Hitching a ride:

A gastropod-associated microbiome community at a Hydrate Ride methane seep and impacts on local biogeochemical cycling

A Thesis

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For Palmer,

A steady rock in changing seas and merging tides

Abstract

Exploration of cold seeps from geological, chemical, and biological perspectives has grown exponentially in recent decades since the first discovery of cold seeps in the 1980s. Symbiotic relationships, often rooted in geochemistry of the environment, have proven to be ubiquitous at cold seeps. However, our understanding of symbiotic relationships at extreme environments is limited. In this thesis, we first review two kinds of cold seep systems—gas hydrate-forming seeps, using Hydrate Ridge as an example, and brine-influenced seeps, with a focus on the Gulf of Mexico—from geological, microbiological, and biogeochemical perspectives. We then report on the composition of microbial communities associated with provannid gastropods as characterized using 16S rRNA gene amplicon and clone libraries as well as Fluorescence In Situ Hybridization. The gastropod shells, collected from seeps at Hydrate Ridge and the Gulf of Mexico are covered with filamentous epibionts on their shells. Large filamentous epibionts were identified as *Candidatus* Marithrix, *Thiomargarita nelsonii*, and a previously undescribed Chloroflexi. Our analysis of three incomplete Chloroflexi's genomes leads us to hypothesize that the Chloroflexi is an acetogen. Environmental samples from a previous sample revealed that the gastropod-associated community differed from the surrounding microbial communities, implying a selection mechanism for gastropod habitation and that the gastropod shell potentially serves as a unique niche. The diverse community of microbes on the shell of these seep-dwelling gastropods may represent a symbiotic relationship made possible by the gastropods motility that provides the attached microbial community with essential metabolites, while the attached community may serve the gastropod by providing it with a source of nutrition, and potentially detoxifying hydrogen sulfide.

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List of Abbreviations

ANME	Anaerobic methylotrophic archaea
AOM	Anaerobic oxidation of methane
ASV	Amplicon sequence variant
F1	Filament 1
F2	Filament 2
F3	Filament 3
FISH	Fluorescence in situ hybridization
SOB	Sulfur-oxidizing bacteria
SRB	Sulfur-reducing bacteria

Chapter 1: A review of cold seep geobiology

Although hydrothermal vents often stand in the spotlight as the habitat that hosts canonical deep sea chemosynthetic communities, a second kind of deep ocean oasis for life exists where near-ambient temperature, hydrocarbon-laden fluids are expelled into the surrounding seawater via fractures in the subsurface. These 'cold seeps', discovered in 1985 (Kenicutt et al.), have unique features that foster a diverse assemblage of life that is comparable to those found at hydrothermal vents. Each methane seep has a slightly different chemistry depending on local geologic history, plate tectonic activity, and sedimentation rates—variables which are dependent on the seep's mode of formation. Most often, seeps form at convergent plate boundaries or passive margins due to heavy sediment loading often at the anticlinal hinge on fault faces (Levin, 2005). These factors manifest as different characteristics of seeps, such as mud volcanoes from enriched fluid dehydration of clays, gas hydrates and other hydrocarbons from high sedimentation and/or organic-rich areas, and distinct rare earth element patterns (Suess, 2014). In this chapter, the formation mechanisms, geochemistries, ecological diversity, and metabolic pathways of/at two varieties of seeps are reviewed to provide context for the novel study that comprises Chapter 2.

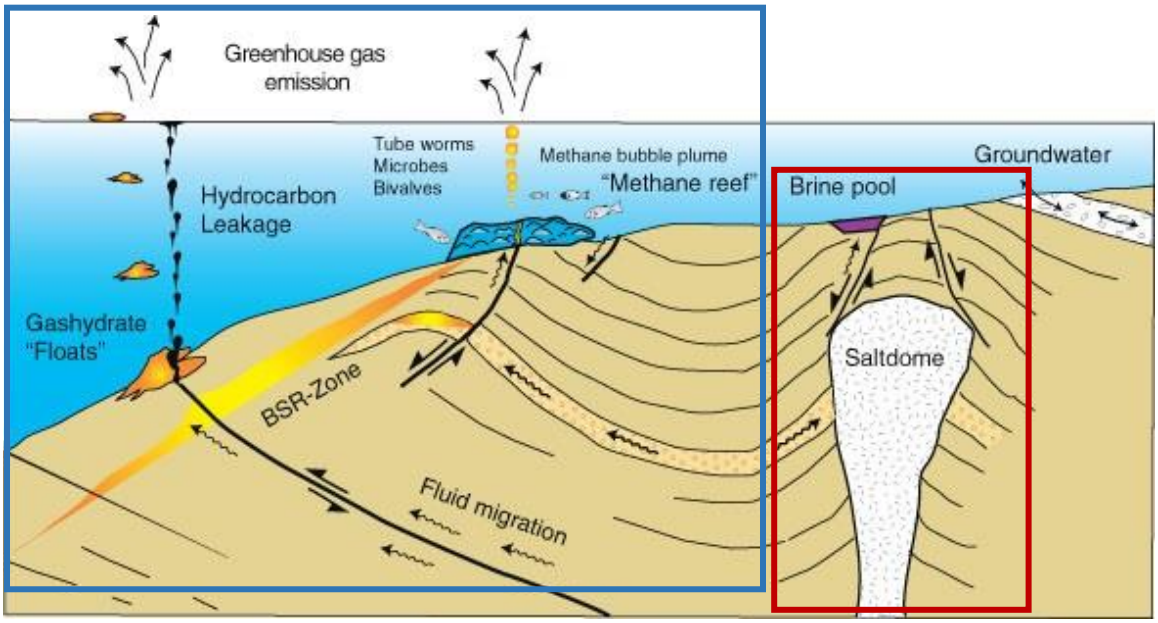

Figure 1: Cold seep schematic | Multiple cold seep environments are condensed and depicted in this summative cold seep schematic by Suess (2014). Gas hydrate-forming cold seeps are boxed in blue and brine-influenced seeps are boxed in red.

1.1 Gas hydrate-forming seeps (Hydrate Ridge, Northeast Pacific Ocean)

1.1.1 *Formation & geological controls*

Gas hydrate-dominated seeps are characterized by their abundance of large-scale gas hydrates, which forms when methane gas molecules crystallize with water molecules in an ice-like structure in specific temperature and pressure settings known as the hydrate stability zone. The example illustrated for the purpose of this thesis is Hydrate Ridge, a well-studied methane seepage site off the coast of Oregon, United States in the Cascadia Margin. 90 km off the coast, there is a subduction zone where the Juan de Fuca Plate collides obliquely with the North American Plate. The subduction of the Juan de Fuca Plate creates an accretionary prism by folding and compression, resulting in diagenesis of

seafloor sediments. However, not all accretionary prisms or wedges result in cold seeps. This area has high methane and other hydrocarbon fluid expulsion due to the compressional stresses of the accretionary prism, creating thrust faults that act as pathways for fluid flow, combined with large quantities of preexisting, mature, buried organic matter (Boetius and Suess, 2004). Lithologies at Hydrate Ridge consist of carbonate mudstones, breccias, and dolomites of several varieties. Almost all carbonates contain methane-derived carbon (Torres, et al., 2002), illustrating the important role of the anaerobic oxidation of methane in carbonate formation.

Hydrate Ridge has a northern and southern summit, which differ from each other not only spatially but in their hydrate stability zones. Hydrate stability is a function of temperature and pressure, and is influenced where there is a transition between hydrate-cemented sediments and free gas below. It was reported that the northern summit's stability zone is shallower with high velocity of methane ebullition and has a lower $\delta^{13}C$ value, but the thermal gradient is homogenous across both summits (Boetius and Suess, 2004).

1.1.2 *Geochemistry*

Generally, gas hydrates may only exist below 500 m (Sloan, 1990). Gaseous portions of the hydrate at Hydrate Ridge have the following composition: > 97% methane, < 3% sulfide, and > 1% CO_2 and short chain hydrocarbons. Two main kinds of hydrates exist, structures I and II. Structure I hydrates occur mostly near the sediment surface and do not have many long chain hydrocarbons or sulfides. Type II structures have a higher concentration of long chain hydrocarbons. Hydrates are found with either porous or

massive fabrics. Stable isotope signatures indicate a biogenic origin of the methane within the gas hydrate (Boetius and Suess, 2004). Decomposition of methane hydrates releases hydrate water that changes the isotopic composition of pore water, decreases alkalinity, and allows for the formation of high magnesium calcite (Torres, et al., 2002).

1.1.3 *Microbial diversity & metabolisms*

There is a steep gradient of chemical species such as sulfate, sulfide, and methane in sediment porewater at methane seeps due to microbial communities in the subsurface. Bacterial mats form at the sediment-water interface on top of hydrates. The substrate at Hydrate Ridge, consisting of unconsolidated sediments and carbonates, is characterized by dissolved methane gas that is in equilibrium with solid hydrate frequently are capped by bacterial mats. Methane gas flux is strongest here at 30 – 90 mmol/m^3day, but the bacterial diversity at these sites is impacted more by substrate than methane or sulfide flux (Torres et al. 2002; Marlow et al., 2014).

Mats have several main principal constituents: sulfur oxidizing bacteria (SOB), sulfur reducing bacteria (SRB), methylotrophic bacteria, anoxygenic methanotrophic archaea (ANME), and heterotrophic bacteria. The metabolisms of these groups are syntrophic, as sulfide concentration is the main determinant of the microbial community distribution here, and sulfide concentration is dependent on AOM and sulfate reduction, which are themselves dependent on methane flux and sulfate concentration. Methane serves as the electron donor for ANME archaea, and sulfate is the electron acceptor for SRB that live in consortia with ANME. The ANME partnership which overall performs anaerobic methane

oxidation, resulting in the production of sulfide and alkalinity, the latter of which contributes to the precipitation of authigenic carbonate that is so prevalent at cold seeps (Sahling et al., 2002; Suess et al., 2002a; Suess, 2002; Luff and Wallmann, 2003).

The most conspicuous SOB at Hydrate Ridge are mainly comprised of the representatives of the family *Beggiatoaceae*, which are broadly referred to as large sulfur bacteria and form the characteristic microbial mats at sites of methane ebullition. Mats at seeps range from thin biofilms to thicker morphologies, likely due to the abundance and eco-physiological characteristics of their constituents (Paul et al., 2017). SOB use oxygen or nitrate to oxidize sulfide produced during anaerobic methane oxidation (AOM) at deeper layers (Teske and Carvalho, 2020). They thrive at cold seeps due to the mixing of reduced sulfur compounds that they use as electron donors, and the availability of electron acceptors such as nitrate and oxygen (McGonigle et al., 2020).

Common taxa within the *Beggiatoaceae* at Hydrate Ridge are *Candidatus* Marithrix and *Thiomargarita nelsonii. Thiomargarita* spp. are 'giant' bacteria and are often recognized as the largest bacteria discovered, reaching cell diameters in excess of 1 mm and visible to the naked eye (Schulz et al., 1999, Salman et al., 2011). The genus is represented by candidate species *T. namibiensis*, *T. nelsonii*, and newly described *T. magnifica* (Volland et al., 2022). While the three are nearly indistinguishable morphologically, *T. nelsonii* cells tend to take a more oblong shape than spherical *T. namibiensis* cells. Since they are non-motile, certain ecotypes of the genus are thought to move passively by currents or attach themselves to motile gastropods and similar mesofauna to access sulfide in the sediments and oxygen and nitrate in the water column (Bailey et al., 2011). *Thiomargarita* stores nitrate within the cell to oxidize sulfide, presumably because sulfide may be spatially or

temporally decoupled from nitrate availability (CITE). In addition to canonical genes associated with sulfur oxidation, *T. nelsonii* found at Hydrate Ridge attached to gastropods also have genes potentially associated with arsenite- and hydrogen-based lithotrophy as well as genes associated with nitrate respiration via denitrification and dissimilatory nitrate reduction to ammonia (Flood, et al., 2016). *Ca.* Marithrix is a filamentous bacterium that dominates seep substrates in thick mats and have been observed being grazed on by gastropods (Salman-Carvalho et al., 2016).

Sulfurovum in the order *Campylobacterales* is another SOB taxon present at Hydrate Ridge. Representatives of this group can oxidize multiple forms of sulfur (elemental sulfur or thiosulfate) via different pathways (Sun, et al., 2020). It is found almost exclusively in active sediments, likely due to sulfur cycling between ANME/SRB producing sulfide for *Sulfurovum* and other SOB to oxidize to sulfate, and repeating the cycle (Marlow, et al., 2014).

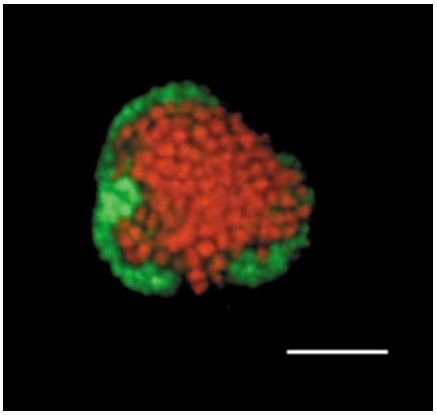

Figure 2: ANME-SRB consortium | ANME (red) in consortium with SRB (green) were discovered in 2000 by Boetius, et al. The consortia mediate anaerobic oxidation of methane and are ubiquitous at cold seeps. Image was taken with confocal laser scanning micrograph by Boetius, et al. (2000).

ANME archaea consist of several taxa—ANME-1, -2, and -3, all related to the orders Methanosarcinales and Methanomicrobiales—but ANME-1 and ANME-2 are most common at cold seeps (Knittel and Boetius, 2009). ANME perform the anaerobic methane oxidation (AOM) though the biochemical pathways, intermediates and syntrophic interactions are debated (refs). ANME are typically found in consortia with SRB in marine settings to partner anoxygenic methanotrophy to sulfate reduction

(Boetius et al., 2000). SRB mainly found in consortia with ANME at cold seeps are endemic and named accordingly: Desulfosarcina clade SEEP-SRB-1. The less common ANME-3 are associated with SEEP-SRB-3, a clade of Desulfobulbus (Teske and Carvalho, 2020). These associations explain the prevalence of specific SRB groups at Hydrate Ridge that live in consortia with ANME archaea, although many taxa of SRB are not known to be associated with ANME archaea.

