Title:

The deep-sea coral, *Callogorgia delta*, associates with bacteria belonging to a novel marine branch of the Mollicutes.

Authors:

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Abstract:

In addition to abundant animal communities, corals from all ocean depths support diverse microbial associates that are important to coral health. While some of these microbes have been classified taxonomically, understanding the metabolic potential of coral-associated bacteria and how they interact with their coral hosts is limited by a lack of genomic data. One example is *Mycoplasma* and other members of the class Mollicutes which are widespread coral associates. Here we investigated the association between two novel members of the class Mollicutes and the deep-sea octocoral *Callogorgia delta.* We screened *C. delta,* a closely related species *C. americana,* sediment, and water for mollicutes using 16S metabarcoding. One ASV was found in most colonies screened (99/108) and often dominated the microbiome (up to 99%). Another ASV was detected at lower abundance and prevalence in these corals. Both were absent in all water and were absent or rare in the sediment. We sequenced metagenomes and metatranscriptomes to assemble and annotate genomes and propose the names *Ca. Oceanoplasma callogorgiae* and *Ca. Thalassoplasma callogorgiae.* The genomes were small, revealed a reliance on the arginine dihydrolase pathway for ATP production, and contained CRISPR-Cas

systems with extensive arrays. CARD-FISH microscopy unveiled an abundant bacterium in the mesoglea which is likely to be *Ca. O. callogorgiae*. These novel mollicutes cluster with others from diverse invertebrate hosts. Altogether, this work describes the association of these novel mollicutes in *C. delta*, provides insight into widespread coral associates, and identifies a novel clade of marine mollicutes whose diversity remains largely undiscovered.

Keywords:

Deep-sea corals, Mollicutes, symbiosis

Introduction:

Corals are important foundation species that support diverse animal communities from shallow waters to the deep sea. In addition to abundant animal communities, corals also associate with a diverse array of microbes (Bourne et al. 2016). Besides the well-studied algal symbionts of the family Symbiodiniaceae, corals associate with a diverse array of microbial taxa that have proven to be important to holobiont function by performing a variety of roles including providing nitrogen through fixation (M. P. Lesser et al. 2007; Michael P. Lesser et al. 2004; S. H. Yang et al. 2016) and causing disease (Baker et al. 2022; J. G. Klinges et al. 2022; G. Klinges et al. 2020; J. G. Klinges et al. 2019). Some of the most widespread taxa commonly found in corals include corallicolid apicomplexans (Vohsen et al. 2020; Kwong et al. 2019; Kirk et al. 2013a; Kirk et al. 2013b), *Endozoicomonas* (Pollock et al. 2018), and *Mycoplasma*.

Mycoplasma and other members of the class Mollicutes are widespread coral associates. While they are most commonly found in octocorals (Gray et al. 2011; Penn et al. 2006; Weiler et al. 2018; Holm and Heidelberg 2016; Kellogg et al. 2016; Porporato et

al. 2013; Shirur et al. 2016; van de Water et al. 2018; van de Water et al. 2017; McCauley et al. 2020), they have also been found in black corals (van de Water et al. 2020) and stony corals (Neulinger et al. 2009; Kellogg et al. 2009). The corals that they inhabit are found in a wide breadth of habitats from shallow-water reefs (Woo et al. 2017; Shirur et al. 2016; McCauley et al. 2020), through the mesophotic (van de Water et al. 2020) and the deep sea (Neulinger et al. 2009; Weiler et al. 2018; Penn et al. 2006) to abyssal depths (Quintanilla et al. 2022). Beyond this, some studies have yielded further insight into the association between mollicutes and corals. For instance, *Mycoplasma corallicola* resides within its host coral's ectoderm in the deep-sea scleractinian, *Lophelia pertusa* (Neulinger et al. 2009). Further, *Mycoplasma* spp. are abundant and dominant in multiple coral species, comprising more than 50% of their associated microbial community (Weiler et al. 2018; Woo et al. 2017; McCauley et al. 2020). Interestingly, the relative abundance of *Mycoplasma* can show substantial variation across the ranges of their host corals. In some coral species, colonies from only a subset of geographic locations are dominated by *Mycoplasma* (Woo et al. 2017; van de Water et al. 2017). Thus, this association may be influenced by environmental conditions or otherwise shaped by geography such as through limited dispersal. *Mycoplasma* may have also coevolved with their coral hosts because they exhibit varying degrees of host specificity. Closely related octocoral species from the same locations differ in the abundance and identity of the *Mycoplasma* phylotypes that they host (McCauley et al. 2020; van de Water et al. 2017; Holm and Heidelberg 2016). Despite these insights, the interactions between mycoplasmas and other mollicutes with their host corals remain unknown.

Almost all other members of the class Mollicutes are symbionts that associate with a wide diversity of plant, fungal, and animal host species including humans (Ing-Ming Lee et al. 2000; Tully et al. 1993). Many members are well-studied parasites (Atkinson 2018), while some are unclear such as the ubiquitous endocellular symbionts of arbuscular mycorrhizal fungi, an ancient association that dates back to the era when plants first colonized the land (Bonfante and Desirò 2017; Desiro et al. 2015; Kuga et al. 2021). Yet others are mutualists with well-established benefits to their hosts such as some *Spiroplasma* spp. that infect insects and confer protection against nematodes, parasitoid wasps, and fungi through the production of unique toxins (Ballinger and Perlman 2019). Recently, many novel and divergent members of this class have been discovered in marine invertebrates from a variety of environments including a holothurian from a deep-sea trench (He et al. 2018), a pelagic photosynthetic jellyfish (Viver et al. 2017; Cortés-Lara et al. 2015), wood-boring deep-sea chitons (Duperron et al. 2013), ascidians from a coastal lagoon (Tait et al. 2007), and crown-of-thorns sea stars from the Great Barrier Reef (Høj et al. 2018). The genome of the holothurian associate has been sequenced and it encodes an extensive CRISPR-Cas9 array leading to speculation that this associate provides viral defense for its host (He et al. 2018). Similar insights about the interactions between corals and their associated mollicutes may be gleaned by obtaining genomes of their associates.

Here we discovered two novel bacteria of the class Mollicutes which are abundant in the deep-sea octocoral *Callogorgia delta. C. delta* is a common coral along the continental slope in the Gulf of Mexico between 400 m and 900 m depth, where it is often the dominant habitat-forming coral (Quattrini et al. 2015; Quattrini et al. 2013). *C.*

delta colonies create habitat for numerous animal species including *Asteroschema* ophiuroids and the chain catshark, *Scyliorhinus retifer*, which lay their eggs directly on colonies (Etnoyer and Warrenchuk 2007). The closely related coral, *C. americana*, is also a dominant habitat forming species. *C. delta* was originally classified as a subspecies of *C. americana* until it was raised to the level of species in 2015 (Bayer et al. 2015). Both species occur in the Gulf of Mexico but exhibit different ecological niches wherein *C. americana* is found at shallower depths (300 - 400m) and *C. delta* is often found near hydrocarbon seeps (Quattrini et al. 2013).

