

The Pennsylvania State University
The Graduate School
Eberly College of Science

**COMPLEMENTARITY IN THE CORAL HOLOBIONT:
A GENOMIC ANALYSIS OF BACTERIAL ISOLATES OF *ORBICELLA FAVEOLATA* AND
SYMBIODINIUM SPP.**

A Thesis in
Biology

by
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Abstract

All holobionts, defined as a multicellular host and all of its associated microorganisms, rely on interactions between its members. Corals, which demonstrate a strong symbiosis with an algal partner, have a diverse holobiont that can be sequenced and analyzed that could reveal important roles of microbes that benefit its health. This microbial community has been predicted to be composed of nitrogen fixers, phototrophs, sulfur and phosphorus cyclers. However, the identity of these microbes responsible for these roles remain uncertain. In addition, there may be complementary roles that are unknown. Predicting these roles is challenging because... A suggested method to overcome this problem is sequencing the genomes of the microbes found in the coral holobiont. I hypothesize that bacterial members of the holobiont play an important role in coral biology via complementary metabolisms in nutrient cycling and aiding the coral in stress response. The complete metabolic capabilities of ten bacteria isolated from the coral holobiont were examined via the sequencing and annotation of the whole genome, followed by pangenomic analysis with 31 whole genomes of closely related strains of bacteria previously sequenced. The metabolisms identified—including denitrification, dimethylsulfoniopropionate biosynthesis and degradation, and cobalamin biosynthesis—potentially complement the coral holobiont via nutrient cycling. These findings demonstrate the importance of genomic sequencing to reveal the interactions between members of the holobiont.

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Chapter 1

Introduction

A holobiont is characterized by a multicellular host interdependently associated with bacteria, archaea, fungi, and a variety of other eukaryotic microorganisms¹. These symbionts can complement the holobiont through metabolic interactions that potentially benefit the host or other holobiont members. There are several well-established symbioses between animals and microorganisms, including nitrogen-fixing bacteria and cellulolytic protozoa within the gut of termites². Corals exhibit a mutualism with single cell algae from the genus *Symbiodinium* which provide photosynthate to the host³. As a source of carbon, the transferred photosynthate can be used by the coral for calcification, a great proportion of the coral's daily respiration needs, general coral growth, and reproduction⁴⁻⁶. This mutualism results in an ability to recycle nutrients, which helps corals thrive in oligotrophic waters. However, the coral-algal symbiosis may not be the only contributors to nutrient cycling in this holobiont.

Akin to other animal holobionts, a thriving and diverse community of bacteria, archaea, and viruses exist in corals⁷. The microbial community can exhibit species-specificity to the host⁸. These holobiont members can be transient, with their abundances susceptible to environmental stress, seasons, climate change, or geographical location⁹⁻¹⁵.

Despite potential fluctuations in community structure, there is evidence that some members of the microbiome are nearly ubiquitous and may represent a core collection of microbes^{16,17}. The core microbiome is centered around the idea of membership; the presence of the certain microbe in each microbiome across the community¹⁸. However, Shade &

1Handelsman suggest a more nuanced approach to defining the core microbiome which would include composition (similar abundance), phylogeny (shared lineages), persistence (shared over time), and connectivity (interactions within community). One example of a potential coral core microbe includes members of the genus *Endozoicomonas* and other *Oceanospirillae* (both are γ -proteobacteria), which are found in high abundance in association with corals and other marine organisms^{19–22}.

In addition to close associations between microorganisms and coral, the coral's endosymbiont may have its own symbioses. *Symbiodinium*-associated microbes isolated from cultures have been reported by Lawson et al (2017). The authors identified a core microbiome comprised of three operational taxonomic units (OTUs) that were present in 18 cultures of *Symbiodinium* species from 5 clades. The OTUs are from the genera of *Labrenzia*, *Muricauda*, and an unclassified *Chromatiaceae*. *Roseobacter*, a genus of the Rhodobacteraceae family, which also includes *Labrenzia*, have been found in association with *Symbiodinium* previously²³.

Discovery of the *Symbiodinium*-associated microbes occurred in part because of the difficulty with growing dinoflagellates in pure cultures free from bacteria²⁴. Use of antibiotics for purification of cultures had inconsistent results in that many cultures would die²⁵. This supports the idea that the bacteria provide a necessary component for the survival of the dinoflagellate²³. Also, these difficulties suggest that the microbes are associated with the *Symbiodinium* and not contaminants.

While the composition and diversity of the coral holobiont are becoming clearer, the metabolic interactions that potentially provide functionality and structure remain elusive.

Sequencing the genomes of microbial isolates from the coral holobiont may begin to provide answers to some of these questions. I hypothesize that these microbial isolate genomes will contain key nutrient cycling metabolic capabilities, such as for sulfur, nitrogen, and vitamins, and these capabilities potentially complement the healthy functioning of the coral holobiont.

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Chapter 2

Metabolic Pathways Identified in Bacterial Members of the Coral Holobiont

Abstract

The coral holobiont is a diverse and complex system. The coral symbiosis with *Symbiodinium* has been heavily studied to reveal its importance to the holobiont. The importance of the other members of the holobiont have yet to be determined. I hypothesize that bacterial members of the holobiont play an important role in coral biology via complementary metabolisms in nutrient cycling and aiding the coral in stress response. The complete metabolic capabilities of ten bacteria isolated from the coral holobiont were examined via the sequencing and annotation of the whole genome, followed by pangenomic analysis with 31 whole genomes of closely related strains of bacteria previously sequenced. The results show the presence of bacterial metabolisms for dimethylsulfoniopropionate biosynthesis and degradation, various nitrogen cycling strategies, cobalamin biosynthesis, and aerobic anoxygenic photosynthesis. Each of these metabolisms could complement coral biology via nutrient cycling or waste management. Discerning more of the metabolic capabilities of the coral microbiome could inform us of its importance for the healthy functioning of corals.

Introduction

Genomic sequencing of bacterial isolates from the coral holobiont allows for a characterization of the metabolic capabilities of bacteria that could provide benefits to the coral holobiont. Although a metagenomic approach can describe a microbiome's phylogenetic diversity and pieces of a microbe's metabolic capabilities, a whole genome affords a clearer depiction of specific microbe's metabolism. Metagenomic sequencing of corals has revealed categories of genes that provide clues about what metabolisms are present within the holobiont, including those for nitrogen fixation, cycling of fixed carbon and nitrogen, dimethylsulfoniopropionate (DMSP) degradation, and production of antibiotics¹⁻⁷. These metagenomic studies present targets for potentially complementary metabolisms in the sequenced genomes of coral holobiont isolated microbes, including those for this study: nitrogen, sulfur, and vitamins.

The oligotrophic waters associated with coral reefs particularly poor in usable forms of nitrogen, like ammonia. However, corals can attain much of their required nitrogen via heterotrophic feeding on zooplankton⁸. Additionally, corals can assimilate particulate matter with associated nitrogen⁹. Despite these heterotrophic methods, fulfilling the high demand for usable nitrogen could be supplemented through nitrogen fixation to compensate for seasonal variation in the availability of food⁷.

Another potential source of nitrogen for the coral holobiont is molecular nitrogen (N_2). However, N_2 needs to be converted via fixation to ammonia before it can be used. This can only be done by a select group of nitrogen fixers, also known as diazotrophs¹⁰. These are found in cyanobacteria, archaea and select lineages of bacteria. This essential ecosystem function is

performed by the nitrogenase protein complex, encoded by *nif* genes. These *nif* genes have been found in multiple shotgun metagenomic studies in corals, suggesting the presence of organisms capable of this function¹¹. However, the taxonomic resolution of the organisms carrying the *nif* genes is unclear.

Other nitrogen cycle pathways could play a role in the microbiome, such as ammonification, nitrification, and denitrification. Complex nitrogen cycling processes have been found in sponge holobiont¹². These nitrogen cycling pathways could provide additional forms of usable nitrogen to the coral or *Symbiodinium* or play a role in the health of the holobiont¹³.

Sulfur cycling could be another component of the functional role microbiome¹⁴. Dimethylsulfoniopropionate (DMSP) is a sulfur-containing compound, a major byproduct of coral reefs (Broadbent et al. 2002; Broadbent & Jones, 2004) and a player in the marine sulfur cycle. *Symbiodinium* spp. are considered heavy contributors to DMSP production which varies by species¹⁷. All classes of Proteobacteria have taxa capable of metabolizing DMSP, with *Roseobacter* clade members as the greatest contributors¹⁸. DMSP can be a source of sulfur and carbon for coral-associated bacteria¹⁴. This metabolism of DMSP results in methanethiol (MeSH) or dimethyl sulfide (DMS). MeSH provides a sulfur source and a precursor for methionine for bacteria, while DMS that reaches the atmosphere can be oxidized and form products that are effective cloud condensing nuclei¹⁹⁻²¹. Since DMSP is found in high concentrations in scleractinian corals²², bacteria with DMSP degradation and utilization gene pathways could benefit from associating with the coral.

