulfur cycling, amino acid and b-vitamin provision, chemical defense, and oxidative and osmotic stress protection. Traits are not shared equally among all members of the symbiotic community, instead clear niche partitioning and metabolic specialization among taxonomically unique symbionts likely contribute to efficient functioning of and co-existence in the healthy octocoral holobiont.

Declarations

Ethics approval and consent to participate

This article does neither contain any studies with human participants nor vertebrate animals or cephalopods performed by any of the authors. This study was exempt from ethical approval procedures according to the current Portuguese legislation.

Consent for publication

Not applicable.

Availability of data and material

The 66 MAGs are available in the European Nucleotide Archive (ENA) under the study accession number PRJEB50578, sample accession numbers ERS10420767 – ERS10420805 (from octocorals) and ERS10422230 – ERS10422256 (from seawater). Assembly accession numbers for each MAG can be found in Table S1 of Additional File 2. All raw metagenome data are deposited under the study accession number PRJEB13222.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TK-C and RC designed the study. RC, AL-L, JMSG, NK and UNR provided resources and materials. TK-C, RT and SGS processed data. TK-C and LK analysed data and prepared figures. TK-C wrote the first manuscript draft. All authors revised and improved the manuscript prior to submission.

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Figures

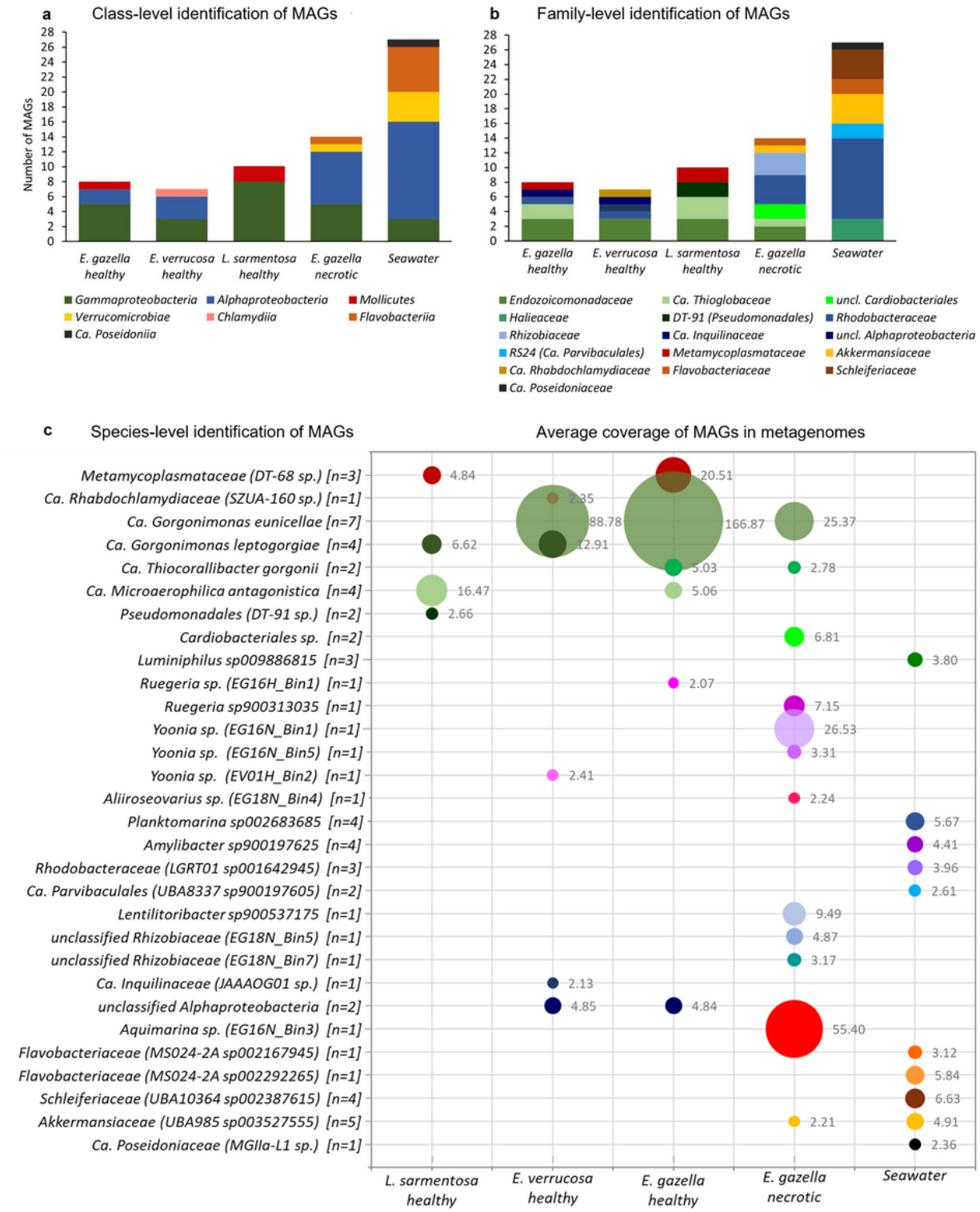

Figure 1

Taxonomic affiliation at **(a)** class- **(b)** family- and **(c)** species-level of the 66 metagenome-assembled genomes (MAGs) obtained from the microbial metagenomes of healthy and necrotic *Eunicella gazella* tissue, healthy *E. verrucosa* and *Leptogorgia sarmentosa* and seawater. Average genome coverage values (inferred by mapping unassembled reads against MAG contigs) represent a relative abundance estimate of the prokaryotes in each microhabitat (**c**).