In order to characterize the association between *Callogorgia delta* and these newly discovered mollicutes, we screened colonies from six sites in the Gulf of Mexico across three years of sampling using 16S metabarcoding to determine the prevalence of these novel mollicutes and quantify their relative abundances. We also screened the closely related species *Callogorgia americana*, sediment, and water from the surrounding environment for comparison. Further, we sequenced metagenomes and metatranscriptomes of *C. delta* to assemble the genomes of these novel mollicutes, assess their metabolic capabilities, and to determine their phylogenetic positions. Finally, we used catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) microscopy to locate bacteria within coral tissue.

Methods:

Collections

Collections are a subset of those described in Vohsen et al. 2020. One hundred and eight *Callogorgia delta* colonies were collected from six sites spanning over 350 km in the northern Gulf of Mexico in 2015, 2016, and 2017 (Fig. 1). The sites are named

after the Bureau of Ocean Energy Management's designations for lease blocks in which corals were sampled and include Mississippi Canyon (MC) 751 (n=19 colonies, 435-443 m, 28.193 N -89.800 W), Viosca Knoll (VK) 826 (n=3 colonies, 547-559 m, 29.159 N -88.010 W), Green Canyon (GC) 234 (n=37 colonies, 505-536 m, 27.746 N -91.122 W), MC885 (n=25 colonies, 624-642 m, 28.064 N -89.718 W), GC249 (n=14 colonies, 789-795 m, 27.724 N -90.514 W), and GC290 (n=10 colonies, 852 m, 27.689 N -90.646 W). To compare *Callogorgia delta* to a closely related coral species, we additionally processed five *Callogorgia americana* colonies that were collected in 2010 from Garden Banks (GB) 299 (n=3 colonies, 358-359m, 27.689 N -92.218 W) and VK862 (n=2 colonies, 352-357m, 29.109 N -88.387 W).

Colonies were sampled using specially designed coral cutters mounted on the manipulator arm of remotely operated vehicles (ROVs). Coral fragments were removed, placed in separate temperature insulated containers until recovery of the ROV, and were maintained at 4 °C for up to 4 hours until preservation in ethanol or frozen in liquid nitrogen.

Sediment samples were taken in close proximity to many of the *Callogorgia delta* collections using push cores with a diameter of 6.3 cm. Upon recovery of the ROV, 1 mL of sediment from the top 1 cm of each sediment core was frozen in liquid nitrogen for later DNA extraction for microbiome analysis. Water was sampled in 2015 using a Large Volume Water Transfer System (McLane Laboratories Inc., Falmouth, MA) which filtered approximately 400 L of water through a 0.22 micron porosity filter with a diameter of 142 mm. One quarter of each filter was preserved in ethanol and another quarter was frozen in liquid nitrogen for these analyses. In 2016 and 2017, 2.5 L Niskin

bottles mounted on the ROV were fired above corals. Upon recovery of the ROV, this water was filtered through 0.22 micron filters which were frozen in liquid nitrogen.

16S metabarcoding

The microbiomes of *Callogorgia delta*, *Callogorgia americana*, water, and sediment were analyzed using 16S metabarcoding. DNA was extracted from coral tissue and sediment samples using DNeasy PowerSoil kits (Qiagen, Hilden, Germany) following manufacturer protocols using approximately 1 cm of coral branches and about 0.25 g of sediment. DNeasy PowerSoil kits were also used for water samples collected in 2015 using 1 cm^2 of filter. For all water samples from 2015, replicate extractions were performed on quarters of the filter preserved in both ethanol and frozen in liquid nitrogen. DNA was extracted from all other water samples using Qiagen DNeasy PowerWater kits.

The V1 and V2 regions of the 16S rRNA gene were amplified using universal bacterial primers 27F and 355R (Rodriguez-Lanetty et al. 2013) with CS1 and CS2 adapters (Illumina, San Diego, CA). PCR was conducted with the following reaction composition: 0.1 U/ µL Gotaq (Promega, Madison, WI), 1X Gotaq buffer, 0.25mM of each dNTP, 2.5 mM $MgCl_2$, and 0.25 µM of each primer, and the following thermocycler conditions: 95 °C for 5 min; 30 cycles of 95 °C for 30 sec, 51 °C for 1 min, and 72 °C for 1 min; and finally 72 °C for 7 min. Libraries were prepared by the University of Illinois Chicago DNA services facility and sequenced on two separate runs on an Illumina MISEQ platform (Naqib et al. 2018). Raw sequence data are available on the NCBI database under BioProject PRJNA565265 and BioSample IDs SAMN12824733 – 4765, SAMN12824767 – 4875.

Amplicon sequence data were analyzed as described in (Vohsen et al. 2020) using QIIME 2 (ver2017.11) (Bolyen et al. 2018). Reads were joined using vsearch and quality filtered using q-score-joined. Deblur denoise was used to detect chimeras, construct sub-operational taxonomic units (later referred to as ASVs), and trim assembled reads to 300bp. An ASV table was exported into R to create bar plots.

Metagenomes and Metatranscriptomes

Metagenomes and metatranscriptomes of *C. delta* were sequenced to assemble genomes of novel mollicutes which were used to assess their phylogenetic positions and metabolic potential. DNA was extracted from eight *Callogorgia delta* colonies as described in Vohsen et al. 2020. In brief, DNA was extracted using DNA/RNA allprep kits (Qiagen, Hilden, Germany) with β-mercaptoethanol added to the RLT+ lysis buffer following the manufacturer's instructions. In addition to these samples, DNA was extracted from an additional 18 samples corresponding to 16 additional colonies.

RNA was extracted from two of those *Callogorgia delta* colonies using an RNeasy extraction kit (Qiagen, Hilden, Germany). The RNA extracts were enriched for bacteria by depleting coral host rRNA using a Ribo-Zero Gold Yeast kit (Illumina, San Diego, CA, USA) following the manufacturer's protocols.

The original eight DNA extracts were sent to the Max Planck Genome Center in Cologne, Germany for library preparation and were sequenced on an Illumina HISEQ2500 platform using 150 bp paired-end reads. The two *Callogorgia delta* libraries with the highest coverage of mollicutes were sequenced a second time. Raw sequence data are available on the NCBI database under BioProject ID PRJNA574146 and BioSample IDs SAMN12856800 – SAMN12856807. Both the additional 18 DNA

extracts and RNA extracts were submitted to the Pennsylvania State University Genomics Core facility for library preparation and were sequenced on a HISEQ with 100bp paired-end reads and a MISEQ platform with 100 bp single-end reads, respectively. Raw sequence data are available on the NCBI database under BioProject ID PRJNA565265 and SRA accession numbers SRR10174410 and SRR10174411.

All eight initial DNA sequence libraries were screened for bacterial SSU rRNA using phyloFlash ver3.3 (Gruber-Vodicka et al. 2019). The two libraries with the highest coverage of mollicute 16S rRNA were sequenced a second time. Of these, the library with higher coverage was chosen for further processing. Reads were trimmed to a quality score of 2 from both ends using BBduk and a kmer frequency analysis was performed using BBnorm. The library was kfiltered to a depth of 1,000 or greater using BBnorm and downsized to 10% using reformat with a single pass. The library was then assembled using SPAdes (Bankevich et al. 2012) with a kmer progression of 21,33,55,77,99,127. Bandage ver0.8.1 (Wick et al. 2015) was used to identify a single circular scaffold of contigs containing the dominant mollicute 16S rRNA gene. The overlaps on the end were manually trimmed off and the scaffold was recentered to the origin of replication predicted by Ori-Finder (Gao and Zhang 2008). This constituted the draft genome for the dominant mollicute and was annotated using classic RAST ver2.0 (Aziz et al. 2008; Overbeek et al. 2005).