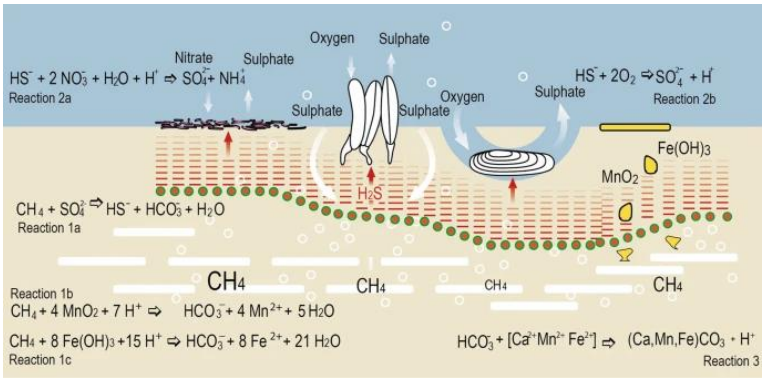

Figure 3: Biochemical cycling of sulfur and methane | The relationships between SOB, SRB, and ANME at cold seeps are mapped. SRB provide ANME with sulfate, who provide SRB with sulfide. SRB provide SOB with reduced sulfur species, who provides SRB with sulphate. Diagram by Suess (2020).

SRB here are mainly represented by groups Desulfobacteraceae and Desulfobulbaceae, both Deltaproterobacteria, which was the most abundant bacterial class at Hydrate Ridge as analyzed by Marlow et al. (2014). The function of sulfur reducing bacteria is the reduction of oxidized sulfur species such as sulfate and elemental sulfur to sulfide. In an SRB-focused study, Desulfosarcina and Desulfococcus in consortium with ANME-2 were present in *all* samples, supporting this relationship as particularly successful at seeps (Knittel et al., 2003). The same study also recovered a high abundance of *Desulfocapsa sulfexigens* from Hydrate Ridge sediments, a sulfur reducer that can disproportionate sulfur, which may contribute to the increased rate of sulfur cycling here (Teske and Carvalho, 2020).

Somewhat surprisingly, methylotrophs, which differ from ANME/SRB consortia in that they perform methane oxidation aerobically, are not abundant here. ANME/SRB consortia

seemingly mediate methane oxidation at Hydrate Ridge instead. At Hydrate Ridge, groups of methylotrophs present include Methylococcales and Verrucomicrobia as reported by Marlow, et al. (2014). These taxa use oxygen to consume methane and convert it to inorganic carbon (CO_2), which is then given to mussels (Teske and Carvalho, 2020). Most methylotrophs operate by producing formaldehyde from carbon substrate via a series of enzymes and cytochromes, which is then partly oxidized to carbon dioxide for energy and partly assimilated into the cell by either the serine cycle or ribulose monophosphate cycle (Ref.)(see reviews by Chistoserdova). Other types of carbon fixation via methylotrophy operate via the CBB cycle (ref.). As the largest biological sink for methane, which is a greenhouse gas, aerobic methanotrophs and ANME archaea are influential on the carbon cycle and the flux of greenhouse gasses to the atmosphere that influences global climate (Rosenberg, et al., 2013).

Heterotrophs, found anywhere there is biomass to gain energy from, are mainly represented by the bacterial taxa Chloroflexi at Hydrate Ridge and also by groups of archaea, constituting most of the biodiversity at cold seeps (Teske and Carvalho, 2020). We demonstrate this with reanalyzed data in Chapter 2 (Figure 19). At another gas hydrate-dominated seep, Blake Ridge, fermentative anaerobic heterotrophs were highly abundant and most prolific near the surface and correlated positively with SRB (Wellsbury et al., 2000).

1.1.4 *Macro- and mesofaunal diversity*

A metazoan abundance and diversity zonation occurs at seep sites, where sulfide toxicity lessens further from the main methane release site. This 'spatial heterogeneity' as described by Levin (2005) is very apparent. In contrast to high-sulfide areas covered in bacterial mats, the adjacent surrounding areas with less sulfide are dominated by vesicomyid clam beds (*Calyptogena pacifica* and *Calyptogena kilmeri*), sea urchins, buccinid gastropods, and cnidarians, and experience less than 1 mmol/m^3day of methane gas flux into the seawater (Levin, 2005; Torres, et al. 2002). Some snails have been found to distinctly dominate seep-*active* carbonate rocks, while other groups of animals such as crustaceans and echinoderms inactive carbonates (Levin et al., 2015). One zone further away, with the least sulfide flux, the *Acharax* genus of bivalve dominates (Boetius and Suess, 2004). Other sponges, anemones, limpets, and chitons also colonize the hard carbonate substrate of this environment, the latter two participating in lithivory of carbonate rock itself (Levin et al., 2015). Ice worms have been found in the hydrate material itself (Fisher et al., 2000; Sibuet and Olu, 1998; Levin, 2005). At lower flux areas where macrofaunal diversity is higher due to less sulfide toxicity and higher oxygen availability (Boetius and Suess, 2004), animal-microbe symbioses arise that are discussed below in Section 1.4.

1.2 Brine-influenced seeps (Gulf of Mexico)

1.2.1 *Formation & geological controls*

Generally, brine-influenced seeps are located near (and are related to the cause of) brine pools. Brine pools are pockets of extreme salinity that are separated from surrounding

seawater due to differing densities, collecting at the bottom of the ocean in depressions and giving the appearance of an underwater lake (refs.). They form by seawater intrusion into underlying salt deposits in the subsurface.

A brine-influenced seep is located in the Alaminos Canyon on the lower slope offshore central Texas, Gulf of Mexico. At this site (AC 601) there is a 180 m wide, 4 m deep brine lake (Teske and Carvalho, 2020). Halite dissolution was confirmed as the brine source here in 2007 by Roberts, et al. by analysis of chloride to sodium ratios. The Louann Salt, a Jurassic-aged halite deposit formed when Pangaea rifted and rejoined repeatedly is the source of this dissolution (Andrews, 1960). This rifting exposed this area to surface conditions and evaporated seawater, leaving salt behind, which solidified into domes whose shape can be seen today via satellite at the bottom of the Gulf (The salt domes are visible my satellite? Or are they being detected though seismic surveys. I thought they were deeply buried beneath an already deep Gulf of Mexico.). The brine pool and nearby mud volcano are results of gas migrations along faulting associated with a local anticline (Roberts, et al., 2010). The interface of brine and seawater formed when sulfate-free, barium-rich brine mixes with sulfate-rich sea water. This is illustrated by a turbid seawater-brine interface with floating barite precipitates. The brine pool at AC601 is surrounded by terraced carbonates that are evidence of changing ‘lake’ levels. The ‘shoreline’ is very reduced (Teske and Carvalho, 2020).

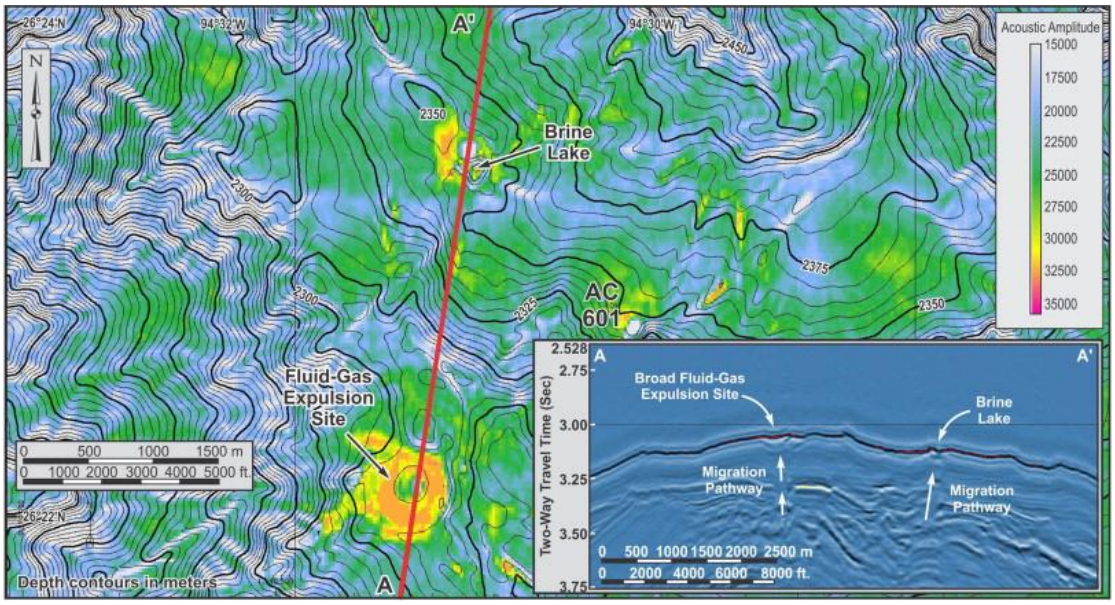

Figure 4: Gulf of Mexico bathymetry | Seafloor topography of and near the brine lake at AC601 was mapped by Roberts, et al. (2010). A cross section was taken along the red line (A – A') and reveals fluid migration pathways to the surface at the brine lake and site of gas expulsion.

There is a nearby low-activity mud volcano nearby, whose source is not completely known. However, hydrocarbon seepage may mobilize sediments to form this feature, and provide a "window into the subsurface" (Teske and Carvalho, 2020). Iron oxidation has stained the mud flows red, giving it the name 'Red Crater'. At the core of this volcano is brine, producing brine flows, as well as dark mud and grey fluidized mud (Teske and Carvalho, 2020).

1.2.2 *Geochemistry*

Since this brine-influenced seep was derived from a salt deposit, it has elevated ammonium and DOC levels, which are reflected in the lake geochemistry. Increased ammonium levels in this type of brine are the result of ammonium desorption when brine and sediments mix. Had the brine environment been hydrate-derived, it would be more enriched in sulfate (Teske and Carvalho, 2020). The brine also contains limiting nutrients such as DOC, iron,

and phosphate, promoting the growth of microbial communities (Teske and Carvalho, 2020). The surrounding sediment of the mud volcano is rich in methane, sulfide from high sulfate reduction activity, and ammonium (Teske and Carvalho, 2020).

1.2.3 *Microbial diversity & metabolisms*

For the most part, microbial diversity here mimics the major groups found at all seeps, including previously described Hydrate Ridge: SOB, SRB, ANME, and aerobic methane oxidizing microbes. However, the zonation of the brine lake geochemical environment results in distinct, zoned microbial assemblages. The bottom of the lake contains the highest salinity and nutrient concentrations, which is reflected in the high diversity of its bacterial and archaeal community (Crespo-Medina et al., 2016). The highest rates of both sulfate reduction and methane oxidation, occur closer to the surface of the lake, producing the most sulfide via the anaerobic oxidation of methane (Crespo-Medina et al., 2016). On the 'shoreline' of the lake, SRB are abundant and produce a high-sulfide microenvironment as well (Teske and Carvalho, 2020). Microbial diversity is positively correlated with DOC, DIC, and sulfide concentrations among other variables, illustrating the effect that the local geochemistry has on microbial ecology in this extreme environment (Crespo-Medina et al., 2016). The mud volcano, just several kilometers away, is a host to mats of SOB typical of a methane expulsion site (Teske and Carvalho, 2020).

1.2.4 *Macro- and mesofaunal diversity*

Meso- and macro-fauna such as urchins and holothurians are more abundant just several meters away from the reduced shoreline of the brine lake because the environment is more oxidized with little to no sulfide toxicity, has higher organic carbon, and higher nitrogen. The abundance of fauna decreases further away from this zone, meaning that their presence is at least somewhat dependent on the microbial life present near the lake for nutrition (Teske and Carvalho, 2020). The mud volcano hosts the greatest concentration of bathymodiolid mussels on the northern Gulf of Mexico continental slope (Teske and Carvalho, 2020). In general, sea anemones, sponges, tubeworms, polychaetes, hydroids, clams, snails, crabs, and mussels can be found in this zone near brine lakes.

1.3 Biogeochemical cycling at methane seeps - overview

Broadly speaking, cold seeps exhibit similarities in their microbial ecology and biogeochemistry. The metabolisms of the above taxa quite harmoniously produce cyclic biogeochemistry at the sediment-water interface of cold seeps, as shown in Figure 5 (Wasmund et al., 2017). Suess (2014) refers to this 'harmony' as "the hydrocarbon-metazoan-microbe-carbonate association", accurately named due to the acute interconnectedness of the -spheres (biosphere, hydrosphere, and lithosphere) of this environment. Because methane is continuously available, the ANME and ANME/SRB consortia continuously oxidize it to carbon dioxide. The ANME/SRB consortia reduce sulfate (SO_4^{2-}) to elemental sulfur (S^0). SRB further reduce S^0 and other intermediate sulfur compounds to sulfide (H_2S). When the sulfide produced fluxes into sediment or water that

contains oxygen or nitrate, SOB oxidize reduced sulfur compounds to sulfate to support chemolithotrophic growth. Due to the foundation of this 'energy web' being methane, it is no surprise that mats of these microbes are most dense at the place of methane release into the sediments and seawater.