Another potential role of the isolated bacteria is the production of vitamins. Cobalamin, also known as Vitamin B₁₂, is biosynthesized only by archaea and bacteria. However, it is a

required vitamin for many bacteria and eukaryotes due to several Vitamin B₁₂-dependent functions, including the synthesis of the essential amino acid methionine²³. Cobalamin has been shown to be a necessary growth factor in culture for a majority of species tested²⁴. Bacteria are the suggested source of cobalamin, which unveils a potential symbiotic relationship between bacteria and algae²⁵. Another study showed that *Symbiodinium* spp. may require vitamin B₁₂ for optimal growth²⁶. Cobalamin synthesis is another promising target for identification within the coral holobiont.

The functional importance of the bacterial members of the coral holobiont have yet to be determined. In this study I examine the metabolic capabilities of bacteria associated with the coral holobiont and whether these metabolisms complement that of the coral or the *Symbiodinium*. Based on previous metagenomic studies of corals, I hypothesize that bacteria play an important role in coral biology via complementary metabolisms in nitrogen, DMSP, and vitamins that benefit nutrient cycling and stress response. To discover the complete metabolic capabilities of the 10 bacteria isolates, I completed whole genome sequencing and annotation, followed by pangenomic analysis with 31 whole genomes of closely related strains of bacteria previously sequenced.

Materials and Methods

Sample Collection of Coral Isolates:

Two healthy colonies of *Orbicella faveolata* from Puerto Morelos, Quintana Roo, Mexico (colonies 452T and 452B) and four diseased colonies exhibiting signs of Yellow Band Disease (YBD, colonies 130DD, 130HD, TwinDD, and TwinHD). Diseased tissue regions exhibited tissue

discoloration. A hammer and chisel were used to collect a 3.5 cm² total area fragment from healthy and diseased tissue via SCUBA, which were then placed in sterile sampling bags. Healthy tissue samples from diseased corals, denoted by HD, were taken 10-15 cm away from YBD lesions. Healthy tissue samples from non-diseased coral colonies are denoted by HH. Diseased tissue samples directly from a YBD lesion is denoted by DD. Samples were handled with gloves and tools sterilized with 50% chlorine. The tissue and skeleton were homogenized using a mortar and pestle in 10 mL of 10X PBS. Bacteria were plated on marine agar and were incubated at 37 °C until microbial growth was observed. Single-cell colonies were re-plated on marine agar and incubated at 37 °C for 1-2 weeks. A total of 284 isolates were obtained from the plates, shown in Table 2-1.

Table 2-1: Isolates collected from *Orbicella faveolata* colonies

<i>Orbicella</i> Colony	Number of Isolates	Number of Isolates Sanger Sequenced (16S rRNA)
452 Top	43	23
452 Bottom	34	34
130 HD	37	6
130 DD	91	25

Sample Collection of Symbiodinium Isolates:

Monoclonal cultures of Symbiodinium were isolated from the Buffalo Undersea Reef Research Culture Center collection of northwestern Atlantic cnidarian hosts, maintained in liquid media (ASP8-A) and stored at 26°C with 80-120 μmol photons • m⁻² • s⁻¹ photosynthetically active radiation via Phillips fluorescent tubes.²⁷

Table 2-2 shows all the bacterial isolates collected from cultures of *Symbiodinium*. Culture samples for the bacterial strains 44A11, 44A6S, 44C2F were retrieved from Dr. Kim

Ritchie at Mote Marine Laboratory. Strains 44A11 and 44C2F were isolated from a *Symbiodinium* Clade B1 culture. Strain 44A6S was isolated from *Symbiodinium* Clade C1 culture.

Bacterial strains 064newd and B7newh were isolated from *Symbiodinium minutum* cultures from the LaJeunesse Lab at The Pennsylvania State University.

Table 2-2: Isolates collected from *Symbiodinium* cultures

Symbiodinium Culture Source	Clades Represented	Number of Isolates	Number of Isolates Sanger Sequenced (16S rRNA)
Kim Ritchie	A1; B1; C1; D2; F2	15	9
Todd LaJeunesse	A2/3/4; B1/2/19/25/26; C1; D1/2; E1; F1	46	36

SANGER Sequencing of Isolates:

Cultures were grown from glycerol stocks. A standard whole colony PCR was done on each isolate, using the 16S rRNA primers 27F and 1492R.

Strains and Cultivation of 10 Selected Isolates:

Ten bacterial isolates from the isolate collections described above, five from *Orbicella faveolata* and five from *Symbiodinium*, were selected for genome sequencing to provide broad phylogenetic diversity based on preliminary Sanger sequencing. The ten strains were cultured on sterile marine agar plates (made with BD Difco™ Dehydrated Culture Media: Marine Agar 2216), applied via streaking, at 26 °C for 48 hours. A single colony was collected and grown in 35 mL of sterile marine broth (made with BD Difco™ Dehydrated Culture Media: Marine Agar 2216) at 26 °C and shaken for 48+ hours.

DNA Extraction and DNA Library Prep:

DNA for whole genome sequencing was extracted from marine broth cultures using a modified CTAB DNA extraction protocol²⁸. A Qubit was used to quantify DNA. Samples were then prepped for DNA shearing using Covaris. The final volume was 52.5 µL and the insert size was 500 bp: 200 µg.

Genomic libraries were created following the TruSeq® Nano DNA Library Prep protocol. First, PCR of TruSeq® Library Prep samples was done, and products stored at -20 C. Running qPCR revealed a high number of strands longer than 1000 bp, so genome library samples were submitted to the PSU Core Facility for Pippin DNA size selection.

Sequencing and Assembly and Quality Assurance:

Samples were sequenced using an Illumina MiSeq. Libraries were first normalized to 4nM according to the MiSeq protocol. Genomes were assembled using default settings of the A5-miseq pipeline²⁹, (Table 2-3 and Table 2-4).

For quality assurance, each scaffold of each genome was blasted to identify phiX sequences and other contamination. Scaffolds that were discovered via local BLAST to be phiX (sequencing contamination) and the human herpes virus (laboratory contamination) were manually removed using nano in command line. Cleaned scaffold files free from these contaminants were then generated.

Table 2-3: A-5 MiSeq assembly stats of the bacterial isolates from *O. faveolata*

	130DD11	130DD58	452B1	452T9	452T16
Contigs	48	74	66	39	21
Scaffolds	37	59	46	27	17
Longest Scaffold (bp)	1368847	536953	980198	1608618	1933708
N50	449574	166141	540149	452110	1819317
Raw Reads	1981978	1898596	4890492	3995210	3317902
EC Reads	1960286	1884365	4853395	3942477	3295077
Reads passing EC (%)	98.91	99.25	99	99	99.31
Raw nt	594554722	568746052	1465946899	1197139073	993736243
EC nt	551511985	526274389	1374509312	1034484884	933805821
Nt passing EC (%)	92.76	92.53	94	86	93.97
Raw Coverage	102.53	116.14	284	222	234.71
Median Coverage	110	121	285	251	241
10 th percentile coverage	75	94	223	221	204
Bases >= Q40	5375079	4530129	4829927	4650296	3976671
Genome Size (bp)	5379100	4531230	4832105	4653730	3978551

Table 2-4: A-5 MiSeq assembly stats of the bacterial isolates from *Symbiodinium*

	064newd	44C2F	B7newh	44A11	44A6S
Contigs	51	69	13	324	88
Scaffolds	40	56	11	313	62
Longest Scaffold (bp)	747501	2557746	1857664	71301	428711
N50	344639	1860162	1371231	20150	180953
Raw Reads	4184504	7127796	2366186	5447558	2193126
EC Reads	4115535	7018121	2346080	5350384	2160611
Reads passing EC (%)	98.35	98.46	99.15	98.22	98.52
Raw nt	1256373613	2138845796	707661577	1635145629	656846780
EC nt	1047046222	1821796494	654566196	1299736818	559834901
Nt passing EC (%)	83.34	85.18	92.5	79.49	85.23
Raw Coverage	216.6	275.23	160.72	389.13	118.44
Median Coverage	268	327	169	512	125
10 th percentile coverage	121	229	137	35	104
Bases >= Q40	4831577	6589410	4070269	3266418	4723243
Genome Size (bp)	4833904	6619201	4072699	3340112	4726937

Pangenomic Analysis with Anvi'o:

A pangenomic analysis of 41 genomes, 10 isolates from this study and 31 genomes from NCBI (Table 2-5), was completed using the open-source analysis and visualization of 'omics tool Anvi'o from the Meren Lab in the Department of Medicine at the University of Chicago ³⁰.