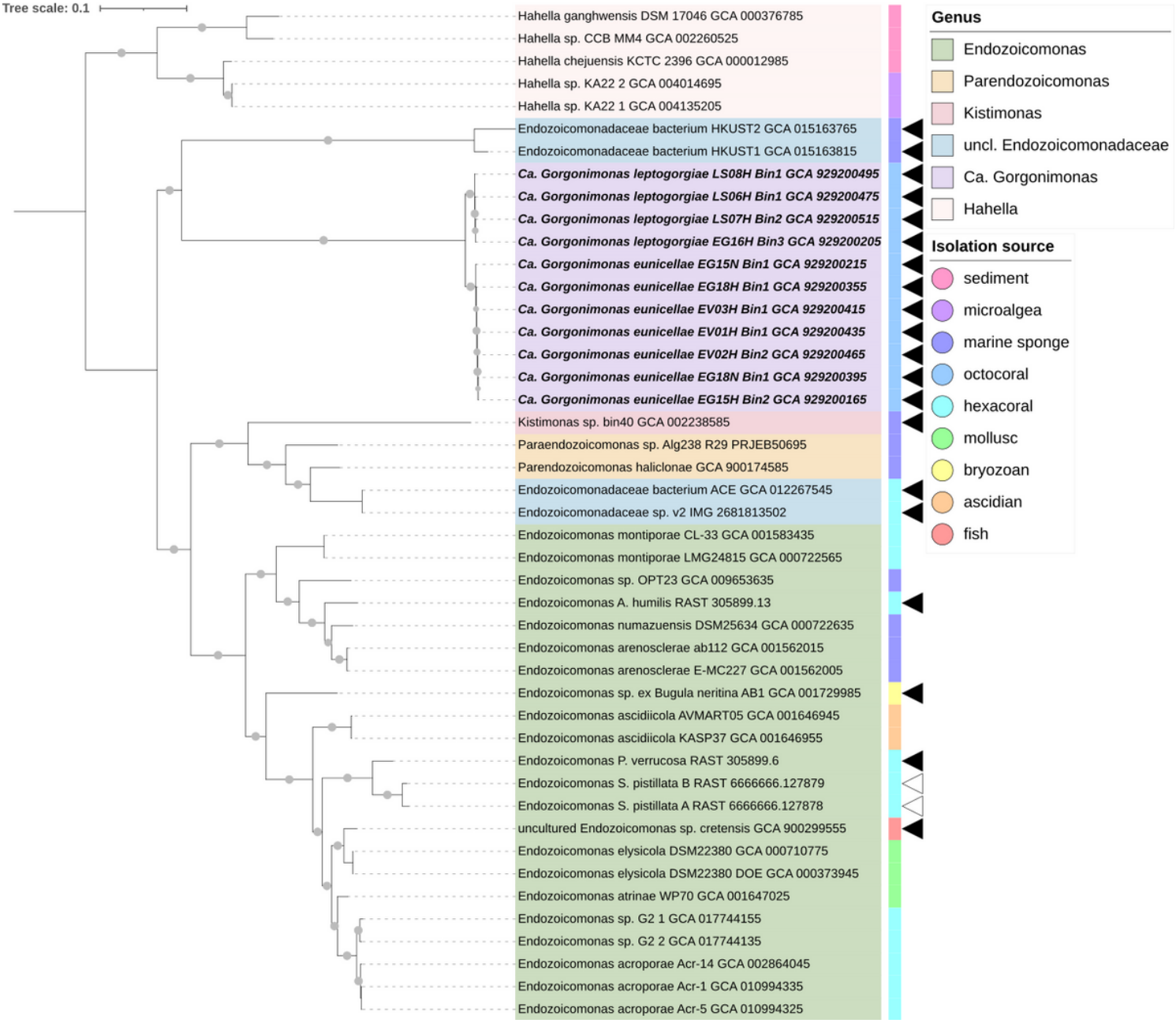

Figure 2

Phylogenomic analysis of the *Endozoicomonadaceae* family using the SpeciesTreeBuilder v.01.0. Evolutionary history was inferred by using a Maximum likelihood method (FastTree2) based on alignment similarity of a set of 49 core, universal genes defined by Clusters of Orthologous Groups of proteins (COG) gene families. Grey dots on the branches indicate bootstrap support of >70%. Black triangles indicate a MAG, white triangles a SAG, the remaining genomes derived from isolates. The 11 *Endozoicomonadaceae* MAGs of this study are highlighted in bold-italics. All other *Endozoicomonadaceae* genomes (n=28) were publicly available on RAST, IMG or NCBI. Assembly accession numbers are given next to the strain names. Note that all *Endozoicomonadaceae* genomes (including MAGs and SAGs) derived from the microbiomes of marine animals, mainly marine

invertebrates. Five *Hahella* spp. genomes of the closely related *Hahellaceae* family were used as outgroup to root the tree. The coloured bar next to the tree shows the isolation source of the genomes. The tree is drawn to scale and was style-edited in iTOL.