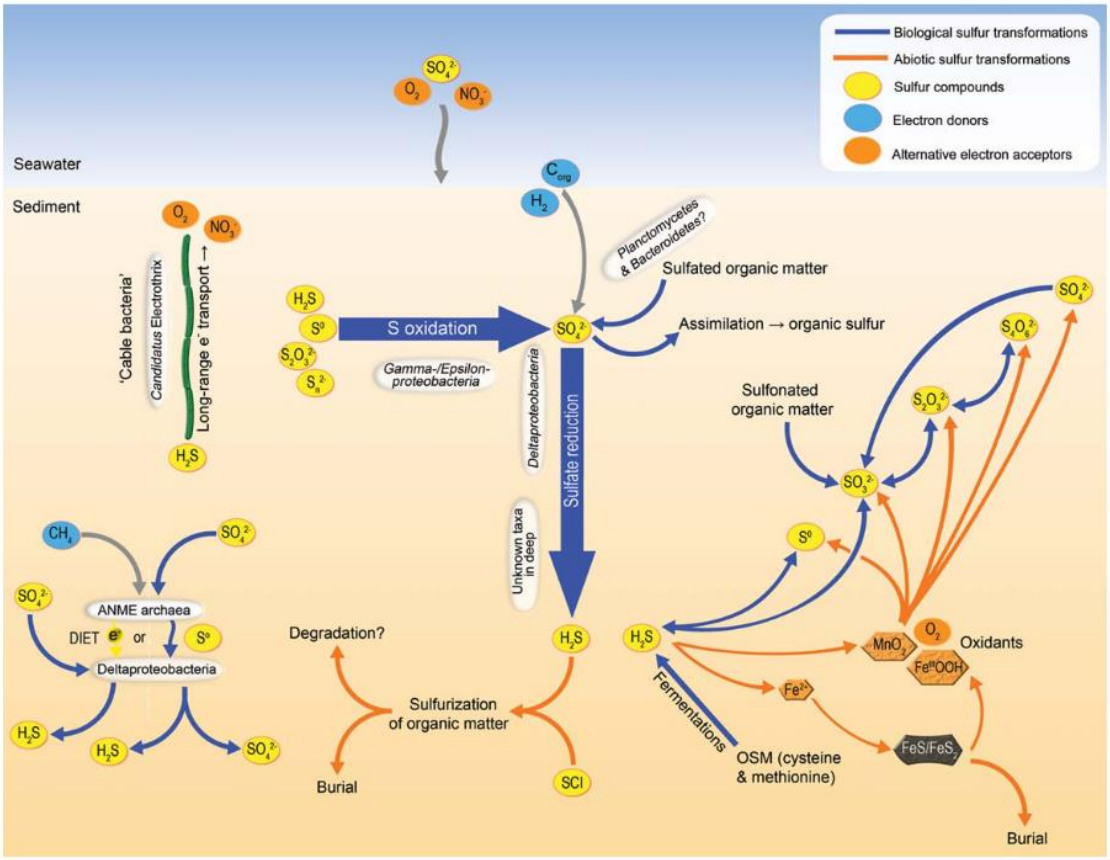

Figure 5: Biogeochemical cycling at seeps | Overall cycling of chemical species in marine sediments with a focus on sulfur created by Wasmund, et al. (2017). Sulfur species are oxidized by Gammaproteobacteria and Epsilonproteobacteria (SOB) and abiotic oxidants. Sulfur is reduced by Deltaproteobacteria both in and out of consortia with ANME, and by unknown taxa in deep sediments. Other processes interact with sulfur species such as fermentations that yield sulfide, presence of methane for ANME to act in consortia with SRB, conversion of sulfide to oxygen and nitrate by cable bacteria, and sulfation of organic matter at the sediment-seawater interface.

1.4 Animal-microbe symbioses at cold seeps

At hydrothermal vents and seeps, there are many established examples of symbiosis, where two or more organisms thrive off each other's life modes either facultatively or obligately. Well known are animals with endosymbionts, where chemosynthetic microbes live inside of the animal, such as in the case of the giant tube worm, *Riftia pachyptila* (Cavanaugh et al., 2006). This endosymbiosis is obligate for the worm as the immense amount of sulfur oxidizing Gammaproteobacteria inside of it provide the host with necessary organic compounds to sustain life at such dark depths. Many clades of SOB often serve as symbionts in these worms, as well as in mussels and clams (Teske and Carvalho, 2020). The case of shrimp *Rimicaris exoculate* and the yeti crab, *Kiwa hirsute*, exemplify a different kind of symbiosis: episymbiosis. Both organisms host assemblages of microbes on their surfaces—the yeti crab uniquely on its arms—that serve as ready-made meals for them and suit the needs of the bacteria by traveling between oxygenic and sulfidic zones often necessary for a sulfur metabolism (Munn, 2019). Non-motile microbes such as *Thiomargarita* spp. may depend on this type of animal-mediated transport to find its electron donor, reduced sulfur, in the sediments, and its electron acceptor, oxygen, in the water column (Schulz, 2006; Bailey et al., 2011).

Provannid snail-microbe gill endosymbioses have been previously published (Borowoski, et al., 2002; Suzuki et al., 2005a, b; Windoffer and Giere, 1997). This relationship is based in the gill of the gastropod and is unique from the potential episymbiotic relationship investigated in Chapter 2. However, these studies provide useful and relevant insights, as gill endosymbiosis is much closer to episymbiosis than gut endosymbiosis, a mode of symbiosis that is very common in the deep ocean.

Substrate-attached mats and unique behaviors of the provannid gastropods were described by Bailey et al. (2011) and reviewed in Section 2.3.1. Attached *Thiomargarita* cells exhibit stalk-like morphologies while free-living daughter cells, which 'bud' off the attached stalks, are spherical. This observation of a dimorphic lifestyle supports an ecological role for attachment surfaces such as the shells of small seep-dwelling snails. The following chapter will expand on the 2011 study by Bailey et al., exploring the role of the provannid snail in relation to its attached mat, and the nature of their relationship.

Chapter 2: Potential symbioses of provannid gastropods and attached microbiomes

2.1 Introduction

There are many established examples of symbiosis, where two or more organisms thrive off each other's life modes in close contact, either facultatively or obligately. At hydrothermal vents and seeps, well known are animals with endosymbionts, where chemolithotrophic microbes live inside of the animal, such as in the case of the giant tube worm, *Riftia pachyptila* (Cavanaugh et al., 2006). This endosymbiosis is obligate for the worm as the immense amount of sulfur oxidizing Gammaproteobacteria inside of it provide the host with necessary organic compounds to sustain life at such dark depths. The case of shrimp *Rimicaris exoculate* and the yeti crab, *Kiwa hirsute*, exemplify a different kind of symbiosis: episymbiosis. Both organisms host assemblages of microbes on their surfaces (the yeti crab on its arms) that serve as ready-made meals and suit the needs of the bacteria by traveling between oxygenic and sulfidic zones necessary to support a sulfur-based chemolithotrophic metabolism (Goffredi et al., 2008). Abalones, such as *Haliotis cracherodii* also have such epibiotic mats of SOB that serve as nutrition for the invertebrate (Stein, 1984). Non-motile microbes such as *Thiomargarita* spp. may depend on this transport to find its electron donor, reduced sulfur, in the sediments, and its electron acceptor, oxygen or nitrate, in the water column (Schulz and Jørgensen, 2001; Bailey et al., 2011).

Currently, only endosymbionts are cited and described in provannid gastropods. Of the five provannid genera, only two genera have been found to contain endosymbionts. Most provannids are deposit feeders, ingesting sediments and filtering for nutrition, i.e. microbes (Waren and Ponder, 1991). The rare cases of endosymbiosis entail SOB and methylotrophs located in the enlarged gill of the snail (Borowoski, et al., 2002; Suzuki et al., 2005a, b; Windoffer and Giere, 1997). In the instance of the provannid gastropod *Ifremeria nautilei*, RubisCo and methanol dehydrogenase genes were detected for autotrophic fixation of carbon dioxide and a methylotrophic pathway, respectively (Borowoski, et al., 2002; Windoffer and Giere, 1997). The provannid genus *Alviniconcha* was also investigated for its SOB Epsilonproteobacteria gill endosymbionts (Suzuki et al., 2005a, b; Windoffer and Giere, 1997). Other, non-provannid marine gastropods such as *Cyathermia naticoides*, *Helicoradomenia* spp., and *Lepetodrilus fucensis* have endosymbionts as well, though these examples are all part of hydrothermal vent communities, not cold seeps (Zbinden et al., 2015; Katz et al., 2006; Bates, 2007a, b).

However, Bailey et al. (2011) observed filamentous microbes on *Provanna laevis* specimens from the Costa Rica margin and Hydrate Ridge. Their collection from Hydrate Ridge in 2010 and cultivation in the lab led to observations of behaviors described in Section 2.3.1 as well as the hypothesis of a symbiotic relationship between the provannid gastropods and their microbial mats. It is noted that invertebrates with attached epibionts have been known to serve as a vessel to transport epibionts between oxygenated and sulfidic pore waters for required metabolic substrates (Ott et al., 1991). We explore this hypothesis, and the microbial community composition of the snails, in this thesis.

Prevalent at Hydrate Ridge as well as other seeps and vents, members of *Provannidae* are strong candidates for episymbiosis due to observed microbial mats on their shells, which we investigate in this study. Several species in the family were first described from the Gulf of Mexico hydrocarbon seeps in 1991 by Waren and Ponder, reporting 1.5 – 2.5 body whorls when contracted and a non-chitinous shell. Since being discovered in 1986 due to the development of deep-sea exploration technology, fossilized provannid gastropods were first recorded in 1995 (Squires) and later were found to have existed all the way back to the Late Cretaceous (Middle Cenomanian) from methane seep deposits in Japan (Kaim et al., 2008). Methane seep deposits such as those in the Paleocene-age Panoche Hills of California reflect Provannidae's prevalence at methane seeps over large time scales, perhaps reflecting how ancient some chemoautotrophic host/symbiont relationships are (Schwartz et al., 2003).

Provannid gastropods were sampled from the Hydrate Ridge gas hydrate-forming seep off the coast of Oregon and observed to have dense accumulations of bacteria attached to their shells, described by Bailey et al. (2011) and Flood et al. (2016) (Figure 6). An investigation began to characterize the microbial community associated with the shell and begin to evaluate the relationship between the snail and its attached community, if one exists. We hypothesized it is likely there is a relationship that has a degree of episymbiosis, due to the mats' constituents' tendency to require transport and the high frequency of these kinds of relationships at vents and seeps, and across the deep ocean.

2.2 Methods

Section 2.2 Methods includes experimental work and other contributions from Verena Carvalho, Beverly E. Flood, Elizabeth Ricci and Sophia Ruben. Environmental sequence data from Hydrate Ridge that was originally produced and reported on by Case et al. (2015), was accessed from the NCBI Sequence Read Archive (accession numbers SRP055767 and SRP049675).

Gastropods and attached microbial communities

2.2.1 *Site description and gastropod sampling*

On cruise AT 18-02 to Hydrate Ridge in 2010, gastropod samples and attached microbial communities were collected and split into the three groups - the first fixed shipboard in sterile ethanol and Instant Ocean (1:1) and stored at -20ºC. Another group of gastropod samples was fixed in 4% paraformaldehyde (PFA) and sterile seawater. The PFA was replaced by 100% ethanol in the laboratory. The final group was refrigerated in seawater as live samples. Additionally, gastropod samples were collected from a brine pool (Stevens et al., 2015) associated with a cold seep in the Gulf of Mexico by DSV Alvin via *R/V Atlantis* (cruise AT 18-02) at 648 m below sea level for comparative analyses with the gas hydrate samples. Mussel beds encircled this brine pool. The gastropod included in this

Figure 6: Gastropod microbial mat images | Images of filamentous epibionts on a provannid gastropod sampled from Hydrate Ridge, originally published by Flood, et al. (2016).

study's data was sampled from the surface of a mussel, and was observed to be feeding on the bacterial mat on the shell of the mussel.

2.2.2 *Laboratory observations of gastropods*

Sediment and seawater collected from the Eel River Basin were placed in an unsterilized plastic container. Three defleshed pig rib bones were placed in the container/tank to seed the simulation of a whale fall community, i.e., a continuous source of H_2S. The live group (about 20 live snails) with visible microbial epibiotic mats were placed in the tank. The tank was aerated for about five minutes daily with an aquarium bubbler. Microelectrodes (Unisense, Denmark) were used to profile the mesocosm sediment water interface to measure sulfide concentrations.

Figure 7: Laboratory gastropod tank | Provannid gastropods collected at Hydrate Ridge were housed in a laboratory-based tank with sediment and seawater from the collection site, pig rib bones, and was aerated daily.

2.2.3 *Molecular characterization of gastropod communities*

DNA was extracted from gastropod samples and their epibiont communities collected at both Hydrate Ridge and the Gulf of Mexico. This was done using the PowerBiofilm™ DNA Isolation kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions. Further, DNA was also extracted from the isolated shell of the gastropod at Hydrate Ridge. 16S rRNA gene amplification was performed on the whole snail using the primer set 27F (5'-AGAGTTTGATCMTG) and 1492r (5'-GGTTACCTTGTTAC) (Lane, 1991). The primers 341f (5'-CCTACGGGAGGCAGCAG) (Muyzer et al., 1993) and

VSO848r (5'-GGATYAATYTCCCCCAACATC) (Kalanetra et al., 2005) were used to target the 16S rRNA gene of some members of the Beggiatoaceae. Cloning was performed on both libraries using a TOPO® TA cloning kit (incl. pCR®2.1-TOPO®Vector and One Shot®Match1TM T1®chemically competent E. coli cells) (Life Technologies Corporation, Grand Island, NY, USA). Plasmids were then extracted using the QIAprep® Spin Miniprep kit (Qiagen, Duesseldorf, Germany). The University of Minnesota Genomic Center performed Sanger sequencing on a 96 capillary 3730xl sequencer (Applied Biosystems) that employed ABI BigDye Terminator version 3.1 chemistry.

2.2.4 *iTag sequencing of gastropod communities*

iTag sequencing of all five gastropod samples and a PCR water control were performed on the V4 region of the 16S rRNA gene with Nextera primers (V4_515F 5'-GTGCCAGCMGCCGCGGTAA-3', V4_806R 5'-GGACTACHVGGGTWTCTAAT-3') (references). V4 libraries were generated by using the amplicon sequencing service at the UMGC (Gohl et al., 2016). The V4 primers included tail sequences to allow subsequent bar-coding (forward primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and reverse primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). The samples were amplified with Kapa HiFidelity Hot Start polymerase (Kapa Biosystems, USA), with an initial denaturation step at 95°C for 5 min and 25 cycles of denaturation (98°C for 20 s), annealing (55°C for 15 s), and elongation (72°C for 1 min). Barcodes and Illumina sequencing adaptors were attached with a second amplification step according to the recommended iTag amplicon sequencing protocols (Illumina, USA), and libraries were size selected (Caliper LabChip XT; PerkinElmer, USA), pooled, and sequenced by using

the Illumina MiSeq system (250-bp paired end), which generated on average 120,000 paired end reads per sample.