In order to generate a genome for the less abundant mollicute, the two libraries with the highest coverage of its 16S rRNA gene were coassembled using megahit (D. H. Li et al. 2015). All libraries were mapped to this coassembly using BBmap with a kfilter of 22, subfilter of 15, and a maxindel of 80. Alignments were converted from SAM to

BAM formats and sorted using samtools (H. Li et al. 2009) and used for binning using metabat (Kang et al. 2015) with default parameters. All scaffolds with contigs belonging to bin 1 constituted a draft genome that was annotated using RAST and used for phylogenomic analysis.

The presence of CRISPRs and associated genes and the number of spacers was determined using RAST annotations and the tool CRISPRfinder (Symeonidi et al. 2021; Grissa et al. 2007). OrthoANI (Lee et al. 2016) was used to estimate average nucleotide identity between genomes. To determine which genes were expressed and to quantify their expression, the two RNA sequence libraries were pseudoaligned to the gene annotations of both genomes using kallisto ver0.44.0 (Bray et al. 2016) using 100 bootstrap replicates, an estimated fragment length of 180 with a standard deviation of 20, and an index with a k-mer size of 31.

Phylogenetic Analyses

Phylogenetic trees were constructed to infer the phylogenetic positions of the mollicutes that were detected in *Callogorgia.* First a tree of 16S sequences was generated to place these novel mollicutes within the wide diversity of mollicutes with available 16S sequences. Full length 16S rRNA genes were assembled from the metagenomes using phyloFlash. These full length sequences were aligned with publicly available Mollicutes sequences that were over 1,000 bp using MUSCLE ver3.8.425 (Edgar 2004). A maximum likelihood tree was constructed using the IQ-TREE web server ver1.6.10 (Nguyen et al. 2014; Trifinopoulos et al. 2016). Within IQ-TREE, ModelFinder (Kalyaanamoorthy et al. 2017) was used to choose a general time reversible model with unequal rates and empirical base frequencies and a FreeRate model (Soubrier et al. 2012;

Z. Yang 1995) for rate heterogeneity across sites with 3 categories (GTR+F+R3) based on the highest BIC score. UFBoot2 (Hoang et al. 2017) was used to obtain ultrafast bootstrap support values (UFBoot) using 1000 replicates.

In order to get a more confident phylogenetic placement of these novel mollicutes, a phylogenomic tree was constructed including genomes from both mollicutes in *C. delta* plus an additional 66 publicly available genomes. One more genome was assembled from a publicly available dataset because an assembled genome was not available. This genome corresponded to the novel bacterium that associates with the jellyfish, *Cotylorhiza tuberculata*, which those authors classified as *Mycoplasma.* PhyloFlash (Gruber-Vodicka et al. 2019) was performed on all 4 available libraries to assess bacterial compositions and the library with the highest coverage of *Mycoplasma* (Library M3) was assembled using megahit (D. H. Li et al. 2015) with a kmax of 241. All 4 libraries were mapped to this assembly using bbmap with a kfilter of 22, subfilter of 15, and maxindel of 80. The alignments were subsequently used to bin the assembly using metabat (Kang et al. 2015) using default parameters. Bin 1 corresponded to the purported *Mycoplasma* genome. This bin and all associated scaffolds were used to represent this genome in phylogenomic analyses. A concatenated amino acid sequence alignment of 43 genes from all 69 genomes was generated with CheckM (ver1.1.3) (Parks et al. 2015). This was used to generate a maximum likelihood phylogenetic tree using IQ-TREE (ver1.6.12) (Hoang et al. 2017; Nguyen et al. 2014).

Both phylogenetic trees were annotated using the revised phylogeny of the class Mollicutes established in (Radhey S. Gupta et al. 2018; Radhey S Gupta and Oren 2020; Radhey S Gupta et al. 2019).

Microscopy

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) microscopy was employed to localize bacteria within *Callogorgia delta.* Colonies collected in 2016 were preserved for microscopy. During subsampling, 0.5 cm long branches were fixed in 2 mL of 4% formaldehyde with 1X phosphate-buffered saline (PBS) at 4 °C overnight (4-8 hours). Samples were washed 3 times and then stored in equal parts ethanol and 1X PBS for up to 21 months. Samples were decalcified in 0.45M EDTA in 1X PBS for 7 days, refreshing the buffer after 4 days, then washed in 1X PBS. Samples were post-fixed in 1X PBS with 1% paraformaldehyde for 1 hour then washed 3 times in 1X PBS and stored in 50:50 1X PBS:ethanol overnight. The next day, the samples were embedded in TissueTek (Sakura Finetek, Maumee, OH) and left at 4 °C overnight. The next day, the TissueTek was replaced and the samples were frozen. Samples were sectioned to a thickness of 4 µm using a Leica (Wetzlar, Germany) cryostat CM3050 S. Adjacent sections were placed on polysine slides and stored at 4 °C before hybridization.

Hybridization began by removing TissueTek from tissue sections via soaking slides in milliQ water for 3 minutes. Autofluorescence was then diminished by soaking slides in ethanol with 0.1% Sudan black B for 10 minutes. Sections were washed three times in 1X PBS and left in 1X PBS for a final 24 minutes. Endogenous peroxidases were inactivated by soaking sections in 0.2M HCl for 12 minutes, then 20 mM Tris-HCl for 20 minutes, lysozyme solution (0.01 g/mL) for 30 minutes at 37 °C, 20 mM Tris-HCl again for 20 minutes, and finally 3 minutes in milliQ water. Slides were then airdried and

sections were circled with a PAP pen. Two probes were used: EUB I-III which targets eubacteria and a control probe (non-EUB) consisting of the reverse complement. Hybridization mixes were created by combining 1 µL of probe (50 ng/µL) with 299 µL hybridization buffer (30% formamide with 0.102 M NaCl, 0.02 M Tris HCl, 10% Blocking Reagent, 0.01% sodium dodecyl sulfate, and 0.1 g/mL dextran sulfate). Each section was covered with approximately 10-20 µL of one of the hybridization mixes. Two adjacent sections were hybridized with the EUB I-III and non-EUB probes separately to produce a negative control of the same tissue region. Each slide was then placed in a humidity chamber consisting of a closed 50 mL tube with a kimwipe soaked in 2 mL of 30% formamide in milliQ water. Hybridization was accomplished by incubating slides at 46 °C for 2 hours within their humidity chambers.

After hybridization, slides were washed for 15 minutes at 48 °C in washing buffer (0.102M NaCl, 0.02M Tris HCl, 0.005M EDTA, 0.01% SDS) followed by 15 minutes in 1X PBS at room temperature. A fresh amplification mix was created by first combining 299 µL 1X PBS and 1 µL H_2O_2, then adding 10 µL of that solution to 1 mL of amplification buffer (1X PBS, 0.01% Blocking Reagent, 2M NaCl, 0.1 g/mL dextran sulfate) and 1 µL of labelled tyramide (1mg/mL ALEXA 488-labelled tyramide in dimethylformamide with 20 mg/mL p-iodophenylboronic acid). Slides were again placed in 50 mL humidity chambers with a kimwipe soaked in 2 mL of milliQ water. Amplification was accomplished by incubating slides at 37 °C for 30 minutes in these humidity chambers.