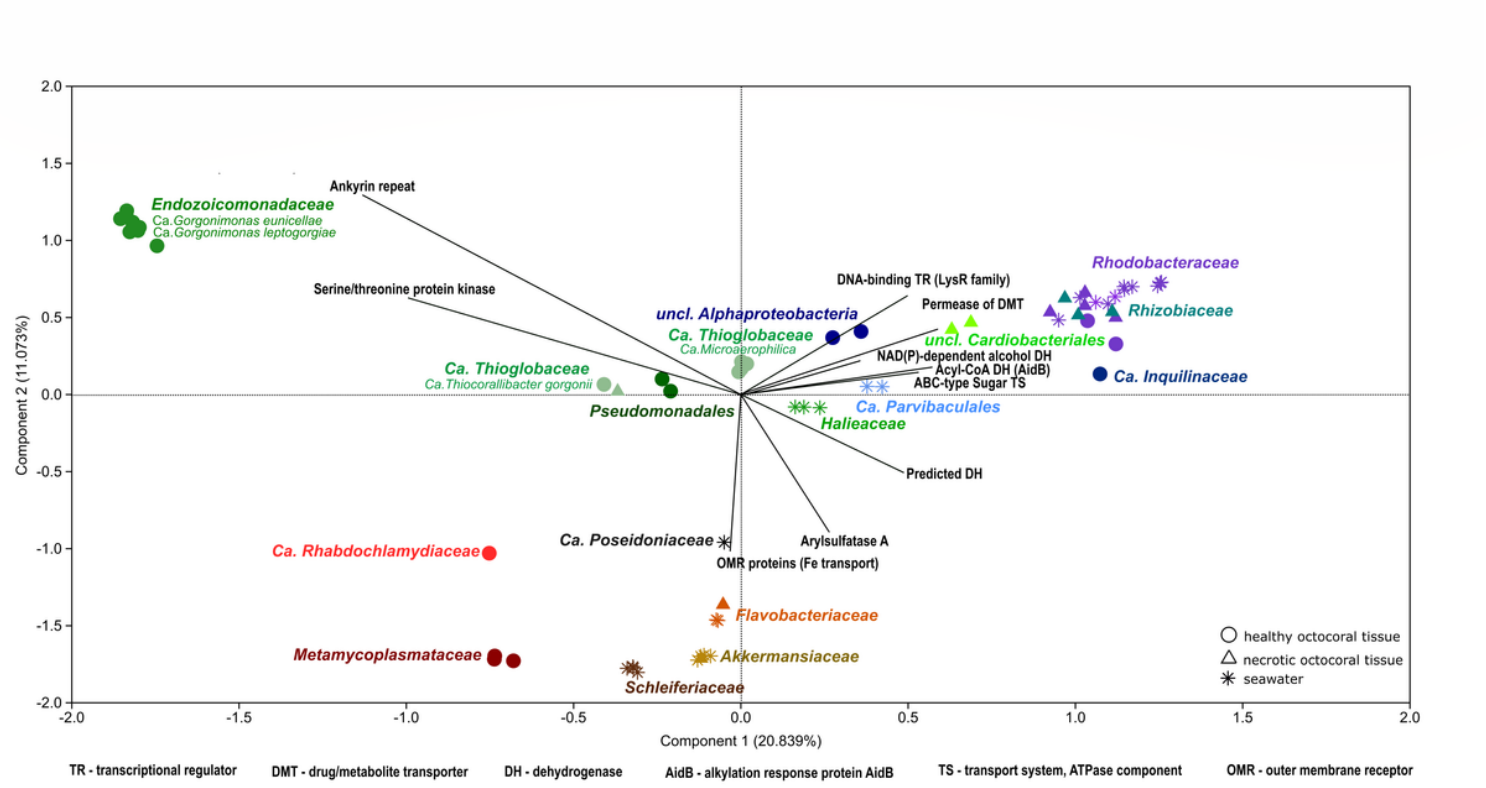

Figure 3

Multivariate analysis of COG profiles of the 66 MAGs of this study. Principal components analysis (PCA) was performed using the Euclidean distance matrix calculated from Hellinger-transformed abundance data. The ordination is shown in Eigenvalue-scale. MAGs from healthy octocoral samples are represented by circles, MAGs from necrotic octocoral samples by triangles and MAGs from seawater by asterisks. Symbols are coloured according to the family-level, taxonomic affiliation of the MAGs with family names written next to them. Black arrows represent the top ten COG functions (i.e., COG0666, COG0515, COG3119, COG1629, COG1960, COG0673, COG0583, COG0697, COG1028, COG3839, in that order) which contributed most to the dissimilarities between these MAGs at order-level, as revealed by a SIMPER test. Note the highly distinct clustering of the *Endozoicomonadaceae* MAGs, statistically supported by a One-way PERMANOVA test ($P < 0.001$) with 999 permutations, and, driven by the high copy number of genes encoding for ankyrin repeat proteins (COG0666) and serine/threonine protein kinases (COG0515). See also Tables S3, S4 (Additional File 2) for complete COG profiles and SIMPER results.