2.2.5 *Fluorescence in situ hybridization of gastropod communities*

To complement the molecular characterization of the gastropod-attached community and determine the identity of some of its constituents, fluorescence in situ hybridization (FISH) was employed. Whole PFA-fixed Hydrate Ridge *Provanna* snails and their attached epibiont communities were dehydrated with ethanol and treated with xylene prior to decalcification via ethylenediaminetetraacetic acid (~ 1 month incubation) or Formical-4™. The snails were then embedded in molten paraffin wax and cut into 4 µm thick slices with a microtome and then applied to a microscope slide and dried overnight. Prior to FISH staining, the slides were heated to 60°C to melt the paraffin wax and then submerged twice in xylene for 5 minutes to remove the residue. Then the slides were placed in a graded series of ethanol for 3 minutes each (2x 100%, 90% and then 80%) and rinsed under running water for 5 minutes and finally stored in deionized water. In addition, fixed large sulfur bacteria were individually removed for identification via FISH.

FISH staining was performed as previously described (Jones et al., 2010). All samples were stained with the universal bacterial probe EUBMIX (1:2 mixture of GCTGCCTCCCGTAGGAGT and GCWGCCACCCGTAGGTGT) (Daims et al., 1999). Probes for following clades were used to explore the microbial diversity: *Archaea* ARCH915 (GTGCTCCCCCGCCAATTCCT) (Stahl and Aman, 1991), *Gamma*- and *Betaproteobacteria* GAM+BET42a (GCCTTCCCACWTCGTTT) (Manz et al., 1992),

Epsilonproteobacteria EP404 (AAATGYGTCATCCTCCA) (Macalady et al., 2006), *Pseudoalteromonas* PSA184 (CCCCTTTGGTCCGTAGAC) (Eilers et al., 2000), and Chloroflexi GNSB-941 **(**AAACCACACGCTCCGCT) (Gich et al., 2001). Positive controls (cultures that were perfect matches to the probes) were included for each hybridization reaction. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted Citiuor AF1 or ProLong®Gold antifade reagent. All probes were synthesized by Sigma-Aldrich®.

Live samples were observed and imaged with an Olympus SZX-16 stereo microscope equipped with a Canon EOS T2i digital camera. Epifluorescence, phase contrast and differential interference contrast (DIC) microscopy were performed on an Olympus BX-61 compound microscope equipped with an Olympus DP72 camera and an Olympus IX-81 inverted microscope equipped with an Olympus DP73 pixel-shifting color camera. Images were compiled with Olympus CellSens Dimensions software.

2.2.6 *Analysis of unidentified filamentous epibiont*

Two of the three morphotypes of large attached bacteria were identified via FISH. Individual filaments of the third, which could not be identified by iTag sequencing nor FISH, were removed from the shell by cutting them close to the attached base with a sterile needle. Each filament was then dragged though sterile 0.2% agar prepared in artificial seawater and added to 2.5 µL of the sample buffer of the illustra GenomiPhi V2 kit (GE Healthcare Life Sciences, Pittsburgh, PA). The filament was disrupted manually in the buffer with a sterile needle before the mix was boiled for 3 minutes at 95°C and supplemented with 2 µL of the reaction buffer and 0.5 µL enzyme mix (Spits et al., 2006).

We assessed the purity of the MDA product by amplifying the 16S rRNA gene sequence and directly Sanger sequencing the PCR product. The whole genome product was then reamplified with the illustra GenomiPhi HY DNA Amplification kit (GE Healthcare Life Sciences, Pittsburgh, PA) yielding final DNA concentrations of 2 µg/µL, which were frozen at -80ºC. The MDA products of three individually amplified filaments were sequenced in separate libraries (6 µg DNA each) using the illumina MiSeq v2 technology, paired-end, 2x250 bp, at the Cornell University Institute of Biotechnology, Ithaca, NY.

The total number of reads were 6,162,192 (Filament 1, F1), 9,354,302 (Filament 2, F2), and 6,118,906 (Filament 3, F3). They were quality checked, trimmed, and artifact/contamination filtered with DUK, a filtering program developed at the JGI (Nordberg et al., 2014) that removes known Illumina sequencing and library preparation artifacts. Subsequently, reads were screened for human, cat, and dog contaminant sequences. The remaining reads (95.3%, 96.8%, and 97.8%) were passed to SPAdes (Bankevich et al., 2012) and assembled into contigs ≥ 2kb. All these steps are part of the Jigsaw2.4.1 pipeline established at the JGI (Nordberg et al., 2014). The assemblies consisted of 2059, 2246, and 2143 contigs, representing 11.1 Mbp, 11 Mbp, and 11.4 Mbp, respectively. These datasets were passed through Metawatt (Strous et al., 2012) and the resulting bins affiliating with the Chloroflexi phylum were extracted. The final datasets were 2.8 Mb (477 contigs, N50=7300) for filament 1, 4.0 Mb (748 contigs, N50=6819) for filament 2, and 4.1 Mb (693 contigs, N50=7551) for filament 3. These draft genomes contain only Chloroflexi-related rRNA genes, and have a G+C content of 49%. According to CheckM analysis, a software designed to assess quality and completeness of (meta)genomes (Parks et al., 2015), the draft genome datasets showed a completeness of

43%, 61%, and 67%, based on 160 *Bacteria*-specific maker genes (marker gene set 109). The estimated contamination level is 0%, 0.92%, and 3.67%, which is in the error range (≤6%) of contamination estimates of incomplete (~70%) genomes (Parks et al., 2015). Strain heterogeneity, tested by the amino acid identity (AAI) between multi-copy genes (Parks et al., 2015), is 0, 0 and 20. At phylum level (*Chloroflexia*), CheckM uses maker gene set 20 with 250 marker genes and results show a slightly lower completion of 30%, 59%, and 66% with contaminations of 0%, 2.01%, and 1.34%. Strain heterogeneities are 0, 0, and 33.33%.

2.2.7 *Phylogenetics*

Raw DNA chromatograms were analyzed and edited using the software BioEdit. Editing included removal of primer binding sequences. The forward and reverse sequences for each 16S rRNA PCR amplicon were assembled using Bioedit's contig assembly program.

Due to its completeness, the assembled 16S rRNA sequence of only Chloroflexi filament 3 was analyzed with the National Center for Biotechnology Information's (NCBI) MegaBLAST algorithm (Morgulis et al., 2008) due to a Basic Local Alignment Search Tool (BLASTn; Altschul et al., 1990) search resulting in no hits. Only 22 hits from the MegaBLAST search had sequence identity greater than 90% and of those they are only from 5 different locations. The 16S rRNA gene sequences of the filament, selected closest hits from MegaBLAST, and several organisms for outgroups were aligned using SILVA's Alignment, Classification, and Tree Service (ACT; Pruesse et al., 2012). The aligned

sequences were used to generate a phylogenetic tree by maximum likelihood analysis within the MEGA-X program (Tamura et al., 2021).

Protein-coding genes of interest (carbon monoxide dehydrogenase medium subunit and propionyl-CoA synthetase) found in one or more of the Chloroflexi filaments' genomes were analyzed with the BLASTp algorithm. For the carbon monoxide dehydrogenase, the top hit was only 74% identical (from another Ardenticatenales bacterium found in the soil of the Mackay Glaciers, isolate MGR_bin47 SD2897-2912_k127_8479746, accession number JACCSL010000345; submitted to NCBI by Leung et al. in 2020) so these BLASTp results were discarded. For the propionyl-CoA synthetase, the top hit was only 81% identical (from a wastewater-derived Anaerolineales bacterium, accession number MBE7468071; submitted by Liu et al. in 2020, unpublished) so only this top BLASTp result was included, and the rest were discarded. Additional amino acid sequences found in studies where the gene of interest was proven to be functional were collected instead. For carbon monoxide dehydrogenase, there were many of these. Contrastly, the only organism that has proven functionality of propionyl-CoA synthetase in the 3-hydroxypropionate bicycle has been *Chloroflexus aurantiacus* (Hügler and Sievert, 2011). Individual genes of the bicycle, which tend to be very multipurpose, have been found in Alpha- and Gammaproteobacteria strains but lack proven functionality (Hügler and Sievert, 2011). Several of these non-Chloroflexi organisms were included to determine potential functionality of F3's propionyl-CoA synthetase. The protein sequence of the gene of interest and those with or without supported functionality in the literature were aligned by MUSCLE (Edgar, 2004) within MEGA-X. The final alignment length for included carbon monoxide dehydrogenase genes was 515 amino acid positions. The final alignment

length for propionyl-CoA synthetase genes was 654 amino acid positions. Each underwent maximum likelihood analysis within MEGA-X was used to generate phylogenetic trees.

Environmental samples

2.2.8 *Collection of environmental samples*

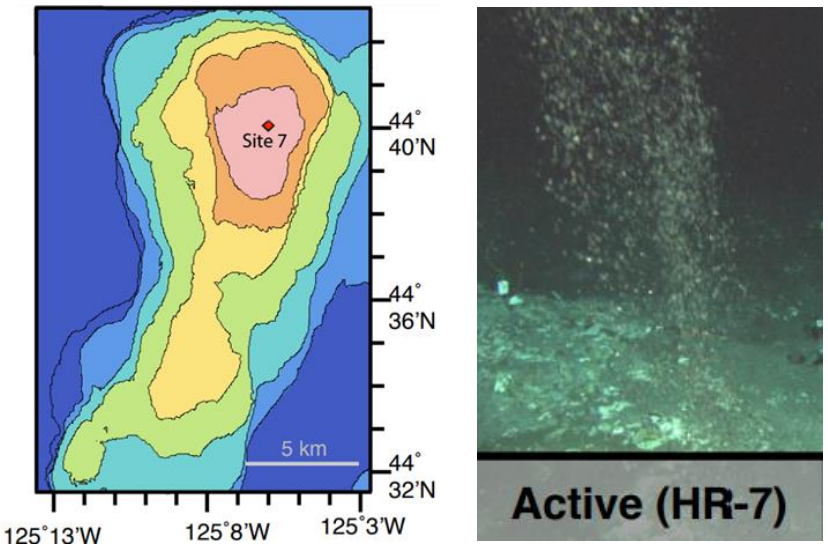

Figure 8: Map and image of Hydrate Ridge | Location and image of environmental samples collection site HR-7 by Case et al. (2015) to characterize microbial communities' substrate specificity. Case et al.'s (2015, Supplementary Fig. 1) data from this site was accessed and reanalyzed using the methods in 2.2.9. Figures by Case et al. (2015).

The environmental samples described and used in this thesis to provide background context to the gastropod analyses reported here were collected by Case et al. (2015) via remotely operated vehicle Jason aboard *R/V Atlantis* (cruise AT 18-10) at Hydrate Ridge North site 7 (44º 40.02687' N 125º 5.99969' W) off the coast of Oregon, USA on the Cascadian margin. Site 7 is categorized by Case et al. (2015) and others (Levin et al., 2015, Tryon et al., 2002, Orphan et al., 2004, Boetius et al., 2004) as an active site due to the indicator biota on and around the seep, such as mats of sulfide-oxidizing bacteria, clam beds, and snails and/or methane visibly escaping the seafloor by bubbles. Sampling was done on

September 3rd, 2011. We downloaded the raw fastq files by Case et al. (2015) from the Sequence Read Archive (accession numbers SRP055767 and SRP049675).

2.2.9 *Re-analysis of environmental samples and comparative analyses*

Environmental samples previously collected and studied by Case et al. (2015) aid this investigation in comparing gastropod communities to substrate samples at Hydrate Ridge. These samples were reanalyzed with updated methods. Raw V4 region Illumina MiSeq 2 x 250 base pair run files from Hydrate Ridge North site 7 created by Case et al. (2015) were downloaded from the Sequence Read Archive. The primers used by Case et al. (2015) were the Earth Microbiome Project V4 primers, which have a Y instead of a C in the fourth base position of the reverse primer. Primers were removed via Cutadapt v. 1.6 (Martin, 2011) and all iTaq sequences were filtered and trimmed for quality utilizing DADA2 1.18.0 (Callahan et al., 2016). ASVs were assembled via DADA2 1.18.0 with a minimum overlap of 20 bp. Updating bioinformatics methods from a previous study allow for a more direct comparison between the seep samples and the gastropod-associated community data first reported here.

All plots were generated in R with Phyloseq (McMurdie and Holmes, 2013), Vegan, DESeq2 (Love et al., 2014), Ggplot2, Dendextend, Tidyr, Viridis, and Reshape packages. Hierarchical clustering to create a dendrogram was done with a Euclidean distance matrix. A multidimensional scaling ordination plot was created using Phyloseq and DESeq2 packages. Alpha diversity indices Shannon and Chao1 were generated with Phyloseq and Vegan packages. Overall taxonomic abundance plots were generated with Ggplot2.

	Name	Experimental Treatment	Type	Location
Case_3544	3544	Native	Carbonate	44.667115N 125.099995W
Case_3625	3625	Native	Carbonate	44.667151N 125.100020W
Case_3626	3626	Native	Carbonate	44.667151N 125.100020W
Case_3628	3628	Native	Carbonate	44.667151N 125.100020W
Case_5102	5102	Native	Carbonate	44.667106N 125.099970W
Case_5103	5103	Native	Carbonate	44.667106N 125.099970W
Case_5104c	5104c	Colonization	Carbonate	44.667088N 125.099995W
Case_5104d	5104d	Colonization	Carbonate	44.667088N 125.099995W
Case_5118N	5118N	Native	Nodule	44.667061N 125.099970W
Case_5118	5118	Native	Sediment	44.667061N 125.099970W
Case_5120b	5120b	Native	Carbonate	44.667079N 125.100020W
Case_5121	5121	Transplantation	Carbonate	44.667079N 125.100020W
Case_5122b	5122b	Native	Carbonate	44.667079N 125.100033W
Case_5123b	5123b	Native	Carbonate	44.667079N 125.100033W
GoMSnail_S70	GoMSnail	Native	Whole Provanna	27.703308N 90.650187W
HRSnail1Whole_S30	HRSnail1Whole	Native	Whole Provanna	44.667113N 125.099995W
HRSnail2Shell_S41	HRSnail2Shell	Native	Provanna shell	44.667113N 125.099995W
HRSnail3Shell_S52	HRSnail3Shell	Native	Provanna shell	44.667113N 125.099995W
HRSnail_S81	HRSnail	Native	Whole Provanna	44.667113N 125.099995W

Table 1: All samples | All samples, including those reported on by Case, as well as the gastropods from HR, and GoM samples that were sequenced and analyzed in this study are listed. The original sample title is followed by its shorthand name, experimental treatment (colonization and transplantations done by Case et al. (2015)), substrate type, and precise sampling location. Sample titles beginning with "Case_" were collected and first analyzed by Case et al. (2015).