Table 2-5: The 31 complete genomes from NCBI used for the pangenomic analysis

Genome ID	Genome Full Name	Genbank ID
A AR33	<i>Agromyces aureus</i> AR33	3289708
A CF514	<i>Agromyces</i> sp. CF514	3708668
A Leaf222	<i>Agromyces</i> sp. Leaf222	2561548
A NDB4Y10	<i>Agromyces</i> sp. NDB4Y10	3191588
B 2102	<i>Bacillus endophyticus</i> 2102	404708
B BDGP4	<i>Bacillus kochii</i> BDGP4	4970868
B DSM13796	<i>Bacillus endophyticus</i> DSM 13796	3702168
B Hbe603	<i>Bacillus endophyticus</i> Hbe603	3218508
B KCTC13922	<i>Bacillus endophyticus</i> KCTC 13922	3049478
L CECT4801	<i>Labrenzia aggregata</i> CECT 4801	2502908
L IAM12614	<i>Labrenzia aggregata</i> IAM 12614	206398
L LZB033	<i>Labrenzia aggregata</i> LZB033	3935448
L RMAR66	<i>Labrenzia aggregata</i> RMAR6-6	4128498
M CCHSB11	<i>Muricauda lutaonensis</i> CC-HSB-11	1711348
M DSM25030	<i>Muricauda zhangzhouensis</i> DSM 25030	3643638
M DSM26351	<i>Muricauda antarctica</i> DSM 26351	3700708
N EBB35	<i>Nitratireductor aquibiodomas</i> EBB 35.1	3366418
N NL21	<i>Nitratireductor aquibiodomas</i> NL21=JCM21793	988978
N RA22	<i>Nitratireductor aquibiodomas</i> RA22	382898
N RR328	<i>Nitratireductor basaltis</i> RR3-28	3986488
R CECT87515	<i>Ruegeria arenilitoris</i> CECT 8715	4846398
R F3871	<i>Ruegeria mobilis</i> F3871	3334368
R G1303	<i>Ruegeria mobilis</i> G1303	3334408
R M4323	<i>Ruegeria mobilis</i> M43-2.3	3334498
R S1923	<i>Ruegeria mobilis</i> S1923	3334668
S DSM22638	<i>Spongiibacterium flavum</i> DSM 22638	3779188
S DSM25885	<i>Spongiibacterium pacificum</i> DSM 25885	5213858
V OCN014	<i>Vibrio coralliilyticus</i> OCN014	1393258
V RE87	<i>Vibrio coralliilyticus</i> RE87	4990488
V S2043	<i>Vibrio coralliilyticus</i> S2043	1717798
V SNUTY1	<i>Vibrio coralliilyticus</i> SNUTY-1	4319058

Genome Annotation and Bioinformatics Analysis:

Genomes were annotated using the fully-automated Rapid Annotation using Subsystem Technology (RAST) version 2.0³¹ tool on the contamination-free genomes; (parameters: Classic RAST, FIGfam version Release 70, errors automatically fixed, frameshifts fixed, metabolic models built, gaps backfilled, debug turned off, verbose level at 0, and replication disabled).

The SEED Viewer³² was used to view and compare subsystems, subsystem features, and analyze KEGG metabolic pathways of the isolates and other published genomes. Subsystems are collections of functional genes that are related via metabolic pathway, protein complex or protein class. Gene counts of subsystems were used to supplement pangenomic analysis.

Results

Genome sequencing and assembly:

Whole-genome sequencing of ten bacteria isolates from *Orbicella faveolata* and *Symbiodinium* spp. was utilized to reveal the metabolic capabilities of the coral holobiont.

The assemblies have averages of 69.55 scaffolds, an N50 over 700,000, 146.82 10th percentile coverage, and 89% of nucleotides pass EC (Tables 2-3 and 2-4). One outlier in quality was, isolate 44A11, which had a much higher number of scaffolds, and much lower N50 and 10th percentile coverage. The quality was deemed sufficiently high for the analyses.

The taxonomic classification of each bacterial isolate was determined by the full-length 16S rRNA gene from the genome. The classification of each isolate can be seen in Table 2-5.

Table 2-5: 16S rRNA classification of bacterial isolates from the coral holobiont

Isolate	Host	16S rRNA ID from Genome	Genome Size (Mbp)	% GC	Label
452T9	<i>O. faveolata</i>	<i>Ruegeria arenilitoris</i>	4.6	58.0	★
130DD11	<i>O. faveolata</i>	<i>Bacillus endophyticus</i>	5.3	36.4	◆
452T16	<i>O. faveolata</i>	<i>Muricauda pacifica</i>	3.9	39.1	●
452B1	<i>O. faveolata</i>	<i>Bacillus kochii</i>	4.8	36.9	▲
130DD58	<i>O. faveolata</i>	<i>Vibrio coralliilyticus</i>	4.5	45.0	■
44A6S	<i>Symbiodinium C1</i>	<i>Ruegeria mobilis</i>	4.7	59.0	★
44C2F	<i>Symbiodinium B1</i>	<i>Labrenzia aggregata</i>	6.6	59.1	◆
064newd	<i>Symbiodinium B1</i>	<i>Nitratireductor aquibiodomas</i>	4.8	61.5	●
B7newh	<i>Symbiodinium B1</i>	<i>Spongiibacterium flavum</i>	4.0	42.5	▲
44A11	<i>Symbiodinium C1</i>	<i>Agromyces indicus</i>	3.3	71.9	■

The pangenome of *O. faveolata* and *Symbiodinium* isolate genomes and each genus:

The pangenomic analysis of the 10 isolate genomes and 31 closely related strain genomes (a total of 150,433 genes) resulted in 24,565 gene clusters. Genes are clustered by Euclidean distance and Ward linkage and shown as presence/absence (Figure 2.1). Singletons and doubletons were removed from the pangenome.

Only 48 genes are shared across all 41 genomes. The COG annotation done by Anvi'o revealed most of the genes to be ribosomal genes.

The pangenomic analysis supports the 16S identification (table 2-5) of 9 of the isolate genomes. The 10th isolate genome, *Vibrio sp. 130DD58*, has a large gap in the genome compared to the NCBI library *V. coralliilyticus* genomes, which suggests that the isolate is a different species.

In addition to the pangenome of 41 genomes, pangenomic analysis was done separately for each of the 7 genera so as to directly compare the isolate genomes with their most closely related, previously sequenced genomes. Table 2-6 shows the number of genes and gene clusters in the pangenome, the number of genes found in all genomes, and the number of singletons found in the coral holobiont isolates.

Table 2-6: Basic stats of Pangenome for each genus. Singletons are shown as number of genes with known COG function within total number of singleton genes found. Isolates listed are those sequenced for this study, while genomes include other genomes that were used for comparative purposes.

Genus	Isolate	Genomes	Total Genes of Genus	Total Gene Clusters of Genus	Genes found in All Genomes	Singletons of Isolates (COG/Total)
<i>Agromyces</i>	44A11	5	18,434	6,508	1,214	189/368
<i>Bacillus</i>	130DD11	7	35,824	9,624	1,173	208/433
-	452B1	-	-	-	-	197/587
<i>Labrenzia</i>	44C2F	5	30,568	8,275	2,511	234/341
<i>Muricauda</i>	452T16	4	14,029	6,922	1,187	737/1371
<i>Nitratireductor</i>	064newd	5	22,181	7,958	1,088	307/702
<i>Ruegeria</i>	452T9	7	31,112	8,671	1,464	859/1219
-	44A6S	-	-	-	-	255/528
<i>Spongibacterium</i>	B7newh	3	11,041	5,366	1,357	351/688
<i>Vibrio</i>	130DD58	5	25,500	7,621	1,700	953/1485

RAST subsystems of the pangenome:

In addition to the pangenomic analysis, RAST was used to give a higher resolution than COG of the functional annotation of gene clusters. With RAST annotation across all 41 genomes, we find patterns within genera of core functional genes (genes found in each genome within a genus) that include nitrogen and sulfur metabolism, as well as genome-unique genes in photosynthesis, and *Bacillus* genomes that dominate the distribution of dormancy and sporulation genes (Figure 2-2).