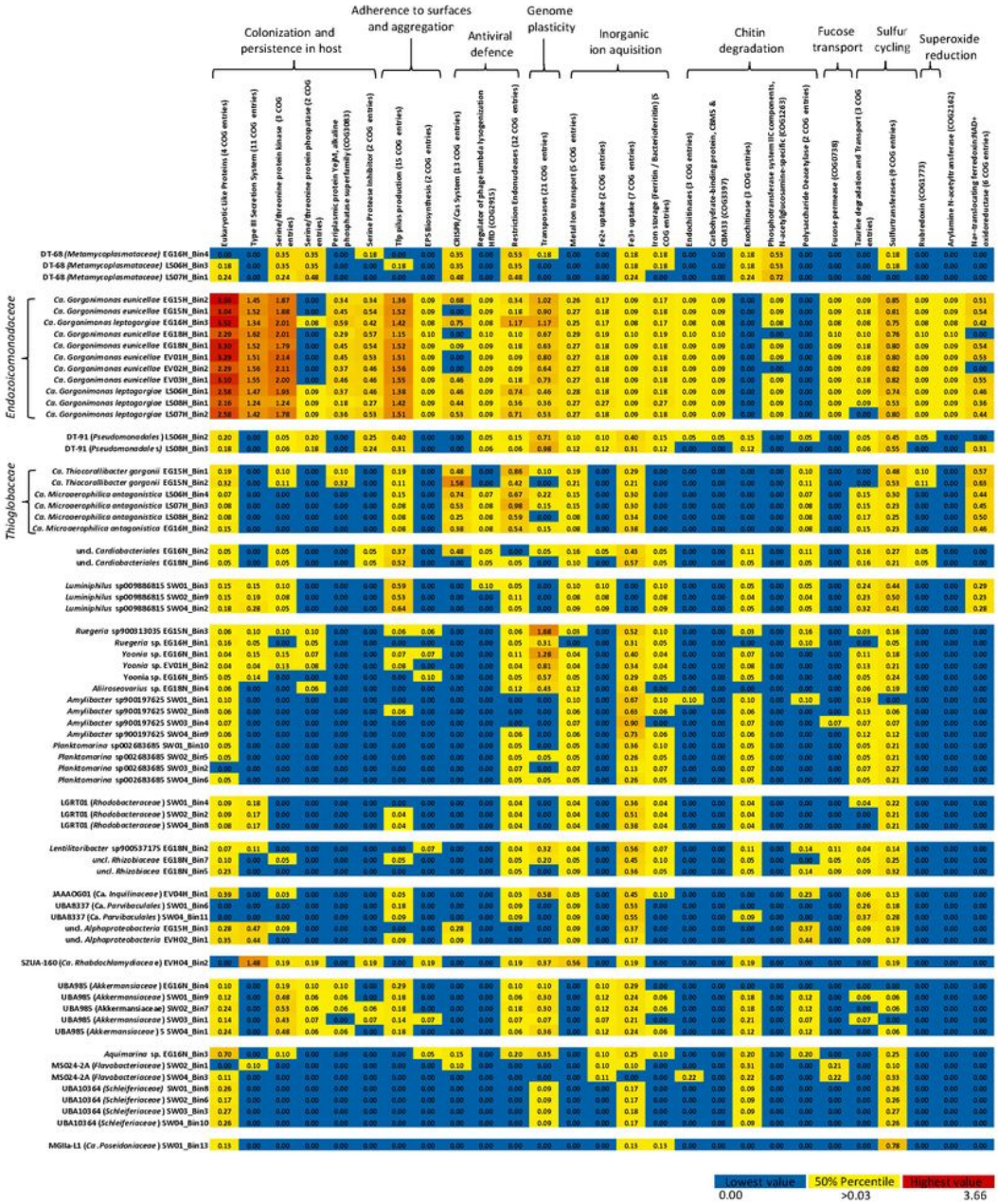

Figure 4

Functional analysis of the 66 MAGs according to their COG profiles. Values for each entry represent the percentage of COGs assigned to a given function relative to the total number of COGs annotated per MAG. The colour code from blue over yellow to dark red reflects an increase in the percentage of COGs related to each function (blue = 0% (minimum value); yellow = 50% percentile; dark red = 3.66 % (maximum value). When functions were represented by more than one COG entry across the data set, the

CDSs counts of these functionally belonging COGs were summed and the number of COGs entries that contributed to each function is given in brackets behind the COG description. Identification of COGs that contributed to this figure can be found in Tables S5A, S5B, Additional File 2.

