

**MICROBIAL INTERACTIONS IN AN ANTARCTIC DESERT: RESPONSES TO
ENVIRONMENTAL CHANGE AND TOOLS FOR LIFE DETECTION**

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Degree
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DEDICATION

To my father, for inspiring my love of science,
and to my mother, for being my biggest cheerleader and my best friend.

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ABSTRACT

Cryosphere microorganisms can provide fundamental insights into individual and community response to a changing climate, and how we may detect past or present life on other planets. The polar desert of the McMurdo Dry Valleys (MDV), the largest ice-free region in Antarctica, supports microbially-dominated ecosystems that experience extremes of temperature, salinity, nutrient availability, and solar radiation. A changing Antarctic climate leads to alteration of these natural gradients, thus, studying how organisms respond to changing stressors can inform predictions of future impacts to ecosystems. This dissertation elucidates how MDV microorganisms interact with their environment through culture-based and multi-omics techniques performed on both environmental materials and bacterial isolates. We describe the ecophysiology of a red pigmented strain of *Massilia frigida* (strain DJPM01) isolated from a microbial mat. Through genomic analyses, adaptations to cold and saline environments, and biosynthesis genes for the pigment prodigiosin were identified. The synthesis of prodigiosin was confirmed using mass spectrometry, and breakdown of this pigment was observed when DJPM01 cultures were incubated under ultraviolet light, a relevant stressor for polar bacteria. To investigate the impact of salinity on whole communities and individual species, we leveraged samples from a natural salinity gradient in MDV soils that forms from permafrost melt. Environmental soil DNA was sequenced to examine differences in community structure and potential correlations with salinity. We then identified changes in transcription and metabolite production in a potentially novel *Gillisia* sp., isolated from this soil gradient, under elevated salt concentrations. A *Shewanella* sp. BF02_Schw, isolated from a Mars analog feature known as Blood Falls, was used to describe biosignatures produced during iron reduction, a plausible metabolic strategy for life on early Earth and Mars. Employing instrumentation relevant to both *in situ* and sample return Mars missions, we identified mineral and molecular biosignatures produced under high iron concentration and confirmed the biogenicity of these signatures via transcriptomics. Collectively, this dissertation describes specific ways that MDV microorganisms interact with their environment that provide insight into the impact of

climatic change on Antarctic communities and inform strategies for the detection of life on other planets.

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CHAPTER ONE INTRODUCTION

The cryosphere and microbial communities

Earth's cryosphere, areas of the planet where water exists in a liquid form, is composed of permafrost, glaciers, ice sheets, and sea ice. Icy regions remain one of the least studied biomes on Earth and can be leveraged to answer broad ecological questions due to the absence of large plants and animals and the susceptibility of these regions to climate change (Shu and Huang, 2022). Organisms from permanently cold environments have unique adaptations that include the production of biomolecules that can be utilized for pharmaceutical or industrial applications (Giddings and Newman, 2015). However, much remains unknown about the diversity, function, and extent of cryosphere microbial communities (Boetius et al., 2015; NAESM, 2024). In the context of a rapidly changing environment, the study of these communities is urgently needed to understand disruption of ecological functions and the threat to biodiversity in the cryosphere (Kennicutt et al., 2014, 2019; Margesin and Collins, 2019; Chown et al., 2022; IPCC, 2022).

In addition to providing insight into terrestrial biology, the cryosphere provides an unparalleled opportunity to investigate adaptive strategies and ground-truth instrumentation relevant to the search for life on other planets. Astrobiological targets of interest in our solar system, such as Mars and ice-covered ocean worlds, host possible biological niches shaped by low temperatures, high salinity, aridity, and UV-radiation (Davila and Schulze-Makuch, 2016; Hand et al., 2020). Terrestrial analog features (i.e., locales on Earth with specific similarities to possible niche spaces beyond Earth) provide crucial insight into the limits for life as we know it, while being considerably more accessible than even our closest planetary neighbors (Cassaro et al., 2021). Thus, investigation of microbial processes and interactions in cryosphere environments, such as the Antarctic polar desert, furthers our understanding of community resilience to climatic change and the possibility of life on other planets.