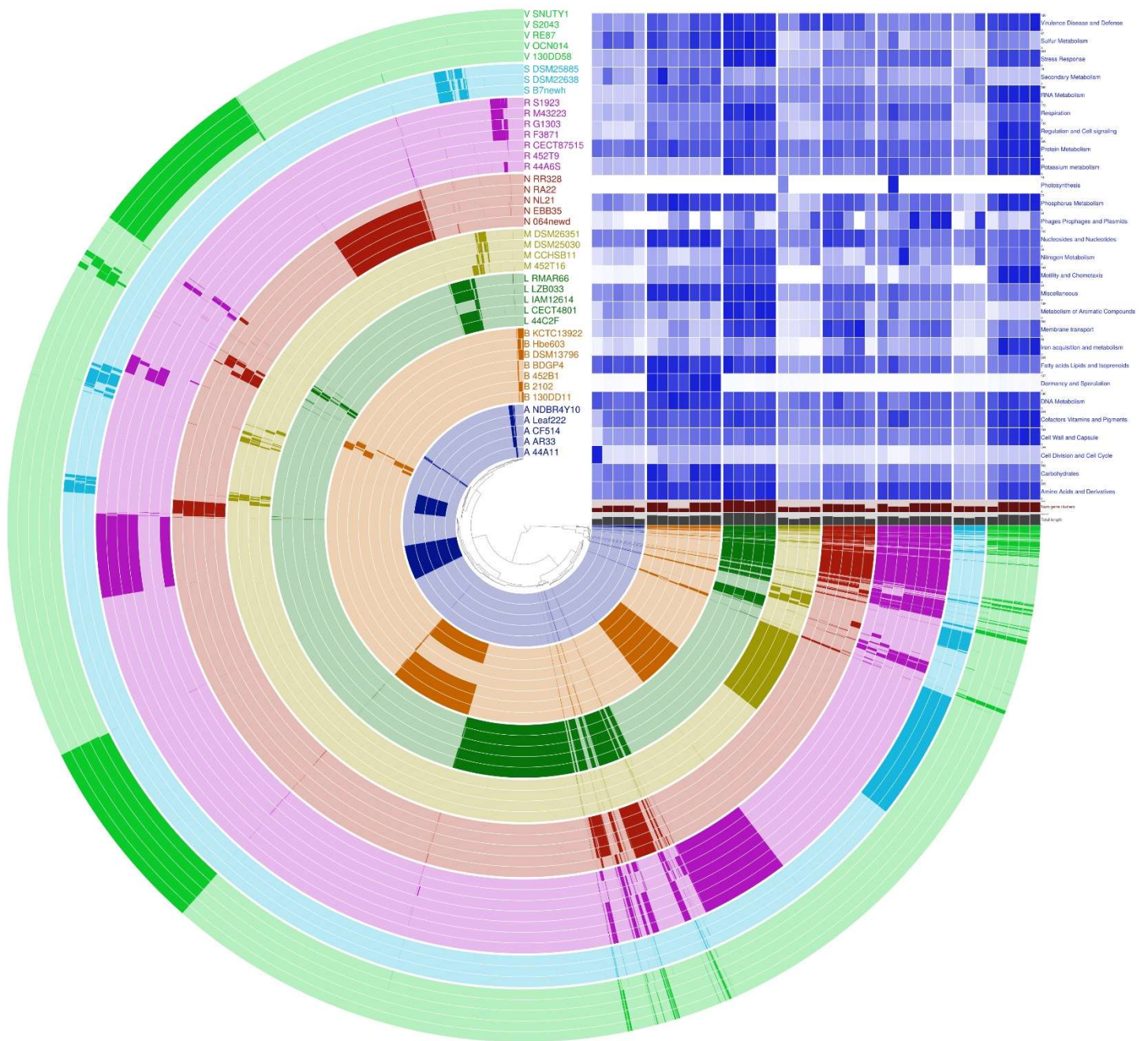


Figure 2-1: The pangenome of coral holobiont isolates and closely related strains

24,565 gene clusters were found across 41 isolate genomes. The isolates sequenced in this study are found in the innermost circle per genus (A 44A11, B130DD11, B 452B1, L 44C2F, M 452T16, N 064newd, R 44A6S, R 452T9, S B7newh, and V 130DD58). Gene clusters group together based on co-occurrence in genomes. The genomes are organized by shared gene clusters, calculated with Euclidean distance and Ward linkage. A darker shade represents occurrence of a gene cluster in that genome. Genomes are colored based on genus. A heatmap of relative gene distribution in RAST subsystem categories is shown in the top right for each genome (white=0, dark blue=max).

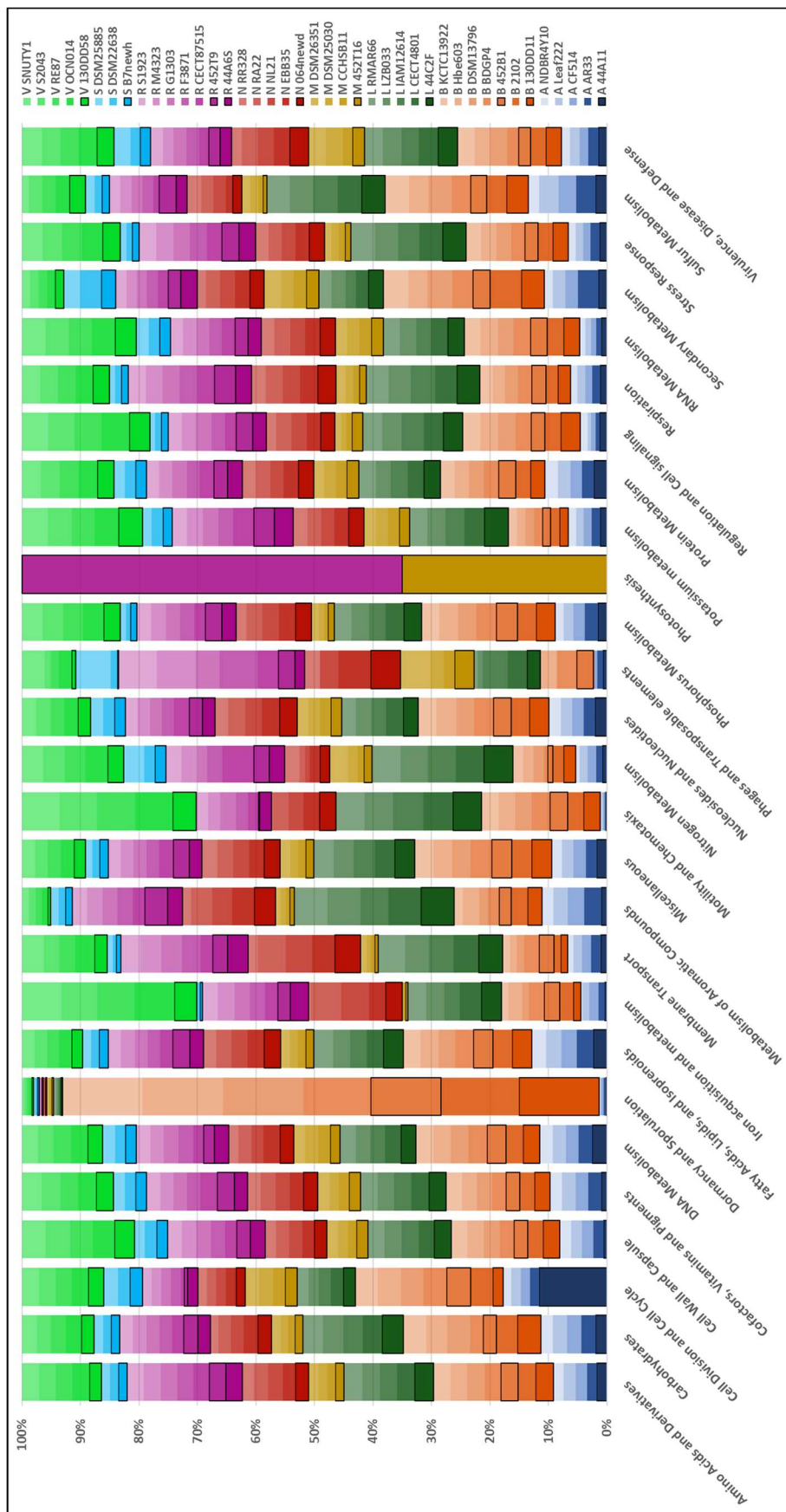


Figure 2-2: RAST subsystem category gene distribution of the pangenome
 Relative gene distribution into RAST subsystem category is shown for each of the 41 isolate genomes (A). Genome names are first letter of genus followed by strain number, and genera are grouped based by color.

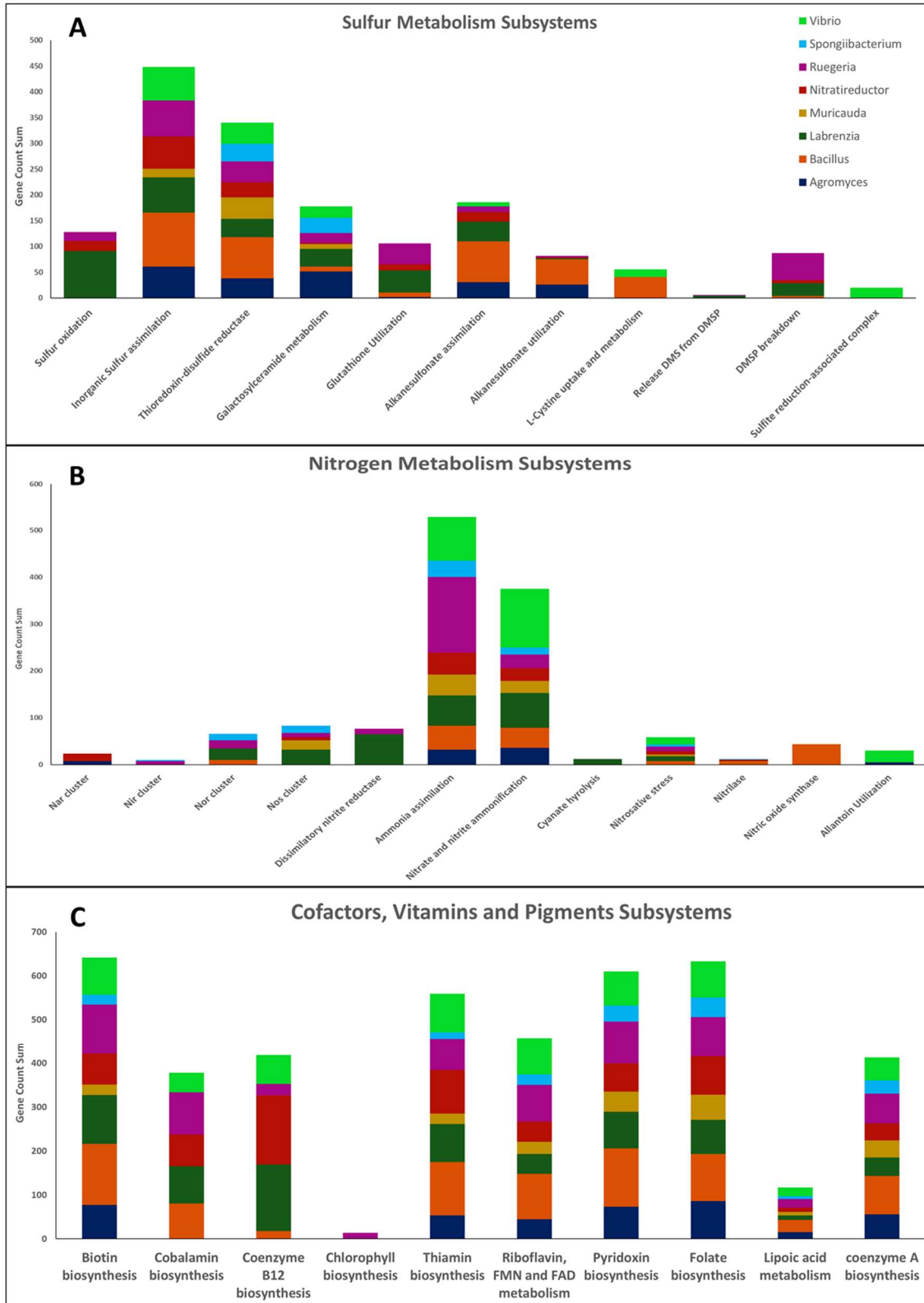


Figure 2-3: Pangenome gene distribution in specific subsystems

Specific subsystems gene distribution for sulfur metabolism (A), nitrogen metabolism (B), and cofactors, vitamins and pigments (C) show gene count sum for each genus.