After amplification, slides were washed by dipping in 1X PBS followed by 10 minutes in 1X PBS, and a final dip in milliQ water. Slides were air dried and sections

were stained with DAPI (1 µg/mL) for 10 minutes in the dark. Slides were washed a final time in milliQ water for 3 minutes. Once airdried, slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA) and viewed on an Axioscope epifluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) with filters restricted to the emission spectra of ALEXA 488 and DAPI. Images were taken with a Zeiss AxioCam camera and visualized using AxioVision software. CARD-FISH signals were considered positive if they appeared consistently within a structure in the coral, were absent in the same structures in negative control sections, and overlapped DAPI signals.

Results:

Occurrence of novel Mollicutes in and around Callogorgia

An ASV (1) belonging to class Mollicutes was dominant in *Callogorgia delta* colonies (Fig. 2). It was detected in 99 out of 108 colonies and was present in samples from all three sampling years (2015-2017) and all six sampling locations (Fig. 2). It averaged 78% of the microbial community among corals which harbored it and reached a maximum of 99% of the community in some samples. It was also present in all five *Callogorgia americana* colonies where it averaged 91% of the microbial community. An additional ASV (2) was identified that differed from ASV 1 by a single base pair among 300. This closely related ASV was detected in 4 of 10 colonies from the deepest site, GC290, and averaged 90% of the microbial community in those samples while ASV 1 was undetected (Fig. 2).

Another ASV (3) was identified that belonged to class Mollicutes. While it was prevalent and abundant in some colonies, it was less so compared to ASV 1 (Fig. 2). This ASV was present in 69 out of 108 *C. delta* colonies (up to 82% of the coral-associated

microbes) and three out of five *C. americana* colonies (up to 15% of the coral-associated microbes). However, this ASV was absent in all *C. delta* colonies from sites GC249 (n=14) and GC290 (n=10).

Neither of these mollicute ASVs were detected in any water sample (n=30). However, ASVs 1 and 3 were detected in 35 and 11 out of 47 total sediment samples with maximum relative abundances of 1.1 and 0.16% of the sediment microbial community respectively.

Phylogenetic positions of the mollicute in Callogorgia delta

Twenty-two full length 16S rRNA gene sequences that correspond to ASV 1 were assembled from separate metagenomes and an additional three were assembled that correspond to ASV 3. Phylogenetic analysis using these sequences revealed that they were distinct from other known mollicutes and clustered with a diverse array of mollicutes recently discovered in association with marine invertebrates (Fig. 3a,b). Interestingly, the two novel mollicutes in *Callogorgia delta* are not each other's closest relative and shared only 84% sequence identity which is below the level which has been suggested to differentiate bacterial families (Yarza et al. 2014). Phylogenomic analysis of shared amino acid sequences among mollicute genomes reiterated this pattern (Fig. 3b). A clade of novel Mollicutes that associates with marine invertebrates was well-supported in both 16S and phylogenomic trees (UFBoot value of 100%). Branch lengths between novel marine mollicutes were long and the average nucleotide identity (ANI) of these novel mollicutes with their closest known relatives were <67% (OrthoANI) suggesting that the mollicutes described here are novel genera and that there is great diversity within

this group. We propose the names *Candidatus Oceanoplasma callogorgiae* gen. nov. sp. nov. and *Ca. Thalassoplasma callogorgiae* gen. nov. sp. nov. for the dominant and less abundant novel mollicutes respectively.

Metabolic capabilities of the novel mollicutes in Callogorgia delta

The genome assembled for the dominant mollicute, *Ca. Oceanoplasma callogorgiae*, was very reduced. It was small (446,099 bp) and encoded only 405 predicted genes. Of these, 265 had functional annotations (65%) and 140 were classified as only hypothetical genes. A single gene was identified that may be involved in carbohydrate metabolism, glyceraldehyde-3-phosphate dehydrogenase. Instead, the genome encoded the three genes that conduct the arginine dihydrolase pathway (*arcA* = arginine deiminase, *arcB* = ornithine carbamoyltransferase, and *arcC* = carbamate kinase). These genes along with *arcD* (arginine/ornithine antiporter) generate ATP through the catabolism of arginine and were among the most highly expressed genes in both RNA libraries (Fig. 4). Like other mollicutes, this genome encoded clustered regularly interspaced short palindromic repeats (CRISPRs) and cas genes (*cas1*, *cas2*, and *cas9*) comprising a Class 2 Type II-C system. It contained more spacer sequences, at 107, than most mollicutes with available genomes (Ipoutcha et al. 2019). It also contained type I, II, and III restriction modification systems.

The genome of *Ca. Thalassoplasma callogorgiae* was very similar to that of *Ca. O. callogorgiae.* It was reduced in size (445,456 bp) and metabolic capabilities with only 416 gene annotations (163 hypothetical). It did not encode pathways for carbohydrate metabolism but did encode glyceraldehyde-3-phosphate dehydrogenase, ribose-5-

phosphate isomerase, ribose-phosphate pyrophosphokinase, and a ribose importer. Like *Ca. O. callogorgiae,* it contained the genes involved in the arginine deiminase pathway, a type I restriction modification system, and a Class 2 Type II-C CRISPR-Cas system. Only 14 spacer sequences were identified however this spacer array spanned a gap between scaffolds and is likely an underestimate.

Localization of bacteria in Callogorgia delta

Fluorescent signals were observed in the mesoglea of two separate *Callogorgia delta* colonies and interpreted as abundant bacteria (Fig. 5 and 6). These signals were present in tissue sections hybridized with the EUB I-III probe but absent in adjacent sections hybridized with the control probe (non-EUB). These signals were <1 µm in diameter and overlapped DAPI fluorescence. These signals were observed in both the mesoglea within polyps (Fig. 5c,e,g) and in mesoglea within the coenosarc surrounding the proteinaceous central axis (Fig. 5a). They formed large aggregates potentially comprised of hundreds or thousands of cells (Fig. 6). Another fluorescence signal was observed in the base of the tentacles in one colony but was far less aggregated (Fig. 6c,d).

Discussion:

Prevalence of novel Mollicutes in Callogorgia delta

The association between *Ca. Oceanoplasma callogorgiae* and *Callogorgia delta* was stable across geography and time. Including both ASV 1 and 2, over 95% of *C. delta* colonies hosted *Ca. O. callogorgiae* including samples from every sampling year and every site. *Ca. T. callogorgia* was less prevalent. While it was detected in samples from every year, it was detected in fewer samples, those samples hosted a smaller relative abundance, and they were completely absent from the deepest two sites. These microbes

may associate with corals in the genus *Callogorgia* more broadly since both *Ca. O. callogorgiae* and *Ca. T. callogorgiae* were detected in the closely related species *C. americana* but were absent or rare in the water and sediment from the same sites.

These results suggest that *Callogorgia* is the primary habitat for these mollicutes. This is corroborated by our observation of abundant bacteria in the mesoglea of *C. delta.* The bacteria living in the mesoglea are likely to be *Ca. Oceanoplasma callogorgiae* given its abundance in the amplicon dataset and in the metagenomes where its genomic coverage is as high as 100 times that of *C. delta*.