The McMurdo Dry Valleys (MDV)

The McMurdo Dry Valleys (MDV) of Antarctica (77–78°S, 160–164°E) are the largest ice-free region of the continent (Lee et al., 2017), and are the coldest and driest

desert on the planet (**Figure 1.1**). The MDV span approximately 4800 km² (ice-free regions; Levy, 2013), and were formed by millennia of glacial and lacustrine processes (Fountain et al., 1999). The MDV receive less than 50 mm of precipitation annually, almost exclusively in the form of snowfall, which frequently sublimates before melting (Fountain et al., 2009). Annual temperature regimes are used to divide the MDV into three regions: Coastal Thaw Zone (mean summer temperature ≥ -5 °C), the Intermediate Mixed Zone (mean summer temperature -10 to -5 °C), and the Stable Upland Zone (mean summer temperature < -10 °C; (Marchant and Head, 2007; Fountain et al., 2014). Areas within the Coastal Thaw Zone, which reaches tens of km inland along the valley floors, are characterized by a seasonally thawing soil active layer, glacial melt streams, and include several large, ice-covered lakes. Permafrost within the Coastal Thaw Zone is near surface (10-60 cm) and ice cemented, thus can contribute to elevated soil moisture through wicking processes (Bockheim et al., 2007, 2008; Ball et al., 2011, Levy et al., 2011). The MDV are also the site of extensive monitoring efforts through a National Science Foundation funded Long Term Ecological Research program (Gooseff et al., 2017; Barrett et al., 2024).

The MDV contain diverse microbial communities (Cary et al., 2010) and are a natural laboratory for studying ecological principles (Monteiro et al., 2022). Food webs within the MDV are microbially dominated and lack both vertebrates and vascular plants. Photosynthetic primary production largely occurs in cyanobacterial mats in stream channels (McKnight et al., 1999; Power et al., 2020), within the lakes (Takacs and Priscu, 1998; Zhang et al., 2015), and in soil biocrusts (Power et al., 2024). Soil bacterial heterotrophs are grazed upon by bacterivorous nematodes (largely *Scottinema lindsayae* and *Plectus* spp.), tardigrades, and rotifers (Adams et al., 2006), which in turn are preyed upon by “apex predator” *Eudorylaimus* nematodes (Shaw et al., 2018). Fungi (Dreesens et al., 2014), viruses (Robinson et al., 2024), and a low abundance of archaea