Subsystems of sulfur metabolism in pangenome

The subsystems of inorganic sulfur assimilation, thioredoxin-disulfide reductase, galactosylceramide metabolism, and alkanesulfonate assimilation have the most number of genes of the sulfur metabolism subsystems and are found in each of the 8 genera (with the exception of inorganic sulfur and alkanesulfonate assimilation in *Songiibacterium*). Sulfur oxidation genes were found in the genomes of *N. basaltis* RR328, *R. arenilitoris* 452T9, and each strain of *Labrenzia*. Each *Labrenzia* strain and *R. arenilitoris* 452T9 contained genes that release dimethyl sulfide (DMS) from dimethylsulfoniopropionate (DMSP). In addition, four genera, *Bacillus*, *Labrenzia*, *Nitratireductor* and *Ruegeria* have multiple genes for DMSP breakdown. *Vibrio* alone had genes in the sulfite-reduction associated complex subsystem.

Model of DMSP breakdown and biosynthesis in isolates of the coral holobiont

Genes involving the breakdown and of biosynthesis of DMSP were found in multiple genomes. The capability for two DMSP degradation pathways were identified: cleavage and demethylation. The capability of cleavage was identified in two isolates, *L. aggregata* 44C2F and *R. arenilitoris* 452T9, which is conferred by two different genes. The *dddL* gene was found in all five *Labrenzia* genomes, and the *dddD* gene was found only in *R. arenilitoris* 452T9 (Figure 2-3). The enzyme DddL splits DMSP into dimethyl sulfide (DMS) and acrylate, while the enzyme DddD results in DMS and 3-hydroxypropionate.

An alternative to DMSP cleavage to DMS is the demethylation and demethiolation of DMSP. This pathway contains the genes *dmdA*, *dmdB*, *dmdC*, and *dmdD*. The *dmdABC* genes were found in the isolates *R. arenilitoris* 452T9 and *R. mobilis* 44A6S, in the 5 other *Ruegeria*

genomes, in four of the *Nitratireductor* genomes (except in *N. aquibiodomas 064newd*), and in two *Bacillus* genomes (including *B. kochii 452B1*, Figure 2-4).

Additionally, *L. aggregata 44C2F* and all the *Labrenzia* genomes have the gene *dsyB*, which is involved in the biosynthesis of DMSP (Figure 2-4). The *dsyB* gene in *L. aggregata 44C2F* had a 1008/1023 bp and 338/340 AA match to the *dsyB* gene of *L. aggregata LZB033*³³.

Within the coral holobiont this *dsyB* gene indicates that the isolate *L. aggregata 44C2F* contributes with *Symbiodinium* and corals to the production of DMSP, which can then be catabolized via the DMSP cleavage pathway by *L. aggregata 44C2F* or *R. arenilitoris 452T9*, or the DMSP is demethylated to acetaldehyde and methanethiol by *R. arenilitoris 452T9* or *R. mobilis 44A6S* (Figure 2-4).

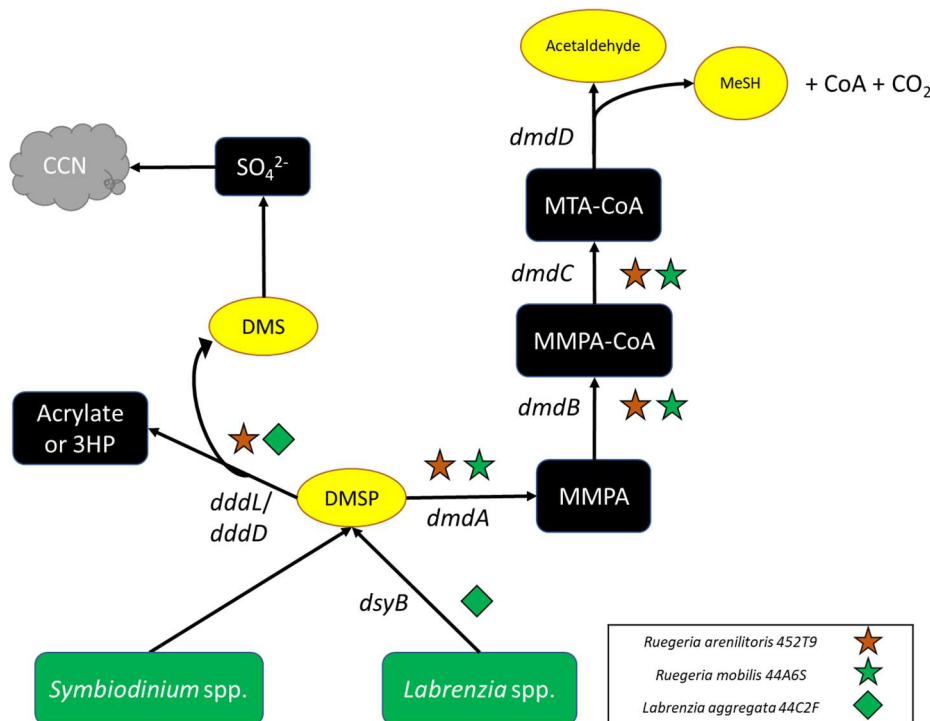


Figure 2-4 | **DMSP biosynthesis, cleavage, and demethylation by isolates.** There are two sources of DMSP in the coral holobiont, *Symbiodinium spp.* and *Labrenzia spp.* Both *Ruegeria* isolates are able to demethylate DMSP. The cleavage of DMS by *R. arenilitoris 452T9* and *L. aggregata 44C2F* leads to sulfur aerosols in the atmosphere which can act as cloud condensing nuclei (CCN).

Subsystems of nitrogen metabolism in the pangenome

Genes involved in denitrification were identified in multiple genomes. All genomes overlapped in the presence of specific genes within the denitrifying reductase gene cluster (Figure 2-3B), which contains genes in four main clusters that include nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*), and nitrous oxide reductase (*nos*). Only two genera, *Nitratireductor* and *Agromyces*, contained *nar* genes, while *nos* genes were found in *Labrenzia*, *Muricauda*, *Nitratireductor*, *Ruegeria*, and *Spongiibacterium* genomes. It appears that denitrification is a common capability for the sequenced genera, suggesting that other bacteria found within the coral holobiont closely related to the strains of this study may also utilize denitrification.

In contrast to denitrification, *Labrenzia* strains possess dissimilatory nitrite reduction genes, which reduce nitrite into released nitric oxide instead of assimilating it. The *Labrenzia* genomes contained the dissimilatory nitrite reductase genes for cytochrome cd1 nitrite reductase (EC 1.7.2.1), heme d1 biosynthesis proteins (*nirDFGHJL*), nitrite reductase associated c-type cytochrome (*nirN*), cytochrome c55X precursor (*nirC*), and uroporphyrinogen-III methyltransferase (*nirE*). Genes for dissimilatory nitrite reduction were also found in the non-coral-associated isolate *R. arenilitoris* CECT 87515, but not the coral-associated isolate *R. arenilitoris* 452T9.

The isolate *N. aquibiodomas* 064newd was unique in having nitrate reductase (*narGHIJKR*). This was a core gene cluster for *Nitratireductor*. Despite other *Agromyces* containing *nar* genes, *A. indicus* 44A11 does not have them.

Nitrite reduction genes were only found in the genomes of one *Spongiibacterium* and four *Ruegeria*, including *R. mobilis 44A6S* has copper-containing nitrite reductase (*nirK*) and nitrite reductase accessory protein (*nirN*).

Three isolates (*R. mobilis 44A6S*, *L. aggregata 44C2F*, and *S. flavum B7newh*) have the nitric oxide reductase genes (*norBCDQ*). In addition, isolates *L. aggregata 44C2F* and *S. flavum B7newh* have the nitrous oxide reductase genes (*nosDFLYZ*).

Subsystems that were common across all genera included ammonia assimilation, nitrate and nitrite ammonification, and nitrosative stress. The nitrosative stress genes protect bacteria from cell damage created reactive nitrogen species acting with reactive oxygen species, and these reactive species are continuously produced in plants³⁴. Some subsystems were unique to a single or two genera including cyanate hydrolysis (*Labrenzia*), nitric oxide synthase (*Bacillus*), and allantoin utilization (*Vibrio* and *Agromyces*).