2.3 Results

2.3.1 *Laboratory observations of live samples*

During incubation and cultivation of live gastropods in the lab, consistent behaviors were observed. The provannid gastropods were observed to climb the walls of their tanks to areas where oxygen was more available, where they stayed for minutes to hours. The gastropods then moved back down into the sediments and flipped onto their dorsal sides and buried their shells in the sediments where sulfide is more available, again staying for minutes to hours. The gastropods fed on the sediments and occasionally the sulfur bacteria near their mantles at the openings of their shells. Bacterial mats disappeared from their shells within 2 months, and the described movement behaviors of the gastropods noticeably decreased with time, coinciding with the disappearance of their attached microbial communities.

2.3.2 *FISH studies*

The FISH images of the provannid gastropods and their attached epibionts can be seen in Figure 9. Epibionts (green), including filamentous *Thiomargarita nelsonii*, are seen in contrast with the auto-fluorescing shell of the gastropod (pink) and its superjacent snail biofilm (dark blue) (Figure 9a). The prevalence of *T. nelsonii* as a ‘hitch hiker’ upon *Provanna* is confirmed in the following taxonomic diversity analyses. Other epibionts, one

of which was identified as *Ca*. Marithrix, are pictured in dark blue in Figure 10A. It is pictured as DAPI stained only, as FISH probes did not hit it successfully.

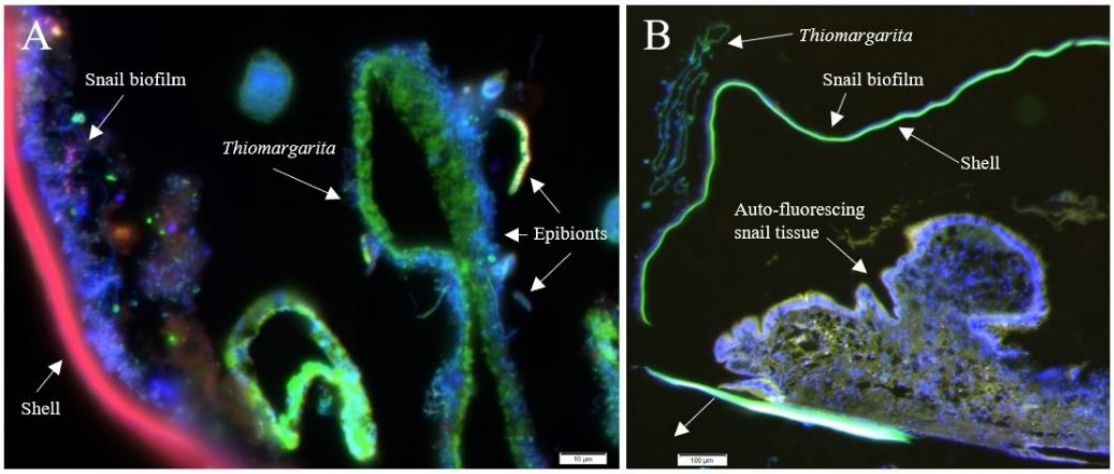

Figure 9A, B: FISH imaging of HR snail shell | A) The snail shell, visible in pink, lines the lower left of the image. The snail's biofilm fluoresces in indigo. *T. nelsonii* was identified as the large, green-colored object, while smaller, epibionts of uncertain identities exist mainly on the right side of the image. **B)** A different section of the snail was imaged featuring snail tissue. Again, the snail's shell and biofilm are visible as a solid green line, while *T. nelsonii* was identified in the upper left of the thin section.

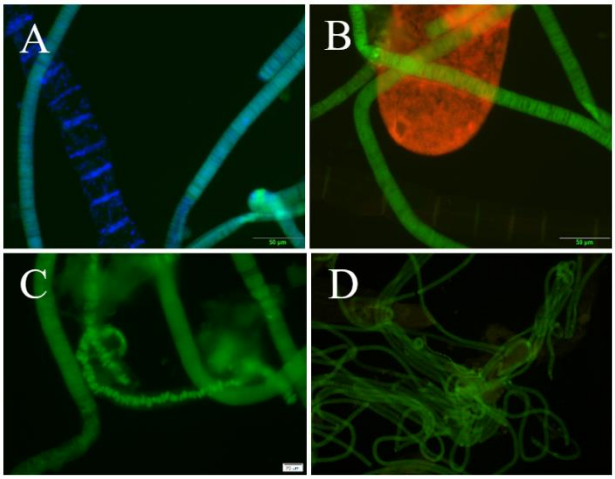

Figure 10A-D: FISH imaging of HR snail epibionts | A) *Marithioploca* in dark blue and Chloroflexi in light blue. B) *Thiomargarita* in orange and Chloroflexi in green. C-D) Chloroflexi.

The third and final epibiont, which is described for the first time here, is imaged in Figure 10. Further described in following sections, these filaments found on the shell of *Provanna* were characterized as an undescribed member of the phylum Chloroflexi by DNA sequencing (Section 2.2.6).

2.3.3 *Novel Chloroflexi*

The highly novel and divergent organism described below is most closely related to the Chloroflexi class *Ardenticatenia,* and was found to branch within the *Ardenticatenia* branch in following phylogenetics analyses (Section 2.3.4). The family *Ardenticatenaceae*, originally proposed by Kawaichi in 2013, include filamentous bacteria that metabolize under aerobic or anaerobic conditions and use nitrate or ferric iron as their terminal electron acceptor. Neither photosynthetic nor fermentative metabolisms have been previously documented of this family. *A. maritima,* the only known member of *Ardenticatenia,* is a filamentous bacterium discovered in an iron-rich coastal hydrothermal field in Japan (Kawaichi et al., 2013). It grows heterotrophically on pyruvate, sugars, starch, and yeast extract using oxygen. It can use iron or nitrate as terminal electron acceptors, facultatively under anaerobic conditions. Its genome was sequenced by Hemp et al. (2015).

All three filaments of this *Ardenticatenia* Chloroflexi (hereafter referred to as F1, F2, and F3) contain genes for carbon monoxide dehydrogenase, an oxygen-sensitive enzyme used to grow on carbon monoxide and/or oxidize lactate to carbon dioxide (Hocking et al., 2015). The partial genomes of F1 and F2 contains genes for the small, medium, and large subunits of this enzyme; F3 contains genes for the medium subunit only. Other genes for oxygen-sensitive enzymes such as CoB-CoM heterodisulfide reductase subunits A, B, and C are present in all three filaments. Oxygen-sensitive FeS-cluster-containing hydrogenase components, a metal cofactor used primarily for electron transfer but also for substrate binding and gene regulation, are also present in all filaments (Ayala-Castro et al., 2008). F1 contains A, B, and C subunits of a heterodisulfide reductase, which, if paired with an Fd:NADH oxidoreductase in the full genome, would pose the possibility of the production

of ATP via chemiosmosis (Sewell et al., 2017). All filaments contain genes for a bacterial F-type ATPase used for ion-gradient phosphorylation. There are also genes for a cytochrome c oxidase, which serves as the final enzyme in the electron transport chain for oxygen respiration.

Genes for proteins associated with assimilatory nitrate reduction to convert nitrate to ammonia are present, i.e., an assimilatory nitrite reductase (ferredoxin) precursor (F1) and a ferredoxin-nitrate reductase (F1). However, the Chloroflexi's closest relative, *A. maritima,* contains genes for proteins associated with dissimilatory nitrate reduction such as a periplasmic nitrate reductase subunit NapA apoprotein and a dissimilatory nitrite reductase (NO-forming), copper type apoprotein.

The F1 and F3 partial genomes contain genes for the C-terminal domain of methylmalonyl-CoA mutase associated with the 3-hydroxypropionate bicycle. F3 also contains genes for malonyl-CoA/methylmalonyl-CoA synthetase and propionyl-CoA synthetase, and F2 contains genes for a propionyl-CoA carboxylase, all of which are also parts of the pathway for a hydroxypropionate pathway. While methylmalonyl-CoA is not unique to this pathway, propionyl-CoA is specific to this pathway (Shih et al., 2017).

F1 contains genes for a 5-metyltetrahydrofolate-homocysteine methyltransferase. F2 contains genes for a NAD-dependent formate dehydrogenase catalytic subunit. These molecules, along with the carbon monoxide dehydrogenases, are part of the reductive acetyl-CoA cycle, or Wood-Ljungdahl pathway, for acetogenesis. The Wood-Ljungdahl and hydroxypropionate pathways are known to co-occur in *C. aurantiacus,* not eliminating the possibility of both carbon fixation pathways occurring here (Tabita, 2009).

2.3.4 Phylogenetic relationships

To more completely identify this Chloroflexi, phylogenetic analyses were employed on the 16S rRNA gene sequence of filament 3 since it was the only complete 16S rRNA gene of the three (Figure 11). Closest relatives were selected from MegaBLAST due to a BLASTn search being inconclusive on this apparently highly divergent strain (Morgulis et al., 2008; Altschul et al., 1990). The filament branches within the Chloroflexi, and within same branches as the *Ardenticatenaceae.* The closest identifiable relative as found by our phylogenetics methods is *Ardenticatena maritima*. As previously discussed, *A. maritima* is the only organism of its genus and was discovered in an iron-rich coastal hydrothermal field in Japan growing heterotrophically using oxygen, or iron and nitrate in anoxic conditions (Kawaichi et al., 2013).

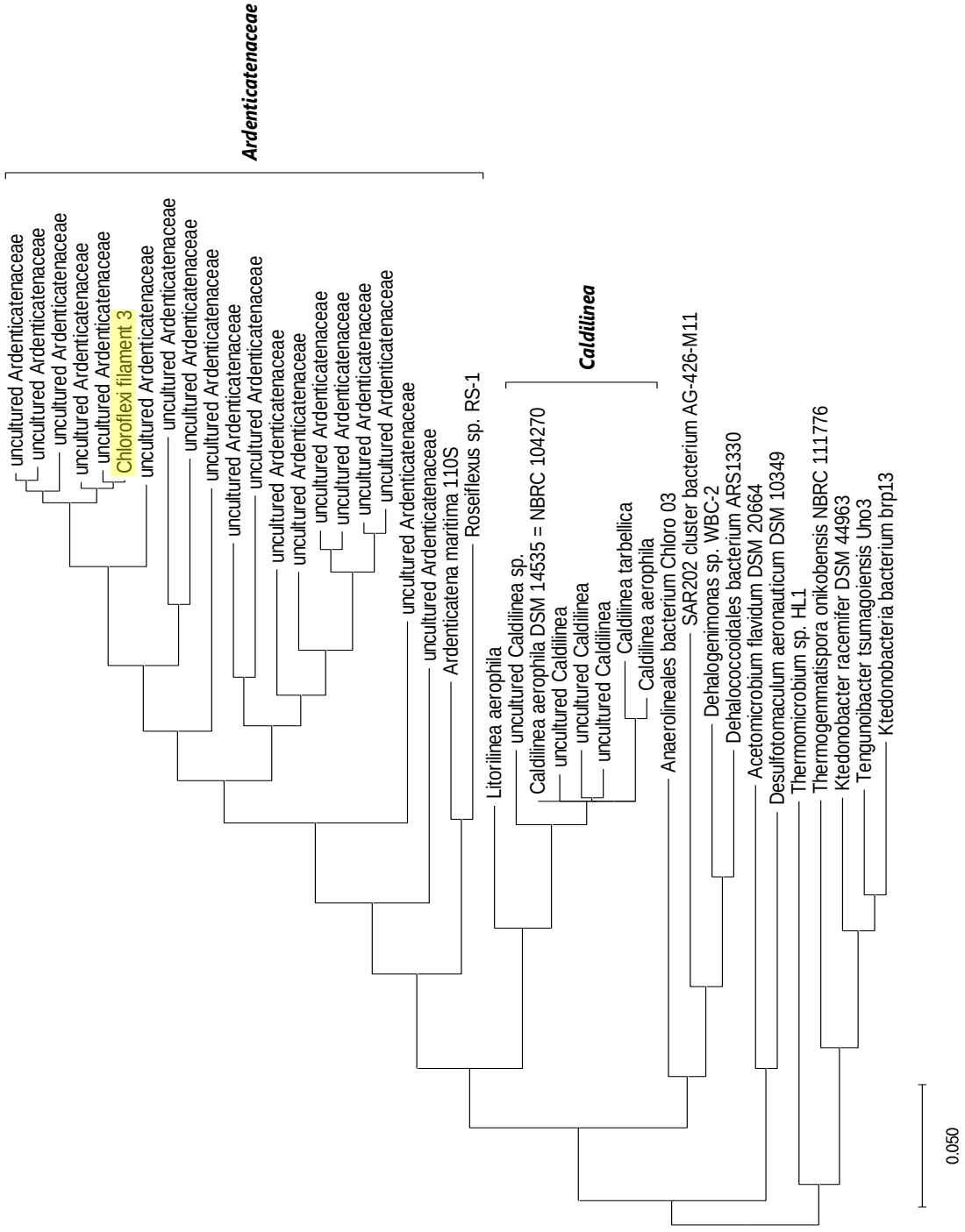

Figure 11: Maximum likelihood phylogenetic tree of the 16S rRNA gene of Chloroflexi filament 3 | Phylogenetic tree created in MEGA-X version 11 (Tamura et al., 2021, Kumar et al., 2018) of Chloroflexi filament 3, closest relatives, and selected outgroups. Closest relatives were selected from MegaBLAST (Morgulis et al., 2008). Alignment completed by SILVA ACT (Pruesse et al., 2012). Chloroflexi filament 3 is highlighted.