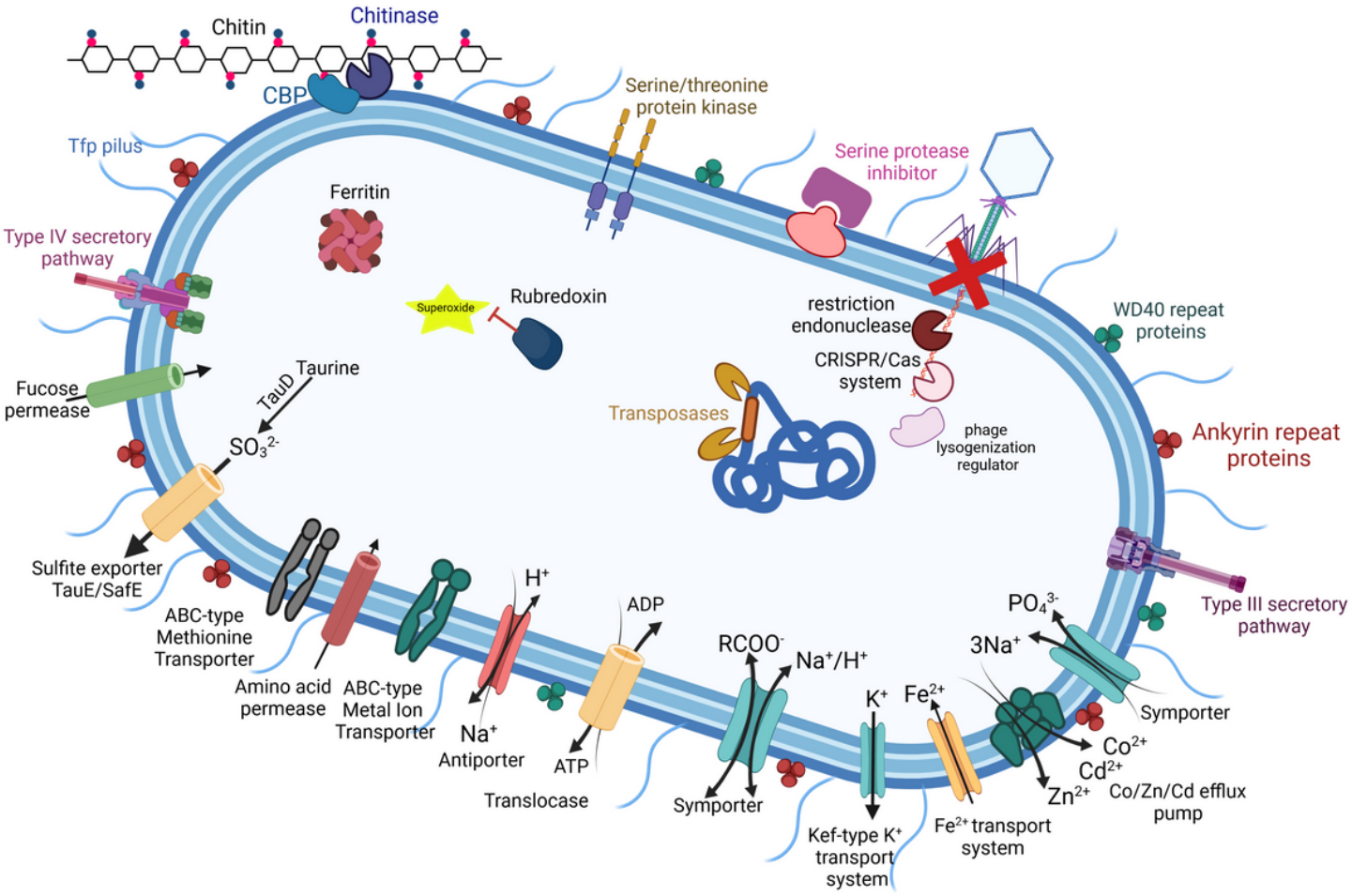

Figure 5

Schematic drawing of the predicted metabolic features (based on COG functional annotations) of the here described Ca. *Gorgonimonas* (*Endozoicomonadaceae*) symbionts of octocorals. Detailed information on the COG functions characteristic of the Ca. *Gorgonimonas* MAGs can be found in Figure S2 of Supplementary File 1 and Tables S4 and S5 of Supplementary File 2. CBP- Chitin binding protein. The figure was created in BioRender.com.