Model of nitrogen cycling by isolates of the coral holobiont

Genes involved in denitrification, nitrate reduction to ammonia, and urea catabolism were found in several isolates, which presents several pathways for nitrogen cycling within the coral holobiont (Figure 2-5). Eight isolates may reduce nitrate to ammonia since they had assimilatory nitrate reductase (EC 1.7.99.4) and nitrite reductase (EC 1.7.1.4) that reduce nitrates to ammonia. *L. aggregata 44C2F* has the ability to reduce nitrites to ammonia but via a dissimilatory pathway.

Genes for urease catabolism were found in *R. arenilitoris 452T9*, *B. endophyticus 130DD11*, *R. mobilis 44A6S*, and *L. aggregata 44C2F*. Urease allows these microbes to utilize possible coral urea to attain ammonia.

Six isolates contained genes within the denitrifying reductase gene cluster subsystem.

Through a complementary pathway of denitrification, the nitrates are reduced to dinitrogen gas that is potentially released from the coral holobiont or makes it available to diazotrophs that can fix the nitrogen into ammonia. Although, no genes for nitrogen fixation, specifically *nif* genes, were found in any of the isolates.

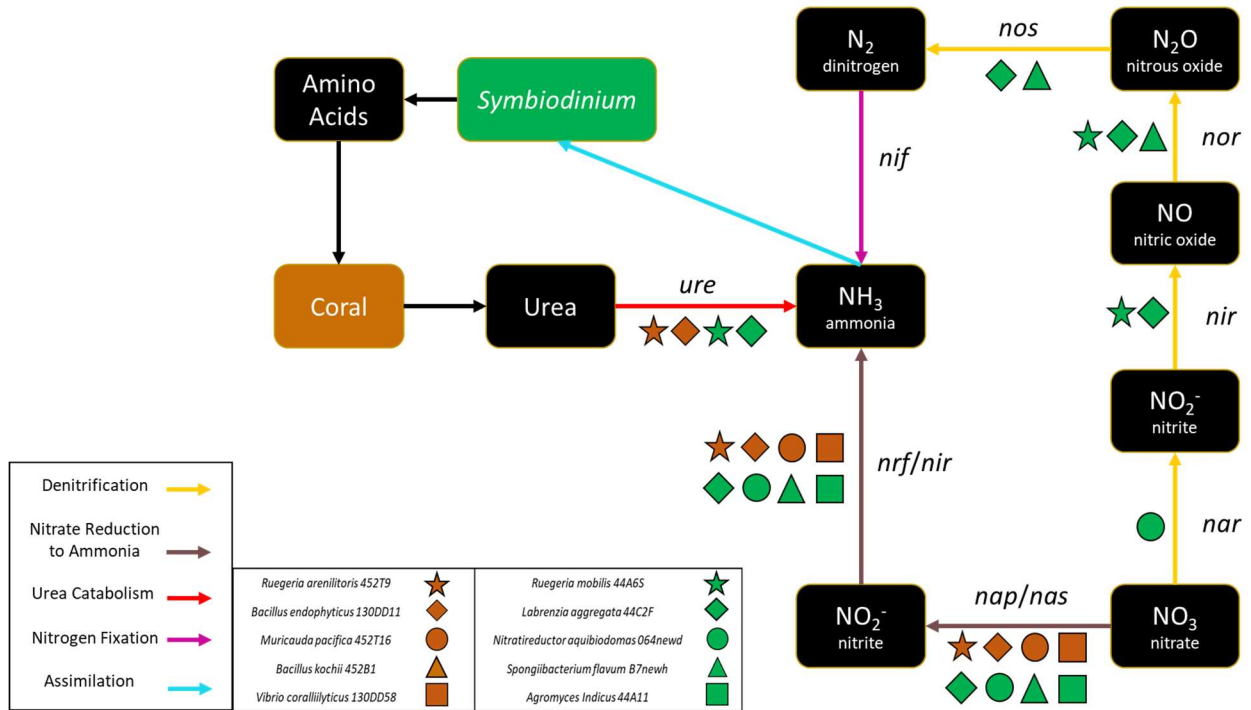


Figure 2-5 | **Nitrogen cycling pathways in bacterial isolates genomes, host, coral, and Symbiodinium.** The metabolic pathways, nitrate reduction to ammonia, and urea catabolism recycle nitrogen within the holobiont. Denitrification results in loss of nitrogen from the holobiont. The gene(s) necessary for a pathway are next to the arrow. Symbols indicate the genome of origin for each gene. Orange symbols indicate bacteria isolated from *O. faveolata* and green symbols indicate bacteria isolated from *Symbiodinium* spp.

Subsystems of cofactors, vitamins and pigments

The distribution of genes for vitamins is fairly uniform across genera in that the number of genes per vitamin per genome was nearly equal for several vitamins (Figure 2-3C). Genes involved in the biosynthesis or metabolism of biotin, thiamin, riboflavin and FAD, pyridoxin, folate, lipoic acid and coenzyme A were found across all 8 genera. The genes involved in

biosynthesis of cobalamin and coenzyme 12 were present in five genera: *Bacillus*, *Labrenzia*, *Nitratireductor*, *Ruegeria*, and *Vibrio*. One genome of *Ruegeria*, *R. arenilitoris* 452T9, contained the bacteriochlorophyll gene *bchC* (as well as 12 other genes) revealing that it had the capability for chlorophyll biosynthesis.

A potential anoxygenic anaerobic phototroph

The genome of isolate *R. arenilitoris* 452T9 contained genes within three subsystems of photosynthesis: bacterial light-harvesting complexes, photosystem II, and chlorophyll biosynthesis (Figure 2-6). The presence of the genes indicates the potential for aerobic anoxygenic photosynthesis, a pathway ancestral to oxygenic photosynthesis widespread in α -proteobacteria. The presence of genes *chlEAE* indicates the isolate is most likely using the aerobic rather than the anaerobic pathway.

The anaerobic anoxygenic photosynthesis (AAnP) marker gene *pufM* of *R. arenilitoris* 452T9 was compared to another anoxygenic photosynthesizing α -proteobacterium (Figure 2-7). The *pufM* genes of *R. arenilitoris* 452T9 are most similar to other Rhodobacteraceae, specifically members of the *Roseovarius* and *Roseivivax* genera.

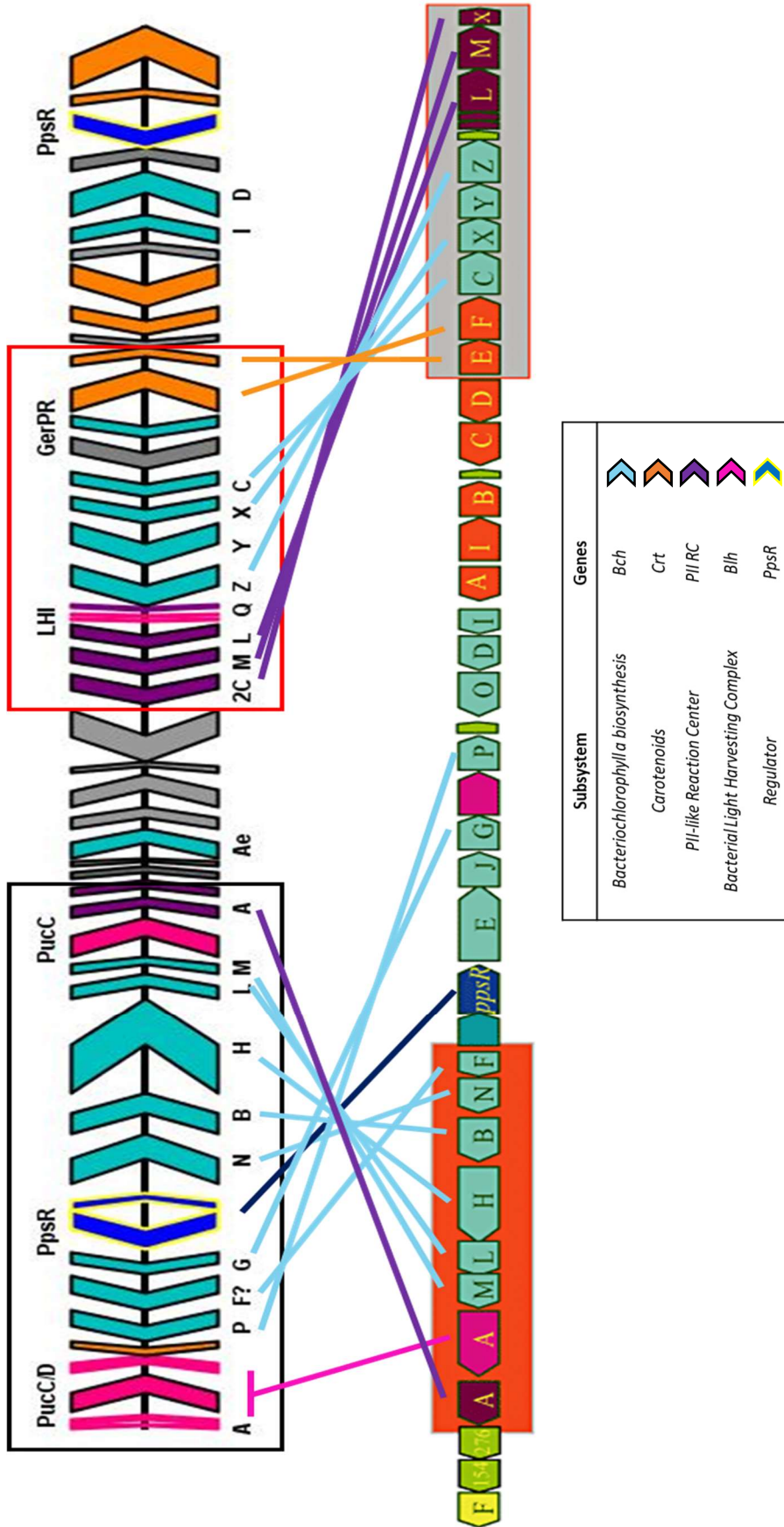


Figure 2-6 | **Aerobic Anoxygenic Photosynthesis Pathway of Isolate *Ruegeria arenilitoris* 452T9.** The isolate's AAnP pathway has complete gene sets to synthesize the necessary enzymes and proteins for bacteriochlorophyll (light blue), carotenoids (orange), photosystem II-like reaction center (purple), and bacterial light harvesting complex (pink). Letters correspond to genes within the operon of that subsystem. The isolate is compared to the α -proteobacterium *Rhodospirillum rubrum*, showing that the genetic repertoire and order of genes are similar.