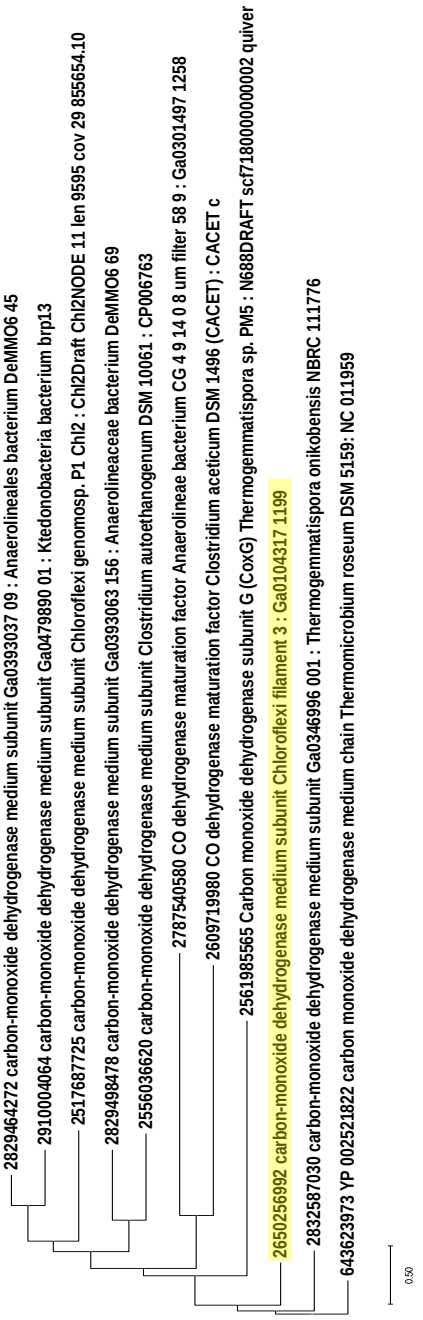

Figure 12: Maximum likelihood phylogenetic tree of Chloroflexi F3's carbon monoxide dehydrogenase medium subunit | Maximum likelihood phylogenetic tree created in MEGA-X version 11 (Tamura et al., 2021, Kumar et al., 2018) of protein sequence for carbon monoxide dehydrogenase medium subunit from Chloroflexi F3 (highlighted) and CO dehydrogenases in other organisms whose functionality have been supported. Protein alignment was performed with MUSCLE within MEGA-X version 11.

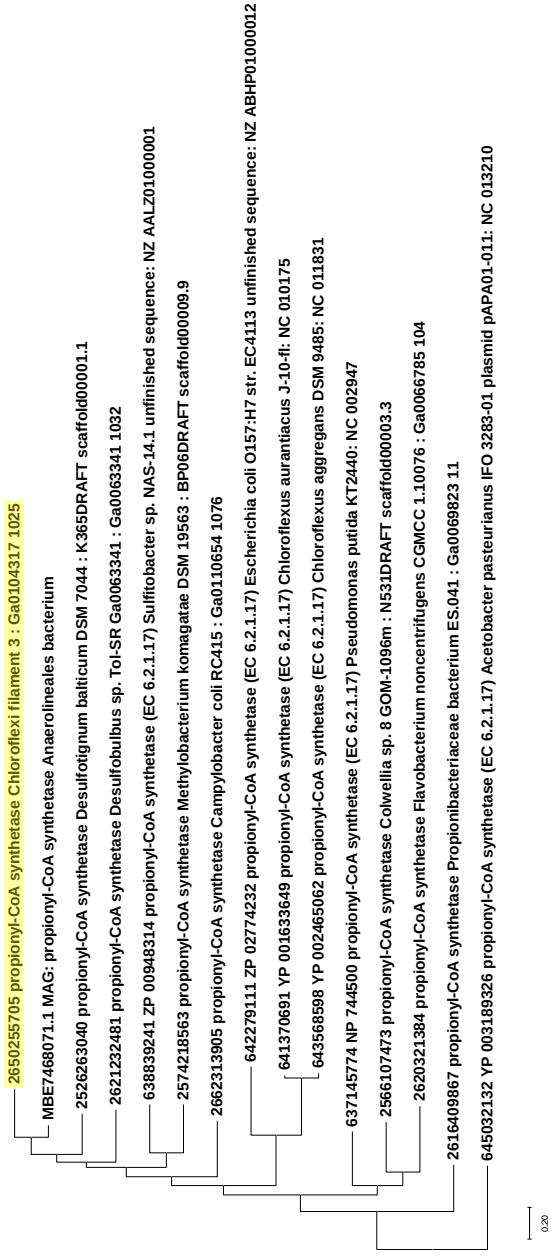

Figure 13: Maximum likelihood phylogenetic tree of Chloroflexi F3's propionyl-CoA synthetase | Maximum likelihood phylogenetic tree created in MEGA-X version 11 (Tamura et al., 2021, Kumar et al., 2018) of protein sequence for propionyl-CoA synthetase from Chloroflexi F3 (boxed) and propionyl-CoA synthetases in other organisms whose functionality in the 3-hydroxypropionate bicycle is supported (*C. aurantiacus*), undetermined, or nonexistent. Protein alignment performed with MUSCLE within MEGA-X version 11.

Phylogenetic analysis was also employed on a gene for a carbon monoxide dehydrogenase medium subunit found in all three filaments (Figure 12). F3's genes encoding a carbon monoxide dehydrogenase medium subunit were found to lie on their own branch of the phylogenetic tree when compiled with relatives known to have a functional CO dehydrogenase. The gene(s) of interest is most closely related to dehydrogenase-coding genes of *Thermogemmatispora onikobensis* and an unidentified species of *Thermogemmatispora* (both class *Ktedonobacteria*). In the genus *Thermogemmatispora*, the dehydrogenase has been shown to allow the oxidation of CO, as members of this genus oxidize atmospheric H_2 and CO (Islam et al., 2019). This genus, along with other Chloroflexi, have been analyzed comprehensively and are well known to oxidize the above gases via CO dehydrogenases.

Phylogenetics analysis was also employed on a gene for a propionyl-CoA synthetase found in F3's genome (Figure 13). It was found to be most closely related to a propionyl-CoA synthetase in an *Anaerolineales* (of Chloroflexi) bacterium. In *Anaerolineales,* the propionyl-CoA synthetase likely does not contribute to a 3-hydroxypropionate bicycle, as investigated members of *Anaerolineales* do not contain all the genes necessary for the pathway (Figure 14; Shih et al., 2017). Interestingly, the propionyl-CoA synthetase gene in Chloroflexi F3 is *not* closely related to two of the three Chloroflexi included in this analysis. One of those included Chloroflexi is *C. aurantiacus,* the only organism on the tree proven to use propionyl-CoA synthetase in the 3-hydroxypropionate bicycle (Hügler and Sievert, 2011; Shih et al., 2017).

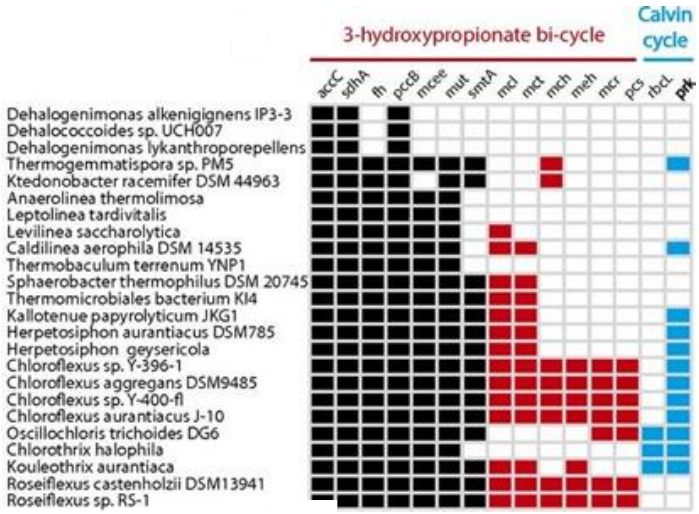

Figure 14: Genes for the 3-hydroxypropionate pathway in the Chloroflexi | Presence of genes for the 3-hydroxypropionate pathway are mapped in various species of the Chloroflexi. While only *C. aurantiacus* has been shown to actively use this pathway, a few other species contain all necessary genes for its use. Figure adapted from Shih et al. (2017).

2.3.5 *Taxonomic diversity*

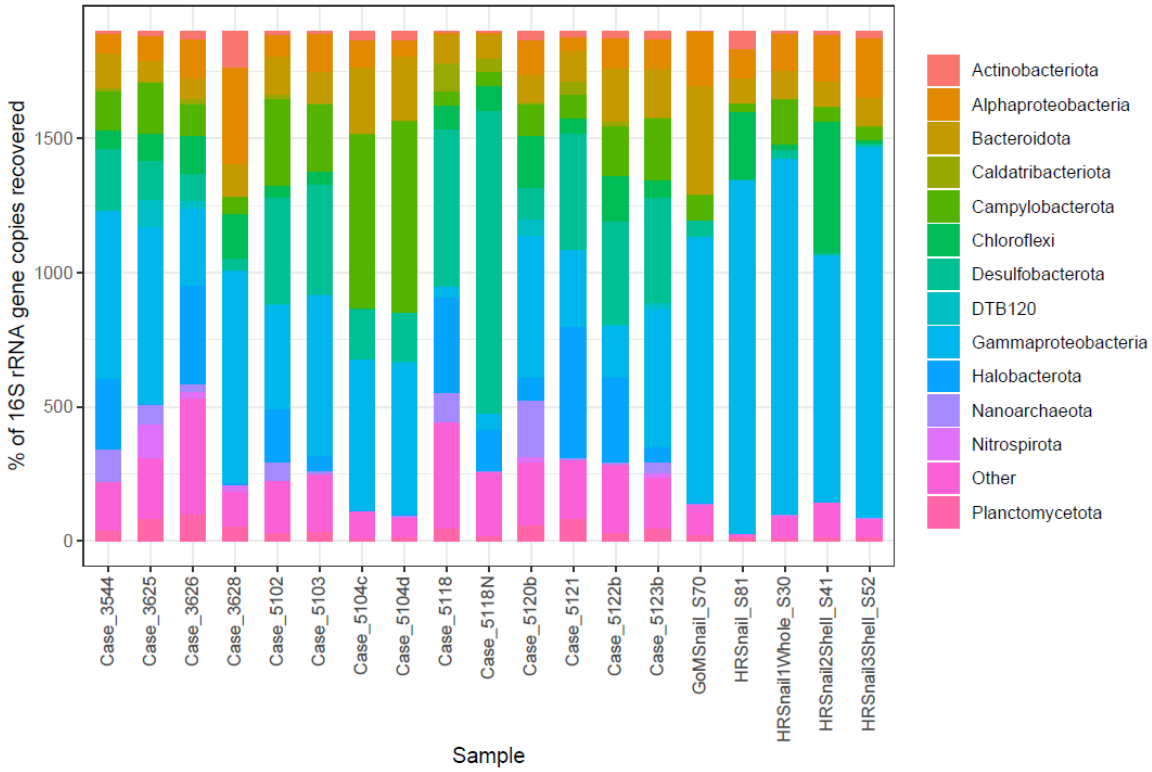

Figure 15: Abundance of prokaryotic phyla, all samples | Gammaproteobacteria comprise the largest proportion of snail-associated samples. Environmental samples have a more even distribution of phyla, some with large amounts of representatives of the Campylobacterota (5104c, 5104d). 5118 and 5118N have extensive ASVs from within the Desulfobacterota and very minimal amounts of Gammaproteobacteria.

Most samples, whether gastropod-associated or environmental, are characterized by large amounts of ASVs assigned to the Gammaproteobacteria. They make up the vast majority of ASVs in the gastropod samples, while environmental samples have a more even distribution of bacterial taxa (i.e., native carbonate sample Case 3626 and transplanted carbonate Case 5121). A few individual samples contain a large proportion of a different bacterial taxon. Namely, the native nodule (Case 5118) and the native sediment (Case 5118N) contain many more representatives of the Desulfobacterota than any other taxon and any other sample (Figure 12).

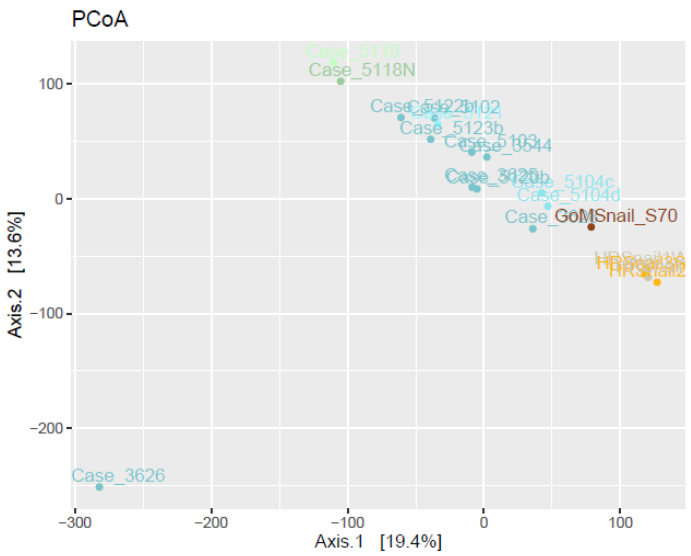

Figure 16: PCoA plot, all samples | Samples are similar except Case_3626, a native carbonate sample from Hydrate Ridge. This sample is significantly dissimilar.

As shown in Figure 15, native carbonate Case 3626 is also an outlier in its similarity to all other samples, gastropod and environmental. At Hydrate Ridge, gastropod samples have exceptionally low Shannon alpha diversity (average $\alpha = 3.9$) compared to environmental samples (average $\alpha = 5.1$). However, the Gulf of Mexico gastropod is comparable to the environmental samples at Hydrate Ridge ($\alpha = 5.1$). Results are similar in terms of Chao1 alpha diversity measures (Table 2).