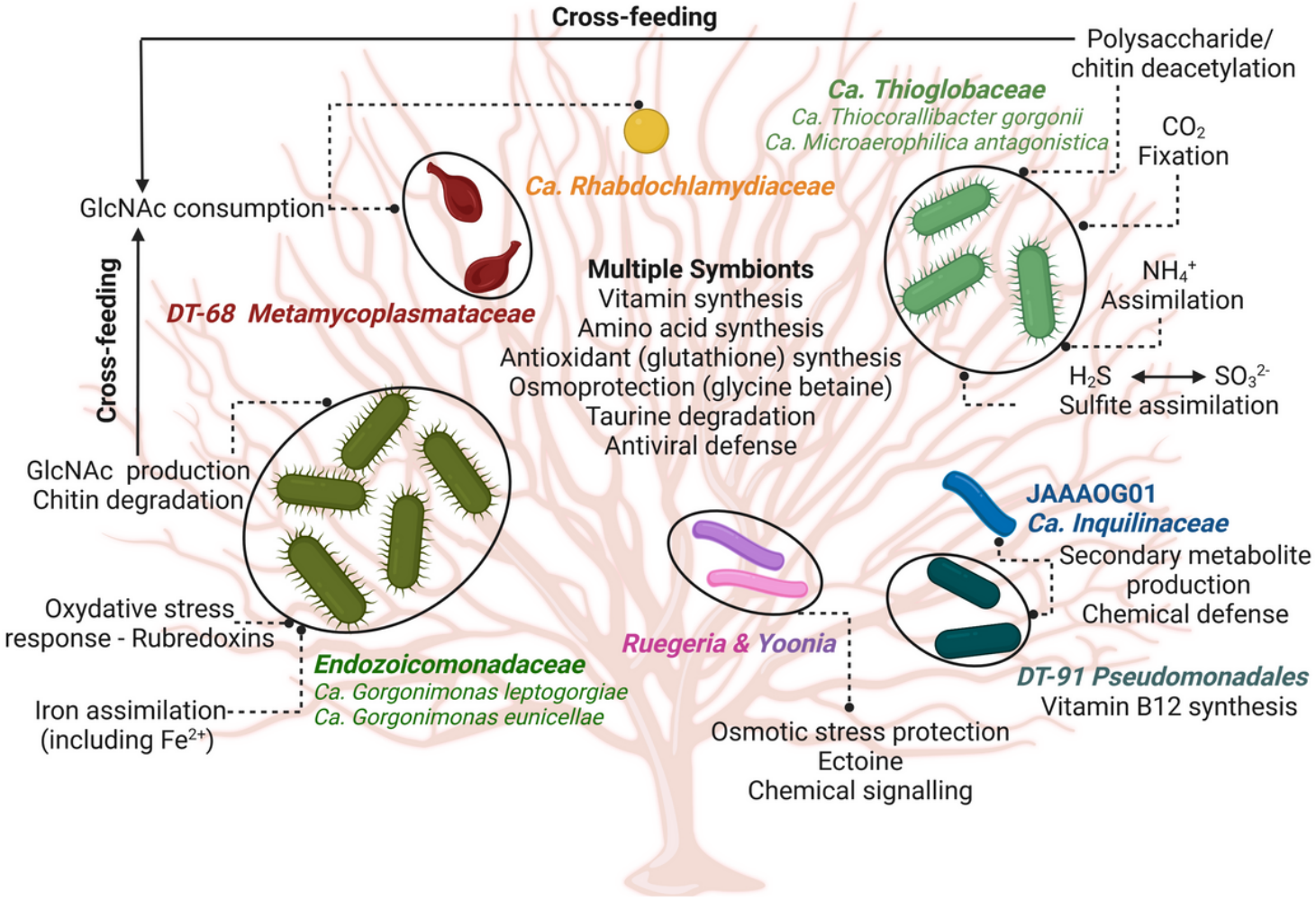

Figure 6

Presumed niche partitioning and metabolic interaction in bacterial symbionts of azooxanthellate octocorals. The schematic overview was inferred from the functions encoded by the MAGs of the 25 bacterial symbionts recovered from healthy *E. gazella*, *E. verrucosa* and *L. sarmentosa* samples. Octocoral symbionts participate in carbon, nitrogen and sulfur cycling, amino acid, and b-vitamin provision, chemical defense and oxidative and osmotic stress protection. While *Endozoicomonadaceae* possess endo-chitinases to break down large chitin polymers, others, including *Metamycoplasmataceae* and Ca. *Thioglobaceae* MAGs, possess exo-chitinase and polysaccharide deacetylase genes, respectively, indicating substrate cross-feeding in the octocoral holobiont. RAST annotation of MAGs showed that B-vitamin biosynthesis capacities, including B1 (thiamine), B2 (riboflavin), B3 (nicotinamide / NAD) B6 (pyridoxin), B7 (biotin) and B9 (folate) are characteristic for *Endozoicomonadaceae* MAGs, while Ca. *Thioglobaceae* MAGs also harbored genes for vitamin B5 (pantothenic acid) biosynthesis but lacked vitamin B1 biosynthesis-related genes. *Pseudomonadales* MAGs possessed biosynthesis capacity for all b-vitamins, including vitamin B12 (cobalamin), demonstrating that *Gammaproteobacteria* symbionts of octocorals can provide all essential b-vitamins. *Metamycoplasmataceae* MAGs do not possess vitamin

biosynthesis genes and rely on supply by other microbiome members. The figure was created in BioRender.com.