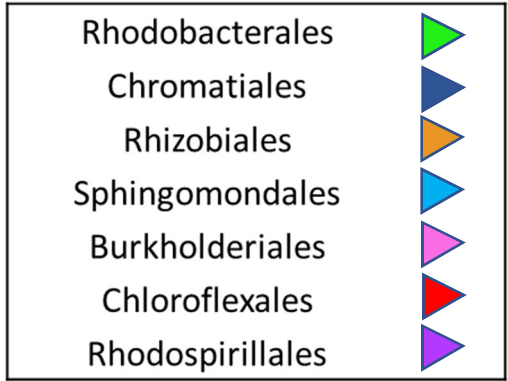
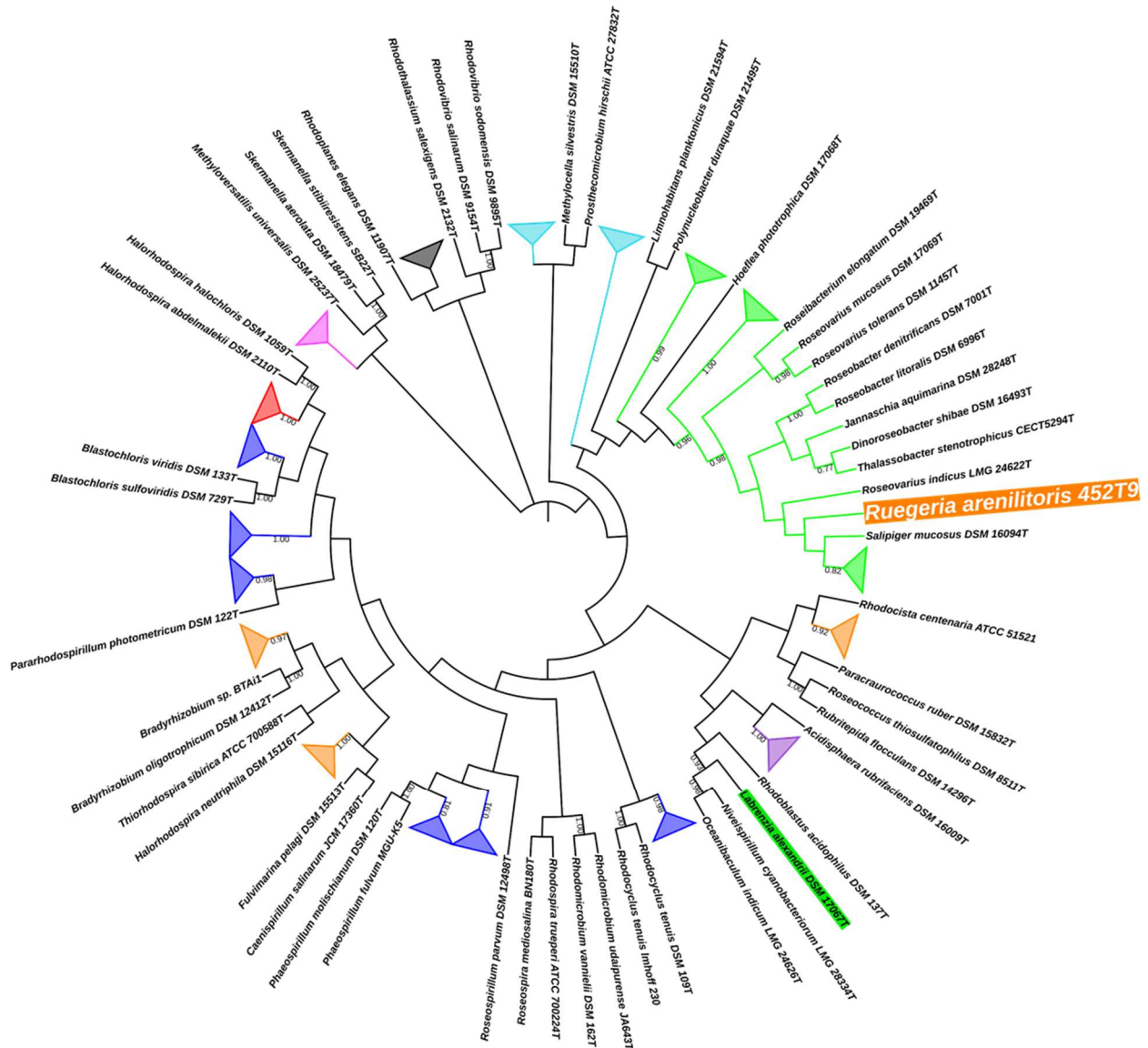


Figure 2-7 | **Phylogeny of marker gene *pufM* in anoxygenic photosynthesizing α -proteobacteria.** The neighboring joining tree (1000 BS) of 197 sequences of the *pufM* gene. Bootstraps values on branches. Evolutionary distance computed using Maximum composite likelihood method. Isolate *R. arenilitoris* 452T9 clusters (orange box) within Rhodobacterales (green).

Discussion

This study used whole genome sequencing of 10 bacteria strains isolated from the coral *Orbicella faveolata* and from cultures of *Symbiodinium* to address two questions: (1) what are the metabolic capabilities of bacteria within the coral holobiont and (2) do these metabolisms potentially complement coral biology? What follows is a discussion on the most interesting metabolisms found in the bacterial genomes that also potentially play an important role to the coral holobiont. This includes the metabolism of DMSP and its role in sulfur cycling, a potential phototrophic isolate, several pathways for cycling nitrogen, and a potential source of vitamin B₁₂.

DMSP biosynthesis, cleavage, and demethylation role in the holobiont

The cleavage of DMSP is an integral part of the global sulfur cycle by releasing DMS, which can enter the atmosphere and transform into light reflecting sulfate aerosols. Once in the atmosphere, DMS can also transform into cloud condensing nuclei, thus contributing to cloud formation which in turn can have a local climate impact by reducing light availability and cooling temperature^{35,36}. These DMS-derived sulfur dioxide chemicals can precipitate while over land and introduce sulfur to the terrestrial environment, thus linking the terrestrial sulfur cycle with the ocean sulfur cycle³⁷.

Two isolates, *R. arenilitoris* 452T9 and *L. aggregata* 44C2F, are capable of cleaving DMSP. The *dddL* gene found in *L. aggregata* 44C2F encodes for the enzyme DddL, the first authenticated DMSP lyase^{38,39}. The *dddL* gene was found in the all *Labrenzia* genomes analyzed.

The enzyme DddL cleaves DMS from DMSP with acrylate as the product. The *dddD* gene found in *R. arenilitoris 452T9* differs instead produces DMS and 3-hydroxypropionate.

Demethylation is another pathway for metabolism of DMSP by bacteria. Two isolates, *R. arenilitoris 452T9* and *R. mobilis 44A6S*, contain the *dmdABCD* genes that comprise this pathway⁴⁰. The demethylation of DMSP provides a source of sulfur via methanethiol (MeSH) and carbon via acetaldehyde. The high availability of DMSP in the coral holobiont may attract DMSP degraders like the *Ruegeria* spp. and thus DMSP may be involved in structuring the holobiont community.

If DMSP-lysing and DMSP-demethylating bacteria are common among coral holobionts, they may play a large role in the global sulfur cycle. All strains of *Labrenzia* and *Ruegeria* contained DMSP-demethylating genes, as well as a few strains of *Bacillus* and *Nitratireductor*, suggesting this may be a common feature for coral-associated bacteria from these genera which would use DMSP as a sulfur and carbon source.

In addition to the potential role of DMSP-cleavage, DMSP itself has been suggested to act as an osmoprotectant, antioxidant, antimicrobial, cryoprotectant, signal molecule, and confirmed as a source of sulfur and carbon to marine bacteria^{21,41-44}.

DMSP and DMS has been recorded in higher quantities surrounding coral reefs than off reef, suggesting coral reefs are a major contributor to DMSP and thus play a role in the marine sulfur cycle^{15,16}.