An ASV identified as the family Thiotrichaceae makes up nearly half of the hits in the whole snail samples at Hydrate Ridge. An ASV for a representative of the family Ardenticatenaceae and another for the family Colwelliaceae follow. The Hydrate Ridge shell is characterized by roughly one half Thiotrichaceae ASVs as well, but a proportion of Ardenticatenaceae larger than that of the whole snail, as well as an ASV for Beggiatoaceae (family), follow. There are several taxa unique to the snail shell: Comamonadaceae, Beijerinckiaceae, and the PS1 clade of Parvibaculales (order). Thiotrichales is also the largest identified ASV in the Gulf of Mexico whole snail sample, and families Flavobacteriaceae and Methylomonadaceae follow in large proportions.

Sample group	Average Shannon alpha diversity	Average Chao1 alpha diversity
Hydrate Ridge, gastropod	3.9	413
Hydrate Ridge, environmental	5.1	891
Gulf of Mexico, gastropod	5.1	870

Table 2: Diversity of samples | Average Shannon and Chao1 alpha diversity measures were calculated and are listed for each grouping of samples.

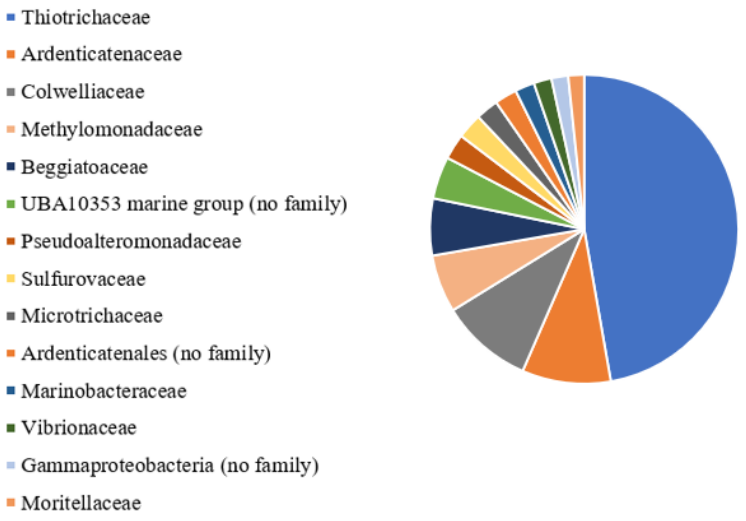

Figure 17: Relative abundance of prokaryotic families, HR whole snail samples | Prokaryotic families present in whole gastropod from Hydrate Ridge samples. Thiotrichaceae ASVs are by far the most abundant in HR whole snail samples.

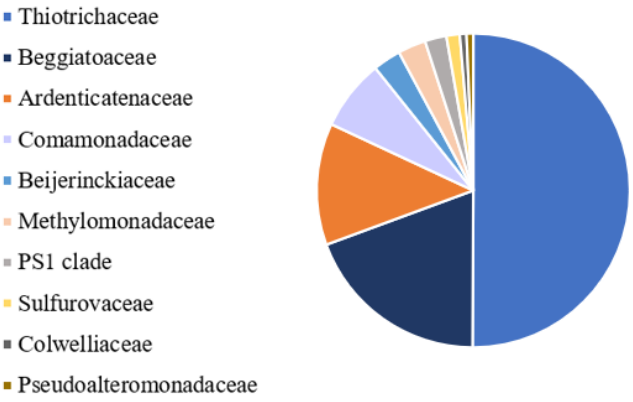

Figure 18: Relative abundance of prokaryotic families, HR shell only samples | Prokaryotic families represented by ASVs recovered from gastropod shell from Hydrate Ridge samples. Thiotrichaceae dominate the shells of provannid gastropods from Hydrate Ridge, followed by ASVs in the Beggiatoaceae and Ardenticatenaceae.

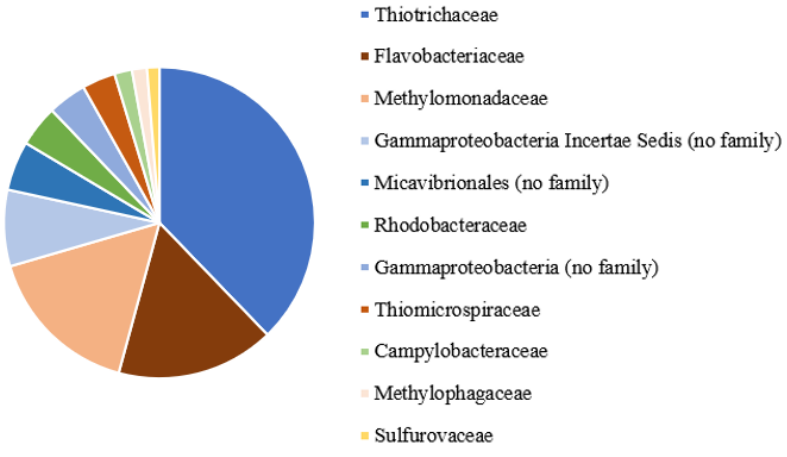

Figure 19: Relative abundance of prokaryotic families, GoM whole snail samples | Prokaryotic families present in whole gastropod from the Gulf of Mexico samples, Thiotrichaceae is by far the most abundant family in HR whole snail samples followed by Flavobacteriaceae and Methylomondaceae.

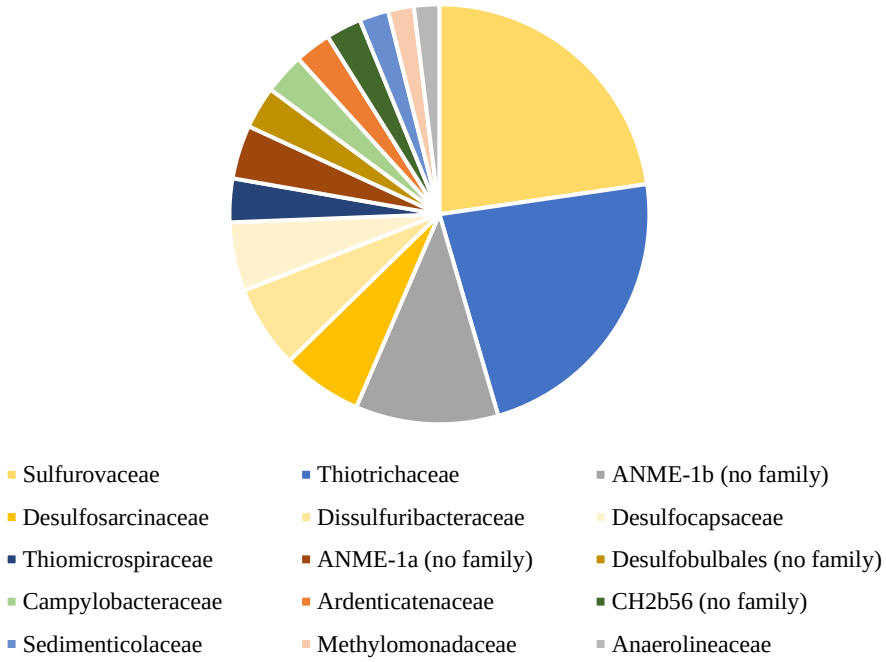

Figure 20: Relative abundance of prokaryotic families, HR environmental samples | Prokaryotic families present in the Hydrate Ridge environment. Sulfurovaceae and Thiotrichaceae are the most common families in the carbonate of Hydrate Ridge.

The microbial community is very different in non-gastropod samples. Instead, the surrounding environment without regard to specific substrate explored in Case et al. (2015) is dominated by Thiotrichales, Sulfurovaceae, ANME-1b archaea, and a suite of sulfur reducing bacteria such as Desulfosarcinaceae SEEP-SRB1, Desulfocapsa, Desulfobulbales, and Dissulfuribacter SEEP-SRB2 (Figure 19). The ubiquity of SRB and ANME archaea in environmental samples compared to the gastropod shell (the latter of which is most enriched in sulfur oxidizing bacteria including representatives of the Thiotrichaceae such as the Beggiatoacaea and Ardenticatenaceae) is supportive of a unique relationship between the snail and its microbiome compared to its environment.

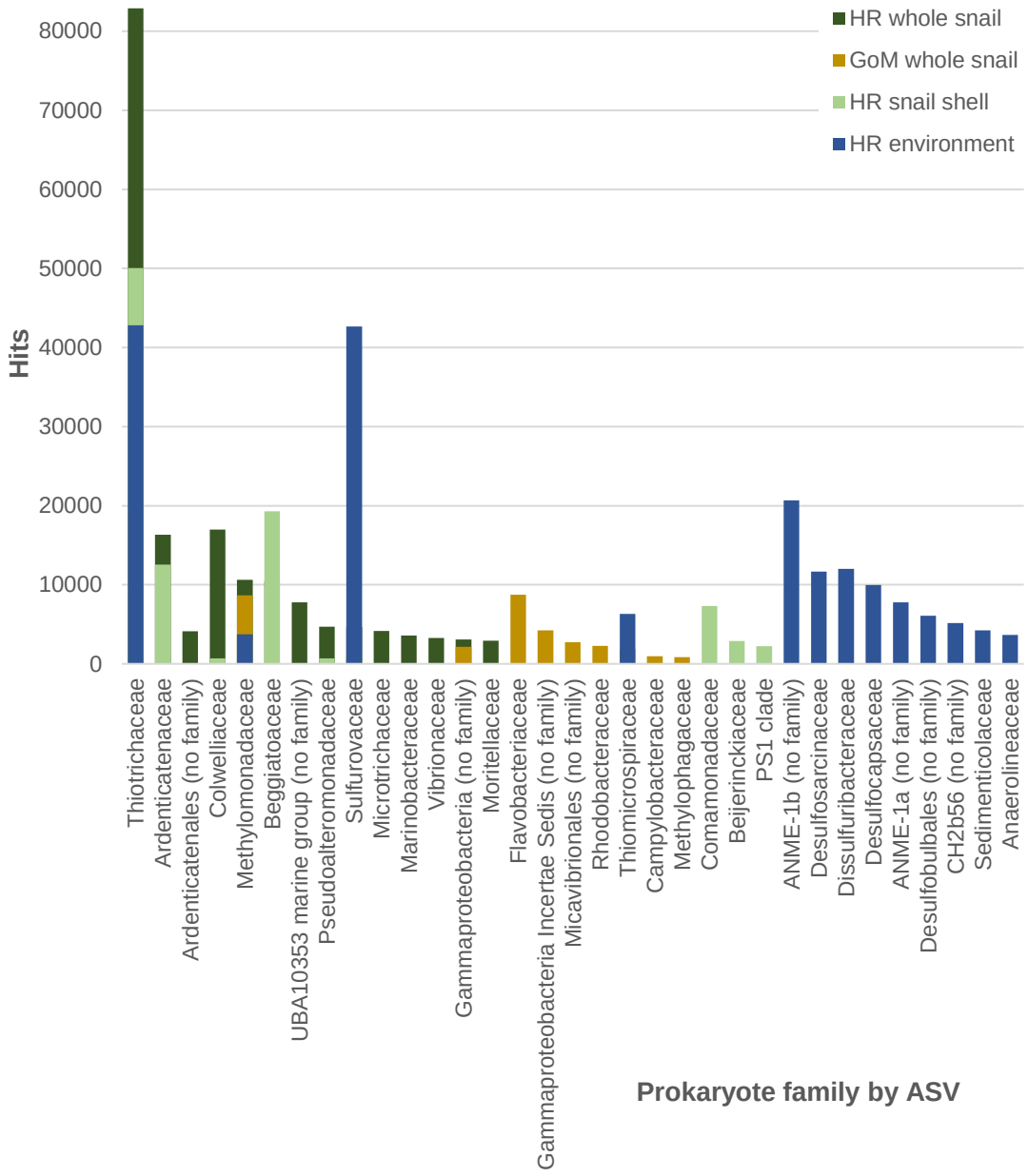

Figure 21: Abundance of prokaryotic families, all samples | Prokaryotic families present in different sample types (whole gastropod from Hydrate Ridge, whole gastropod from the Gulf of Mexico, gastropod shell only from Hydrate Ridge, and the surrounding Hydrate Ridge environment. The SOB families Thiotrichaceae and Sulfurovaceae include the most abundant ASVs in the HR environment, followed by several SRB and ANME (two groups not found in any snail samples). The Thiotrichaceae is by far the most abundant family in HR whole snail samples. Beggiatoaceae is the most abundant family on HR snail shells and is also unique to those samples. Flavobacteriaceae is the most abundant family in GoM whole snail samples, and is not found in any other sample(s).

2.4 Discussion

2.4.1 *Microbial diversity*

When comparing the ASVs identified through iTag analyses in each sample type, distinct communities associated with different habitats can be observed. Snail communities at both Hydrate Ridge and the Gulf of Mexico are largely comprised of sulfur oxidizing bacteria, namely of the family Thiotrichaceae. Methylotrophs are more prominent in the Gulf of Mexico snail. The microbial diversity of the shell community is lower than that of the whole snail likely due to the subtraction of gut contents. Beggiatoaceae—presumably *Ca.* Marithrix that was then identified via FISH—are more prominent on the shell than in the whole snail, as is Candidatus *Ardenticatenaceae,* the isolated and sequenced Chloroflexi in section 2.6. Environmental samples from Hydrate Ridge are also enriched in Thiotrichaceae, but are nearly matched in number of hits by ASVs of the sulfur-oxidizing family Sulfurovaceae. Among the samples analyzed here, a number of taxa of sulfur reducing bacteria are unique to the environmental samples, including Desulfosarcinaceae, Dissulfuribacteraceae, Desulfocapsaceae, and Desulfobulbales. ANME archaea are also limited to the environment and absent from the snail samples. The stark contrast between the snails, regardless of collection site, and the seep environment lies in the sulfur oxidation taking place on the snail and sulfur oxidation *and* reduction and ANME taking place in the surrounding carbonates/sediments. Part of this is likely because many of the environmental samples record anoxic conditions, while snail samples are inherently oxic due to the animal's respiration. The distinctive enrichment of sulfur oxidizing-bacteria colonizing provannid gastropod shells suggests that the snail serves to promote their growth. Observed behaviors of these snails in the laboratory provide additional support for this idea.