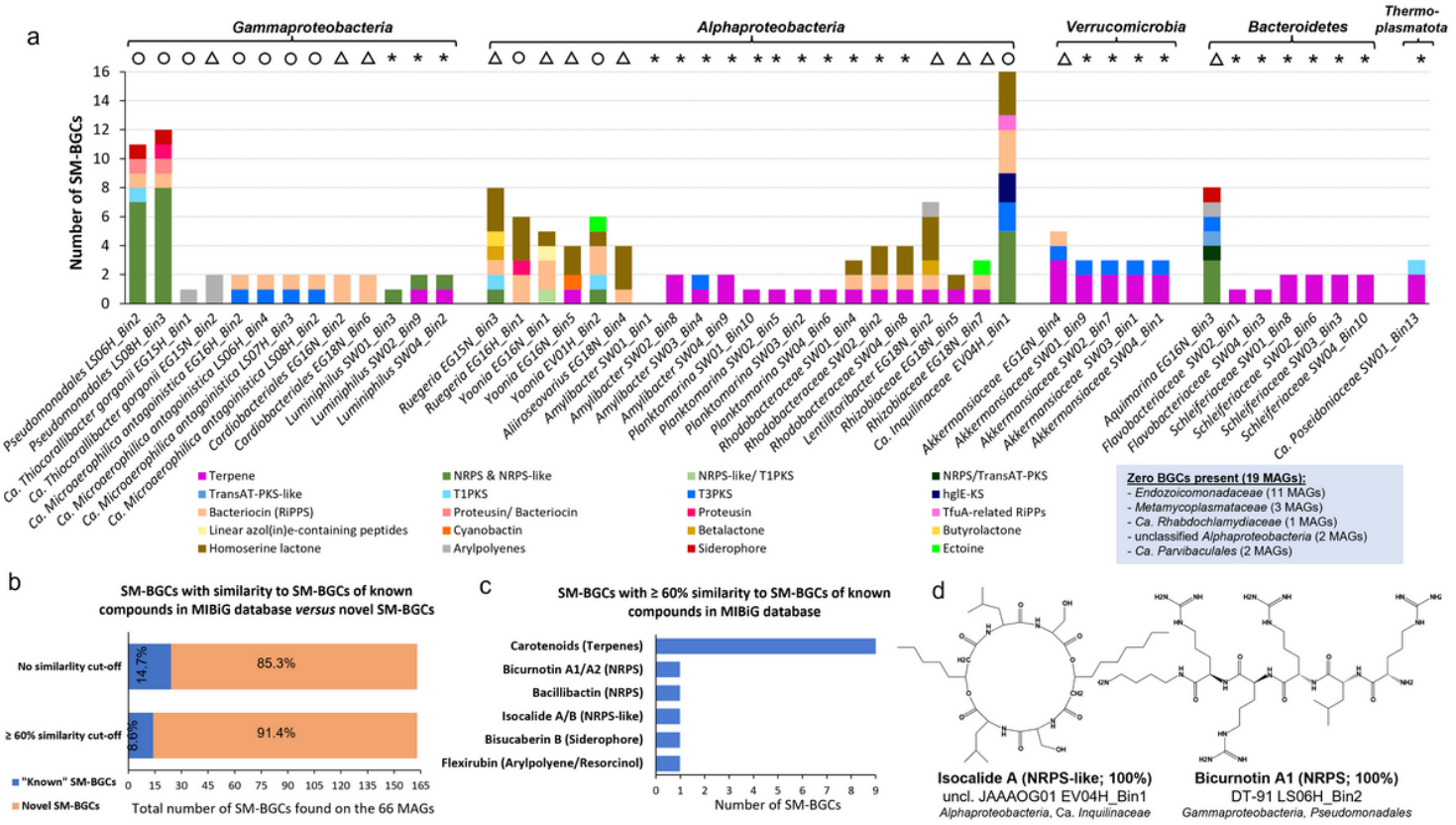

Figure 7

Secondary metabolite coding potential in each MAG obtained from microbial metagenomes of healthy and necrotic octocoral samples and seawater. **(a)** Distribution of secondary metabolite biosynthesis gene clusters (SM-BGCs) across the 66 MAGs of this study. Symbols above bars indicate the origin of each MAG (same as in Figure 3). SM-BGC counts per compound class were obtained using antiSMASH v.5.0 with default detection strictness (and all extra features on). PKS - polyketide synthase; hglEKS - heterocyst glycolipid synthase-like PKS; NRPS - nonribosomal peptide synthetase cluster; TfuA-related, TfuA-related ribosomal peptides. **(b)** Percentage of SM-BGCs, identified on the 66 MAGs, with a hit to SM-BGCs of known compounds present in the MIBiG database. Only 14 of the 165 SM-BGCs obtained in this study shared 60% or more similarity to SM-BGCs of known compounds, highlighting the high degree of novelty encoded in these MAGs. **(c)** Predicted compounds among the SM-BGCs identified in the MAGs. **(d)** Chemical structures of isocalide A and bicurnitin A1, encoded in the octocoral-derived MAGs of JAAAOG01 EV04H_Bin1 (Ca. *Inquilinaceae, Alphaproteobacteria*) and DT-91 LS06H_Bin2 (*Pseudomonadales, Gammaproteobacteria*). Structures were drawn with ChemDraw v. 12.0.2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1.docx
- AdditionalFile2.xlsx