Recent research has identified a bacterial method for DMSP biosynthesis via the expression of the *dsyB* gene³³. The *dsyB* gene is hypothesized to work similar to the

transamination of methionine pathway. Curson found that *dsyB* conferred DMSP production via methylation of 4-methylthio-2-hydroxybutyrate (MHTB) to 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB). This methylation of MHTB (MHM) is most likely the rate-limiting step in DMSP biosynthesis^{45,46}. Curson et al. found *dsyB* conferred DMSP production with no input of precursor compounds, but there was increased production of DMSP when Methionine or intermediates of the transamination pathway are supplemented.

Due to the *dsyB* gene found in *L. aggregata 44C2F*, *Labrenzia* could be providing other bacteria with DMSP and its metabolites and thus potentially influencing the structure of the community. Or *Labrenzia* could be providing the coral holobiont with DMSP as a tool for protection against salinity fluctuations or as an antioxidant. DMSP concentration in scleractinian corals is temporally variable and has been tied to certain stressors⁴⁷⁻⁴⁹. It has yet to be shown if bacteria-produced DMSP influences overall DMSP production in the coral holobiont or if it links to certain stressors.

It was previously thought that only the *Symbiodinium*, corals, and other algae produced DMSP in coral reefs, but new evidence indicates that α -proteobacteria like *Labrenzia* spp. need to be considered in that calculation⁵⁰. It is possible that α -proteobacteria contribute to high concentration of DMSP in coral reefs.

AAnP role in the holobiont

AAnP is dissimilar to oxygenic photosynthesis in several ways, including use of bacteriochlorophyll, a lack of carbon fixation, and no production of oxygen. However, the AAnP

pathway provides ATP to the organism via a proton gradient similar to oxygenic photosynthesis. Most bacteria that perform AAnP do not have RuBisCO and thus don't fix carbon and are not autotrophs. These bacteria utilize other sources of carbon, which in the coral holobiont there is plenty of reduced carbon available. Therefore, an AAnP organism can utilize light for energy and survive while attaining carbon from other sources.

Light availability is limited since *Symbiodinium* dominates the coral holobiont and absorbs most of the available light. However, endolithic algae, organisms that live in the coral skeleton are able to compete with *Symbiodinium* with a slightly shifted light absorption spectra for their chlorophyll A. Utilizing wavelengths of light unutilized by *Symbiodinium* could be one method of making a living in the coral holobiont.

AAnP bacteria may use a similar strategy. AAnP uses bacteriochlorophyll which absorbs light in the wavelength range of 700-850nm most efficiently which is longer than light collected by chlorophyll. Bacteriochlorophyll uses bacterial light-harvesting complexes and the reaction centers of which specialize in absorbing ~750 nm, 800-850 nm, and 870 nm. It is feasible that AAnP bacteria can utilize the near-infrared light to survive in the coral holobiont. There is enough near-infrared radiance available in corals to support anoxygenic phototrophic bacteria⁵¹. Thus, *Ruegeria* may be performing AAnP by specializing on under-utilized wavelengths of light.

The results of this study show that the isolate *R. arenilitoris* 452T9 has the genetic potential for aerobic anoxygenic photosynthesis (Figure 2-6) despite *Ruegeria* as a genus being previously described as non-photosynthetic and missing bacteriochlorophyll A⁵². These results

challenge that description of the genus. A complete collection of operons for bacterial light harvesting complex, photosystem II-like reaction center, and bacteriochlorophyll are present in *Ruegeria arenilitoris* 452T9. The arrangement of the present genes closely resembles other α -proteobacteria AAnP operons (Fig. 2-6). The operon arrangements for *bchCXYZ + pufLM + crtEF* and *bchBFHLMN + blhA + puhA* are conserved in Proteobacteria. The order of these two operons in *R. arenilitoris* 452T9 also resembles the α -proteobacteria *Rhodobacter sphaeroides*, but there are few differences within one operon. This supports the idea that *R. arenilitoris* 452T9 has an α -proteobacteria-like AAnP and evolved its own pathway rather than acquiring it via horizontal gene transfer.

In addition, a phylogeny based on the anoxygenic photosynthesis marker gene *pufM*, indicates *R. arenilitoris* 452T9 groups with other α -proteobacteria, and more specifically with other Rhodobacterales (Fig. 2-7). The coral holobiont potentially harbors AAnP bacteria, including *R. arenilitoris* 452T9, that can take advantage of the available infrared light in the coral.

Nitrogen metabolism role in the holobiont

Since ammonia is a form of nitrogen that *Symbiodinium* prefers to use, ammonifying bacteria could be an additional source to the coral host. Eight isolates contained the nitrate and nitrite reductases to form and assimilate ammonia. Alternatively, the *L. aggregata* 44C2F genome utilizes the dissimilatory nitrite reductase which releases ammonia. This potentially provides ammonia that can be assimilated by *Symbiodinium* or other holobiont microbes which

is needed for forming chloroplasts and amino acids. Although, *Symbiodinium* may also obtain ammonia through the recycling of coral urea. This waste product can be broken down by urease to form ammonia. The urease enzyme was found in four isolates, and thus indicates a potential function for bacteria within the holobiont.

In contrast to recycling nitrogen, nitrifying and denitrifying bacteria could compete for available nitrogen within the holobiont. Nitrifying bacteria oxidize ammonia to nitrite, while denitrifying bacteria oxidize nitrates to dinitrogen. Nitrification could control *Symbiodinium* growth by competing for available ammonia and oxidizing it to less usable nitrates⁵³. Likewise, denitrifying bacteria could limit available nitrogen or contribute to nitrogen loss from the holobiont. The actual role of nitrifying and denitrifying bacteria could be more complex by structuring the bacterial community of the holobiont.

Cobalamin Biosynthesis

Genes for cobalamin synthesis (also known as vitamin B₁₂) were found in six isolates of this study: *R. mobilis* 44A6S, *L. aggregata* 44C2F, *N. aquibiodomas* 064newd, *R. arenilitoris* 452T9, *B. endophyticus* 130DD11, and *V. corallilyticus* 130DD58. These bacteria could be a source of vitamin B₁₂ to the holobiont.

The inability to synthesize cobalamin, termed cobalamin auxotrophy, is widespread in algae. Bacteria are the suggested source of cobalamin, which unveils a potential symbiotic relationship between bacteria and algae²⁵. These studies included *Dinophyta*, but only one species of *Symbiodinium* was represented, which did not require of vitamin B₁₂.

Looking at the coral holobiont more closely, there is evidence of the presence of cobalamin in the coral holobiont and of the importance of its availability on *Symbiodinium* growth. High concentrations of vitamin B₁₂ were measured by radio assay in the gastric cavity fluid of *Galaxea fascicularis*, as were high concentrations of bacteria (Agostini et al. 2012). These bacteria were likely responsible for high concentrations of cobalamin, and it is possible these bacteria produce cobalamin for coral and/or *Symbiodinium* uptake⁵⁵.

Another study tested the effect of cobalamin on the growth of *Symbiodinium*. *Symbiodinium* spp. from clade A were isolated from *Cassiopea* and cultured in 4 different mediums, with combinations of presence/absence of cyanocobalamin and antibiotics. The results showed that the *Symbiodinium* cultures without cyanocobalamin and antibiotics had significantly lower abundances than the culture with cyanocobalamin and antibiotics during the final stages of growth²⁶. In addition, the cultures treated with no cyanocobalamin and no antibiotics had equivalent growth to cultures treated with cyanocobalamin and no antibiotics. These results suggest that antibiotics successfully reduce a source of cobalamin by eliminating bacteria and that bacteria can be a sufficient source of cobalamin.

The *Symbiodinium* bacterial isolates in this study that are capable of cobalamin synthesis could act as a source for the *Symbiodinium* host. The bacteria isolated from coral colonies could be sources of cobalamin for the coral, but also for the *Symbiodinium* depending on the location of these bacteria. By acting as a provider of cobalamin, these bacteria could be conferring a significant, maybe essential, nutrient to the coral holobiont.

Cobalamin producing bacteria could be selected by the coral in favor of non-vitamin B₁₂ producers. One study showed that non-cobalamin producing *Salmonella* strains were more virulent than cobalamin producing strains, suggesting the host responds differently to infecting bacteria based on the ability of cobalamin synthesis⁵⁶.

Conclusions

The pangenome of the bacterial members of the coral holobiont has revealed several common metabolisms within and across genera, including the biosynthesis and metabolism of DMSP, denitrification, and cobalamin biosynthesis. Due to the coral's need to be highly efficient in nutrient acquisition and retention due to the oligotrophic habitat, it is feasible that the microbiome plays a role in nutrient cycling. The metabolic capabilities of the isolates potentially contribute to the production of beneficial nutrients like cobalamin, an osmoprotectant to help coral survive salinity fluctuations, and by recycling and retaining nitrogen. The ability to synthesize DMSP is potentially a core function of *Labrenzia*. Additionally, DMSP catabolism and availability could influence the bacterial community within the coral holobiont. Likewise, cobalamin synthesizers could provide an essential coenzyme for several processes in bacteria (methionine synthetase) and the hosts (growth promotion). My analyses also revealed that a member of the genus *Ruegeria* may be an aerobic anoxygenic phototroph despite the genus being described as non-phototrophic. This research supports the argument for further genomic sequencing of holobiont members to gain a higher resolution of the metabolic capabilities of the members and to help elucidate their potential roles within the coral holobiont.

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