Metabolic capabilities and interaction with the coral host

The genomes of both *Ca. Oceanoplasma callogorgiae* and *Ca. Thalassoplasma callogorgia* were reduced like many symbiotic bacteria and possessed almost identical metabolic capabilities. Both genomes revealed a dependence on arginine to generate ATP which they likely receive from their coral hosts. However, it has been suggested that the arginine dihydrolase pathway alone is not efficient enough to support the ATP demands of mollicutes (Romano et al. 1980). The energy demands of some non-fermentative mollicutes are additionally supported by the generation of ammonium which generates a proton gradient that in turn drives ATP-synthases (Romano et al. 1980; Smith et al. 1993). This likely occurs in these mollicutes since the arginine dihydrolase pathway produces ammonium and ATP-synthases were among their most highly expressed genes.

The catabolism of arginine may be a key pathway at the center of the interaction between *Callogorgia delta* and its dominant mollicute. Arginine is an important regulator in the tripartite symbiosis between plants, their mycorrhizal associates, and the endosymbiotic mollicutes which are found within the mycorrhizae. Arginine constitutes

over 90% of the amino acids in arbuscular mycorrhizal fungi and serves as the primary intermediate in nitrogen exchange between fungal and plant partners (Govindarajulu et al. 2005; Tian et al. 2010). Abundant arginine may similarly be provided by *Callogorgia delta*, however it is unclear how either coral or mollicute may process the resulting ammonia and ornithine.

Another commonality between these novel mollicutes was the presence of CRISPR-Cas systems in both of their genomes. *Ca. Oceanoplasma callogorgiae* possessed substantially more spacer sequences than most other mollicutes (Ipoutcha et al. 2019). This suggests that they possess extensive viral defenses and/or often encounter foreign DNA. Other metabolic processes may also underly the basic functioning of these novel mollicutes since many genes were unannotated. In fact, some of the most highly expressed genes had unknown functions. Further study is required to describe the complete metabolic potential of these mollicutes.

While there is strong evidence that the dominant mollicute is a symbiont, it is still unclear if the mollicute provides any benefit to its coral host or if it is commensal or a parasite like many other members of the Mollicutes. The metabolism of arginine by the mollicute may provide the coral with an alternate pathway to process nitrogenous waste or may even function to recycle nitrogen which may be useful for corals which rely on a nitrogen-poor diet of marine snow (Kriest 2002). However, these possibilities are speculative without a complete understanding of the capabilities of both the mollicute and the coral host. Another possible benefit that the mollicute may provide is protection from viruses using their CRISPR-Cas systems as suggested for the symbionts of hadal sea cucumbers (He et al. 2018) which are the closest relatives of *Ca. O. callogorgiae*.

additional experiments are required to fully understand the metabolic interactions between these mollicutes and their coral host and any potential benefit this may provide for the coral.

Phylogenetic position of the novel mollicutes in C. delta

Ca. Oceanoplasma callogorgiae and *Ca. Thalassoplamsa callogorgiae* were both novel and highly divergent members of the mollicutes. Phylogenetic analysis grouped them with other novel mollicutes that were isolated from other marine invertebrates with high support. These other invertebrates were phylogenetically diverse, exhibited diverse lifestyles, and originated from wide ranging habitats including holothurians from hadal trenches, sea stars from the Great Barrier Reef, wood-boring chitons from the deep sea, ascidians from a coastal lagoon, and a pelagic jellyfish with algal symbionts (Cortés-Lara et al. 2015; Duperron et al. 2013; Tait et al. 2007; Viver et al. 2017; He et al. 2018). The diversity of these related associates suggests that this novel clade associates with marine invertebrates broadly and is not restricted to hosts with specific lifestyles or from specific habitats. Further, the large phylogenetic distances between mollicutes in this clade suggests that a substantial amount of diversity remains undiscovered. Interestingly, this novel clade of marine mollicutes clusters alongside the endocellular symbionts of mycorrhizal fungi so these two clades may be more closely related to each other than to other mollicutes.

In addition to the novel mollicutes in *C. delta*, the metabolic capabilities of two other members of this clade were able to be characterized: the associate of hadal sea cucumbers named *Spiroplasma holothuricola* and the associate of a pelagic jellyfish classified as *Mycoplasma*. These two genomes showed many similarities with the

genomes of the novel mollicutes in *C. delta.* All four genomes were very small ranging between 380 kbp and 450 kbp placing them among the smallest genomes of any bacteria and smaller than other members of the class Mollicutes (Faburay and McVey 2022). All four genomes were very restricted in metabolic capabilities. All encoded the arginine dihydrolase pathway and no more than four genes involved in carbohydrate metabolism. The mollicute from the jellyfish encoded lactate dehydrogenase, ribulose-phosphate 3-epimerase, and two genes in common with *Ca. Thalassoplasma callogorgiae*: ribose-5-phosphate isomerase and ribose-phosphate pyrophosphokinase. *Spiroplasma holothuricola* encoded only phosphate acetyltransferase and acetate kinase. All four genomes encoded Class 2 Type II-C CRISPR-Cas systems. The two genomes with scaffolds that spanned the entire CRISPR array contained 107 and 76 spacers. This is more than any other member of the Mollicutes except for *Mycoplasma liparidae,* a gut associate of a hadal snailfish, which encoded 118 (Lian et al. 2020). The extent of CRISPR arrays in these deep sea mollicutes may reflect a common adaptation to deep-sea environments.

Conclusion

Here we discovered two novel members of the Mollicutes that associate with the coral *Callogorgia delta* and propose the names *Ca. Oceanoplasma callogorgiae* and *Ca. Thalassoplasma callogorgiae.* We demonstrated their prevalence across sites, years, and in a congener, generated genomes, characterized their metabolic potential, located abundant bacteria within the mesoglea of the coral host, and uncovered their phylogenetic position among a novel clade of marine mollicutes that has not yet been recognized. This work advances our understanding of the widespread coral associates of the class

Mollicutes by providing genomic resources and metabolic inferences but also reveals that a large diversity of these novel marine mollicutes remain undiscovered.