2.4.2 *Gastropod behaviors*

The potential significance of the oscillating motility behaviors described in 3.1 becomes clear when considering the assemblage of sulfur oxidizers on the shell. We hypothesize here that the microbiome hosted by the gastropod shell is dependent on the snail's movement between the water and the sediment-water interface to access reduced sulfur in the sulfidic sediments and oxygen in the water column as the electron acceptor. Because the snail was observed feeding on the microbiome as it encroached on its rostrum and the detection of SOB in the snail samples, it is likely that the proliferating microbial mat on its shell serves as a food source in return. These observations, in conjunction with the known metabolisms of these bacteria, local biogeochemistry, and other well-cited examples of eukaryote-prokaryote mutualism, make this hypothesis plausible. These behaviors should be further investigated by isolating the contents of the gut of the gastropod to confirm that it benefits from its 'hitchhikers' as a food source.

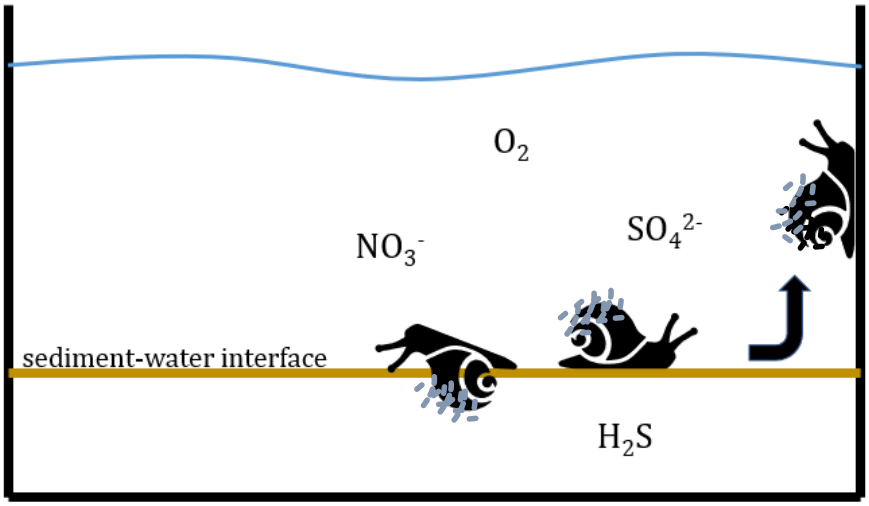

Figure 22: Snail behavior | Spatial and temporal snail behavior is depicted. Oscillation between the sediment-water interface and the simulated water column was repeated behavior and is suspected to allow access to electron

2.4.3 *Chloroflexi description*

Based on our analyses of genomes with limited completeness, the previously undescribed Chloroflexi of family Ardenticatenaceae in this study is likely an acetogen that grows on carbon monoxide, performs oxidative phosphorylation, assimilatory nitrate reduction, and operates via both the Wood-Ljungdahl pathway and potentially the 3-hydroxypropionate bicycle pathway for carbon fixation.

The genes for a carbon monoxide dehydrogenase present in all three filaments are required for growth on carbon monoxide, as it catalyzes the conversions between carbon monoxide and carbon dioxide (Hocking et al., 2015). Alternatively, some carbon monoxide dehydrogenases aid the acetyl-CoA pathway in oxidizing lactate to carbon dioxide (Hocking et al., 2015). The gene for a CO dehydrogenase medium subunit in F3 is most closely related to CO dehydrogenase genes from members of the Chloroflexi genus *Thermogemmatispora.* These genes have proven functionality in respiring CO (Islam et al., 2019), supporting its functional operation in our bacterium. All filaments contain essential genes for ion-gradient phosphorylation such as an F-type ATPase that are typical of bacteria, making it likely for this organism. It may perform assimilatory nitrate reduction due to the presence of genes for an assimilatory nitrite reductase (ferredoxin) precursor and a ferredoxin-nitrate reductase.

The presence of genes for a 5-methyltetrahydrofolate-homocysteine methyltransferase and a NAD-dependent formate dehydrogenase catalytic subunit along with the carbon monoxide dehydrogenases support the use of the reductive acetyl-CoA (Wood-Ljungdahl) pathway for this organism. Acetogenesis has been documented in other Chloroflexi, such as a deep sea Chloroflexi that performs homoacetogenesis (Sewell et al., 2017) and in

subseafloor-dwelling Chloroflexi which is supported by the finding of genes for pathway-specific enzymes, electron bifurcation, and abundant ferredoxins (Fincker et al., 2020). Because this pathway operates under anaerobic conditions only, we can confirm that the Chloroflexi is at least partially anaerobic. Because there are genes for a cytochrome c oxidase present in at least one filament, we can infer that this bacterium can respire oxygen via the electron transport chain. Although the Wood-Ljungdahl pathway is strictly anaerobic and our Chloroflexi contains other oxygen-sensitive genes, its capability for oxygen respiration opens up the possibility for this bacterium to be facultatively anaerobic, or at least oxygen-tolerant, and able to thrive in oxic environments as well.

Partial genomes from all filaments contain genes for the hydroxypropionate pathway for carbon fixation, and, although not complete, the filament genomes together demonstrate the possibility of this, especially due to propionyl-CoA's exclusivity to the hydroxypropionate pathway (Figure 22). Despite its distance from *C. aurantiacus'* gene for propionyl-CoA synthetase that would imply the use of the 3-hydroxypropionate cycle, Hügler and Sievert (2011) among others suggest that an operating 3-hydroxypropionate bicycle is likely present in Chloroflexi other than *C. aurantiacus*. possibility remains although not supported by this phylogenetics analysis. Several genes within this pathway are oxygen-sensitive, which supports that this organism has anaerobic capabilities. Whether this is facultative or obligate cannot be determined from this data, but this bacterium is likely an oxygen-tolerant anaerobe due to the anaerobic nature of acetogens. One of the genes suggestive of a hydroxypropionate metabolism, methylmalonyl-CoA mutase, was also assigned to another Chloroflexi, *C. aurantiacus*, a filamentous, anoxygenic phototroph (Tang, et al., 2011). This further supports a primarily anaerobic life

mode for the *Ardenticatenaceae* Chloroflexi studied here. *C. aurantiacus* also potentially fixes carbon via the Wood-Ljungdahl pathway, possibly because 3-hydropropionate is secreted as a metabolite but it could possibly serve as an intermediate for the Wood-Ljungdahl pathway, helping to fix carbon and produce pyruvate as well (Tabita, 2009). We propose that our Chloroflexi bacterium is utilizing its available resources more efficiently by using both carbon fixation pathways.

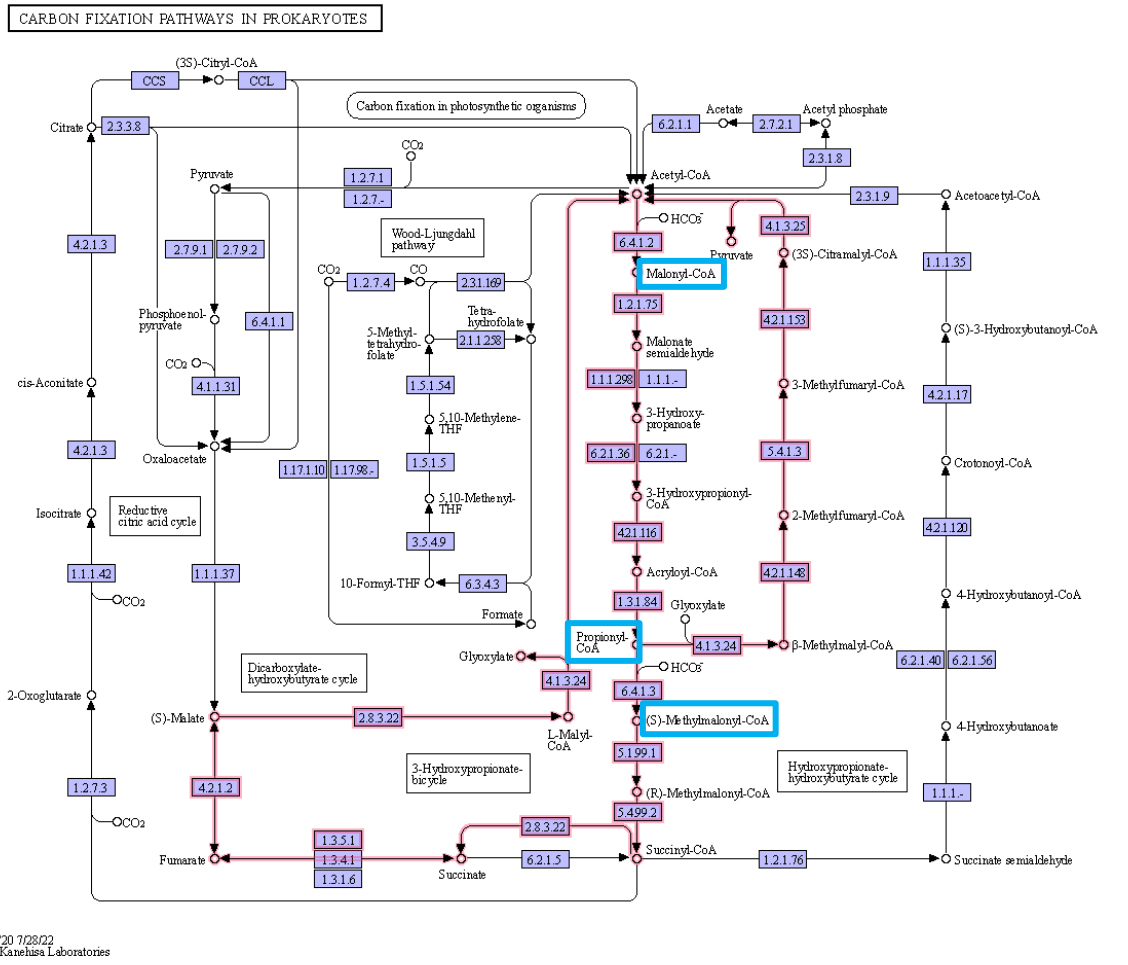

Figure 23: Present 3-hydroxypropionate bicycle molecules | Figure of carbon fixation pathways in prokaryotes with enzymes involved in the 3-hydroxypropionate bicycle boxed in red. Enzymes present in one or more filaments are boxed again in blue and include malonyl-CoA, propionyl-CoA, and methylmalonyl-CoA.

2.4.4 *Potential epibiotic function of shell community*

Since filaments on the shells were isolated and analyzed to be *Ca.* Marithrix, *T. nelsonii*, and an undescribed Chloroflexi of family *Ardenticatenaceae* with predicted metabolisms, we are able to illuminate the potential function of this gastropod-attached epibiotic community. As explored from a bioinformatics standpoint, the Chloroflexi is an acetogen that grows on carbon monoxide and performs assimilatory nitrate reduction, as well. *Ca.* Marithrix is a filamentous, non-motile, seep-dwelling bacterium that has been observed being grazed on by gastropods (Salman-Carvalho et al., 2016). It is a member of the 'giant sulfur bacteria' family Beggiatoaceae, a family of sulfur-oxidizing bacteria within the Gammaproteobacteria. *T. nelsonii* is a filamentous, non-motile marine bacterium also in the family Beggiatoaceae. This filamentous bacterium described in section 1.1.3 is ubiquitous at cold seep environments, accessing sulfur in the sediments and nitrate/oxygen in the water column.

Symbiotic relationships are common at cold seeps as explored in Chapter 1, and some basic principles of these relationships can be considered when discussing the potential role that our microbiome plays in the cold seep environment. However, the epibiont microbial community described here serves in a potentially *episymbiotic* relationship with the gastropod, so using existing relationships as estimations is limited since they are mostly endosymbiotic. One known episymbiotic relationship at vents is between the yeti crab and microbial mats on its claws. The microbial mat grows on methane and hydrogen at hydrothermal vents and the crab feeds only on its external microbial mat. Because invertebrates live in narrow redox zones that may be close to their physiological limits, they are largely reliant on chemolithoautotrophic bacteria as their only nutritional source

(Roterman et al., 2018). Similarly, we hypothesize that the symbiotic function of the microbial mat is serving as the gastropod's main food source. This is supported by the observation of the *Provanna* feeding on the shell-attached mat. The mat, comprised of *Ca.* Marithrix, *T. nelsonii*, and a novel Chloroflexi of family *Ardenticatenaceae*, hypothetically requires the movement between redox zones considering the life mode of at least one of its members (*T. nelsonii*). Assuming this is the case, the Chloroflexi is a facultative anaerobe.

By considering the functions of the three bacteria—*Ca.* Marithrix, *T. nelsonii*, and *Ardenticatenaceae*—found colonizing the surface of the *Provanna* shell, the behavior of the gastropod, the surrounding environmental microbial diversity at Hydrate Ridge and in the Gulf of Mexico, and what is known of the biogeochemical cycling at cold seeps, we contextualized the *Provanna* and its 'hitchhikers' in the framework of cold seeps and hypothesized an explanation of their roles in these unique environments.

2.5 Conclusion

The methane seeps at Hydrate Ridge North host copious amounts of microorganisms living via modes and pathways not commonly thought of outside of the scientific community, truly expanding our common definition of life. Methylotrophs, ANME archaea, and sulfur bacteria all proliferate in such conditions, living off of abundant methane and cascading, biologically mediated reactions.

We presented evidence that contributes to the collective illustration of life at methane seeps that has been amassing since their discovery in 1985. A potential symbiotic relationship between provannid snails and their shell microbiomes is suggested by this study as the snail possibly provides motility to the attached microbes, while the microbes serve as a nutrient source for the snail. This potential relationship expands our knowledge of what symbiotic relationships and unique life modes can look like under deep ocean conditions. The growing catalogue of these relationships at vents and seeps suggest that perhaps symbiosis is the rule, not the exception.

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