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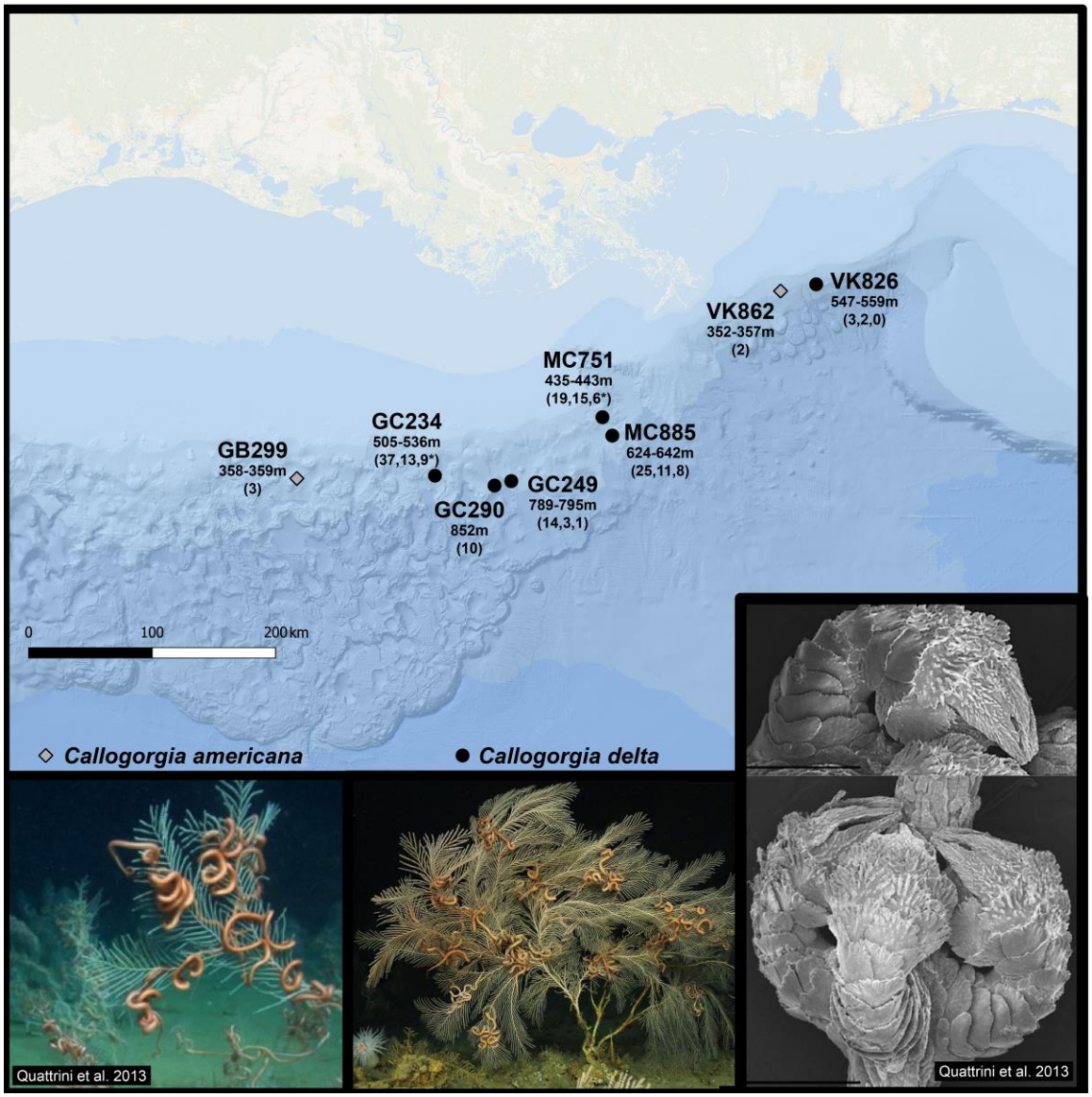


Figure 1. Map of sampling locations. Black circles mark locations where *Callogorgia delta* was sampled and gray diamonds for *C. americana*. Below site names, the depth range of collected samples is shown followed by the number of colonies, sediment, and niskin water samples in parentheses. If no water or sediment were collected, only the number of colonies is shown. A star signifies whether a 400L water sample was taken in addition to water collected with niskins. Bathymetric map obtained from BOEM (credit:

William Shedd, Kody Kramer). Image of *C. americana* and electron micrographs taken from (Quattrini et al. 2013).

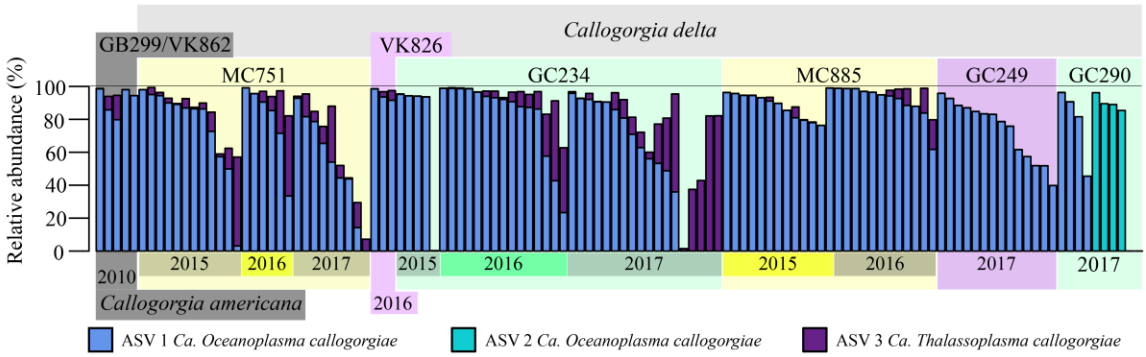


Figure 2. The relative abundances of three ASVs corresponding to novel mollicutes in *Callogorgia delta* and *C. americana*. Each bar represents the microbial composition of a single colony and are organized by species, site, and sampling year.

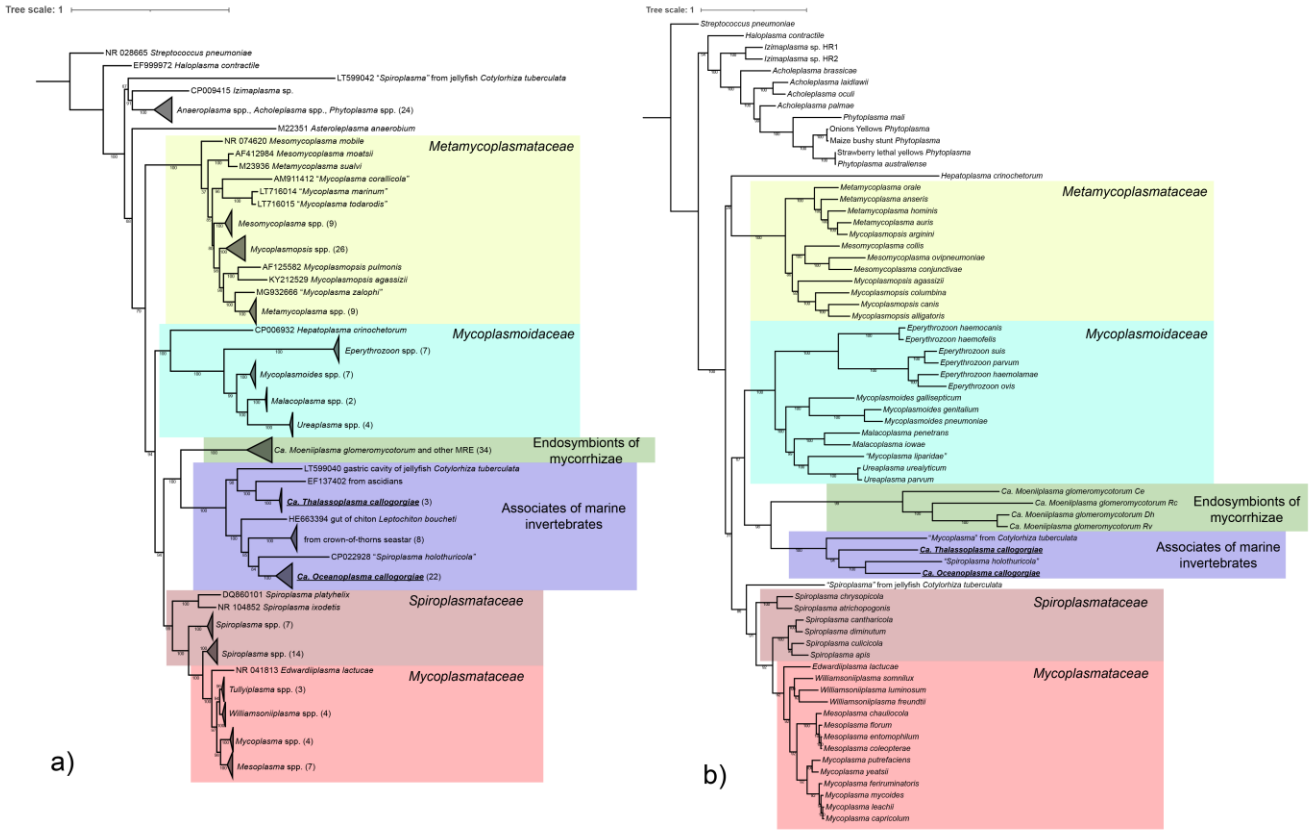


Figure 3. Maximum likelihood phylogenetic trees of class Mollicutes using the 16S rRNA gene (a) and amino acid sequences of 43 genes (b). UFBoot support values are reported at each node. Numbers in parentheses designate the number of sequences within collapsed nodes. *Ca. Oceanoplasma callogorgiae* and *Ca. Thalassoplasma callogorgiae* are presented in bold text and underlined.

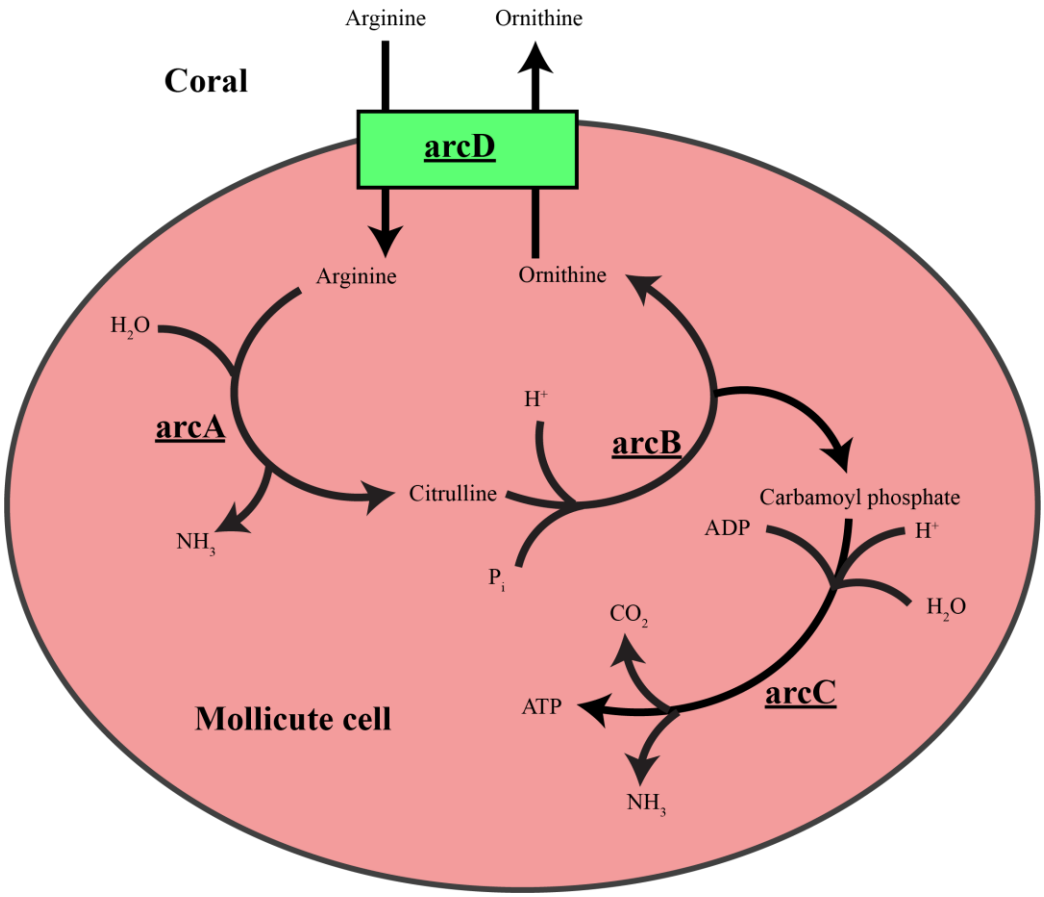
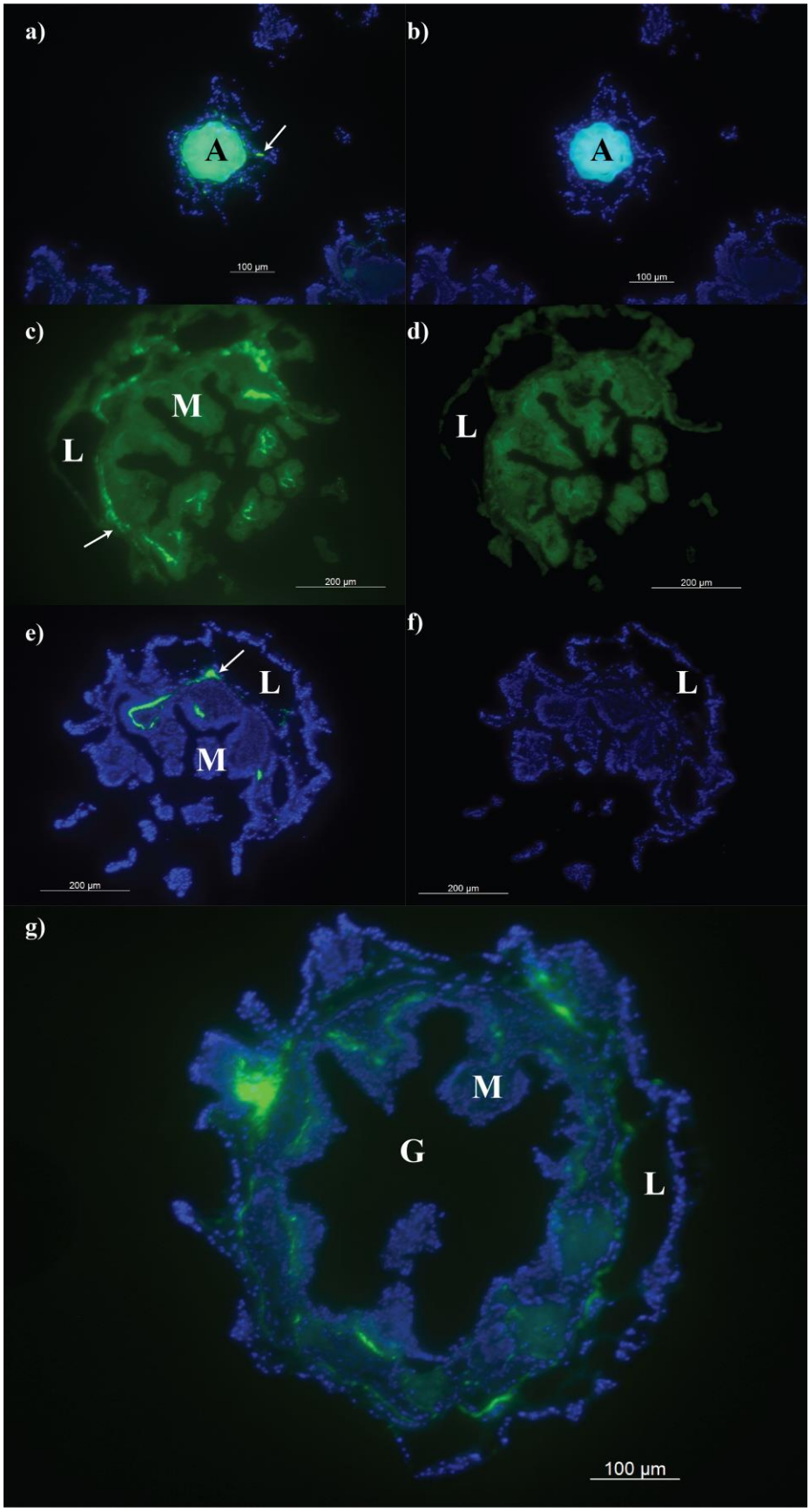


Figure 4. Metabolic map of the arginine dihydrolase pathway encoded in the genomes of both *Ca. Oceanoplasma callogorgiae* and *Ca. Thalassoplasma callogorgiae.* Genes are in bold and underlined text. arcA = arginine deiminase, arcB = ornithine carbamoyltransferase, arcC = carbamate kinase, arcD = arginine/ornithine antiporter.


a)
A
100 µm
b)
A
100 µm
c)
M
L
200 µm
d)
L
200 µm
e)
L
M
200 µm
f)
L
200 µm
g)
M
G
L
100 µm

822

Figure 5. Bacterial signals in the mesoglea of *Callogorgia delta* using CARD-FISH with a general eubacterial probe (EUB I-III) and DAPI staining of DNA. Fluorescence in the emission range of DAPI is shown in blue while green shows emission in the range of the fluorophore ALEXA 488 used by both EUB I-III and control probes. Paired images compare adjacent sections where left images display sections hybridized with EUB I-III and show bacterial signals (a,c,e) while right images display sections hybridized with a negative control probe and only show autofluorescence (b,d,f). Signals were observed in transverse sections through the central axis (a,b) and transverse sections through a single polyp (c,d,e,f,g). Arrows in (a) and (c) show examples of EUB I-III fluorescence. A = central axis, G = gastrovascular cavity, M = gastric mesentery, L = sclerite lacuna.

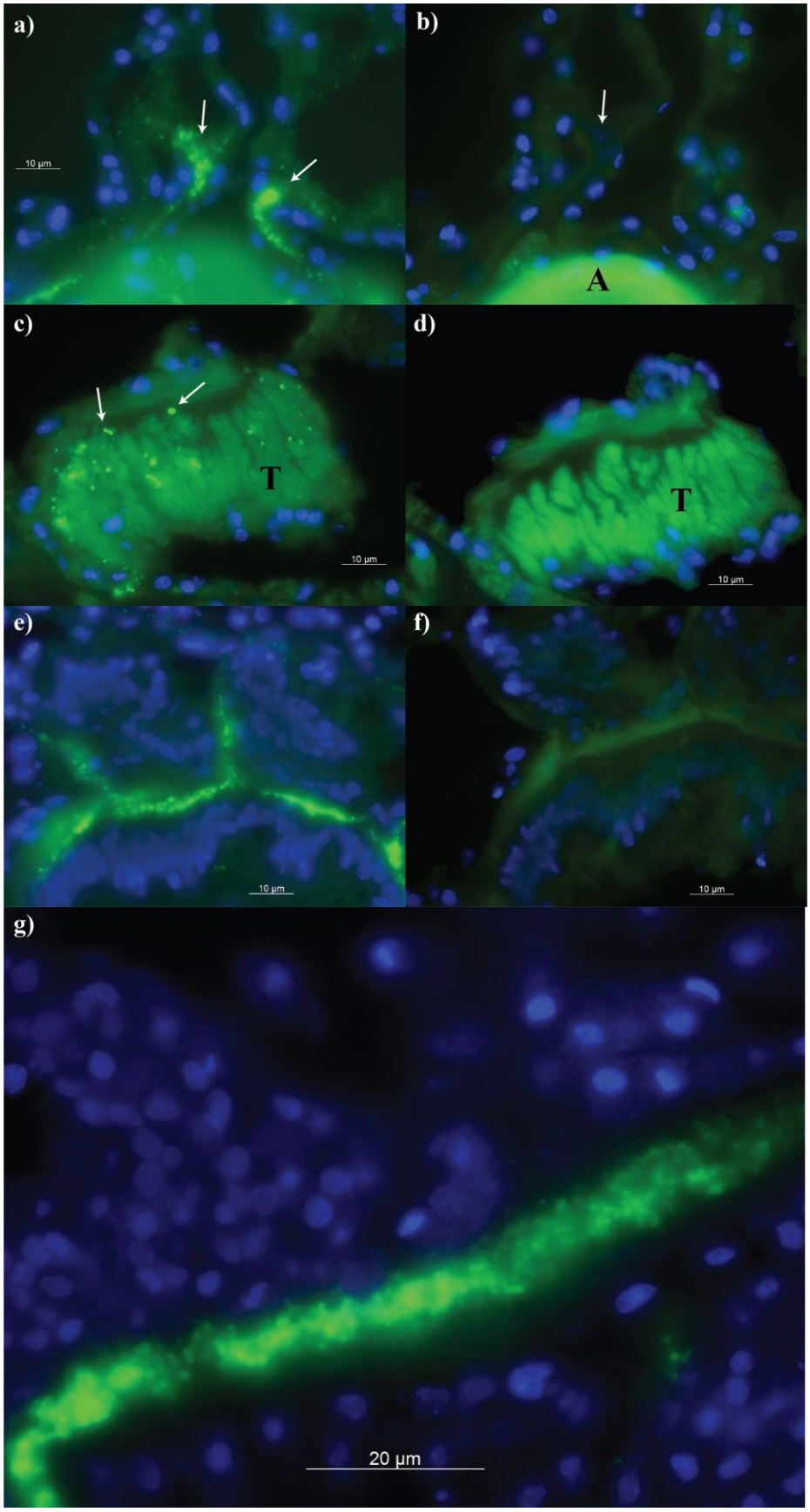


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Figure 6. Higher magnification of bacterial signals in the mesoglea and tentacles of *Callogorgia delta* using CARD-FISH with a general eubacterial probe (EUB I-III) and DAPI staining of DNA. Fluorescence in the emission range of DAPI is shown in blue while green shows emission in the range of the fluorophore ALEXA 488 used by both EUB I-III and control probes. Paired images compare adjacent sections where left images display sections hybridized with EUB I-III and show bacterial signals (a,c,e) while right images display sections hybridized with a negative control probe and only show autofluorescence (b,d,f). Transverse sections through the central axis (a,b) exhibit EUB I-III fluorescence (a, arrows) in the surrounding mesoglea and an overlap of DAPI (c, arrow). (a, arrow). Transverse sections through a polyp (c,d) show EUB I-III fluorescence (c, arrows) in the base of a tentacle. Transverse sections through the body wall of a polyp show granular (<1µm) EUB I-III fluorescence in the mesoglea (e,f,g). A = central axis, T = tentacle.