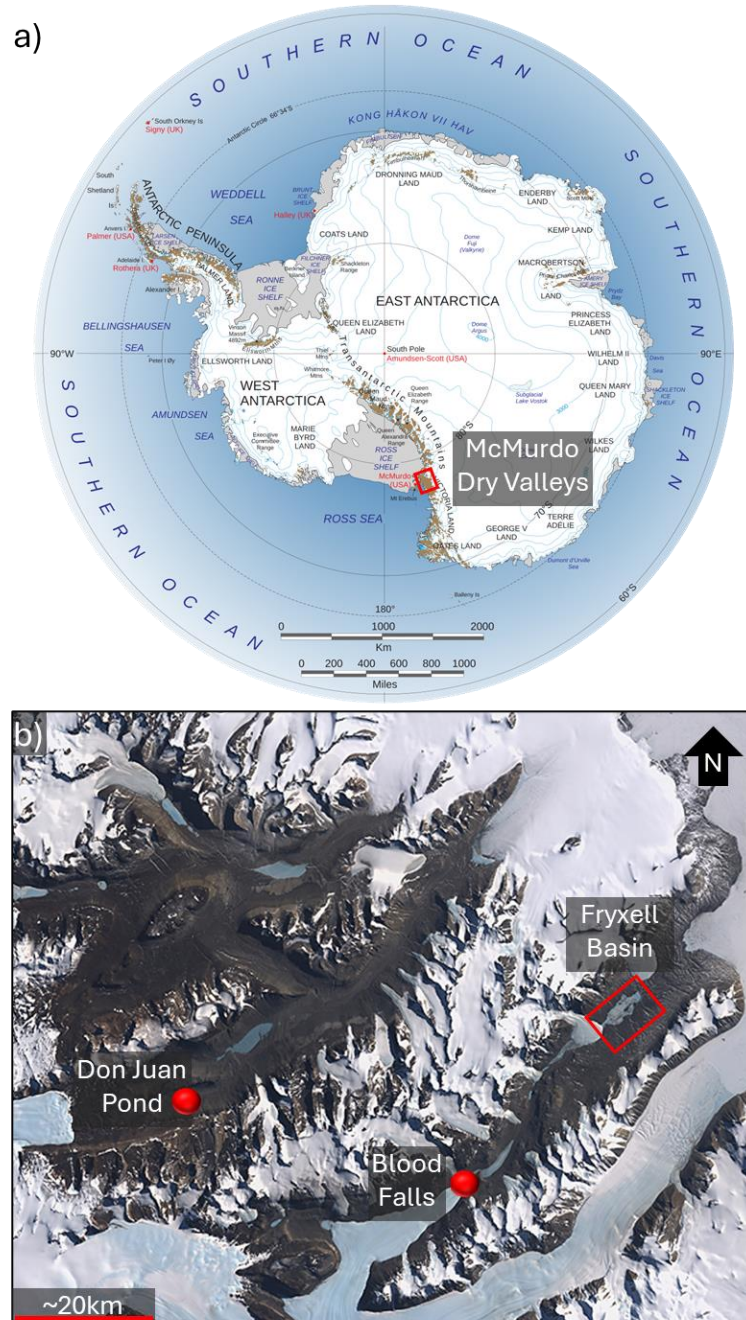


Figure 0.1 McMurdo Dry Valleys, Antarctica. (a) Distribution of the ice free regions of Antarctica, with the location of the MDV boxed. *Figure adapted from NASA.* (b) Satellite image of the MDV, indicating relevant field sites. Blood Falls (Chapter Four) and the Fryxell Basin (Chapter Three) are both within Taylor Valley, and Don Juan Pond (Chapter Two) is within Wright Valley. *Image credit: NASA.*

(Richter et al., 2014) are present throughout the MDV, though have received considerably less attention over the decades. Community assembly within the MDV is believed to be largely shaped by abiotic factors; for example, local variations in moisture, salinity, and pH have been posited as the major drivers of soil community structure (Hogg et al., 2006; Lee et al., 2012; Feeser et al., 2018; Bottos et al., 2020). Abiotic factors are heterogeneously distributed in gradients at both local and regional scales, in part due to the unique geophysical legacy of MDV soils (Bockheim, 2002). These naturally occurring gradients can thus be exploited to understand the impact of specific abiotic factors on microbial community structure and function in a trophically-simple ecosystem.

Microbial adaptations for survival in the MDV

Microbial life in the MDV is subjected to a wide array of extreme conditions. Soil temperatures can range from a summer maximum 25 °C to a winter minimum of -60 °C (Obryk et al., 2020). The MDV receive 24 hours of daylight during the austral summer and are thus exposed to large quantities of UV radiation (Obryk et al., 2018), while experiencing 24 hours of darkness during the winter “dormant” season. Soil moisture can range from saturation to < 1% gravimetric water content, leading to desiccation stress on resident organisms (Treonis et al., 2000; Salvatore et al., 2023). Soil water is most visibly sourced from the melting of glaciers and snow patches (Gooseff et al., 2011), though soil moisture is also influenced by the melt of shallow permafrost (Ball et al., 2011; Ball and Virginia, 2012), deliquescence by soil salts (Levy et al., 2011; Gough et al., 2017), and the minimal precipitation (Fountain et al., 2009). Additionally, extensive groundwater has been identified within the MDV, though hydrological connectivity remains poorly constrained (Mikucki et al., 2015; Dugan et al., 2022). Salinity can range from freshwater equivalent to hypersaline, including the notable extreme of Don Juan Pond (DJP) located in Wright Valley. DJP is a CaCl₂-dominated pond that can reach ~ 40% (g g⁻¹) salinity, depending on water level (Dickson et al., 2013; Toner et al., 2017), and yet hosts a viable bacterial community within its sediments (Mikucki et al., *in prep*). The wide range and distribution of stressors present throughout the MDV provide numerous opportunities to

study both extremophilic microbial adaptations and the role of abiotic factors in shaping community structure and function.

Organisms capable of growth at low temperatures (i.e., psychrophilic and psychrotolerant organisms) possess a variety of adaptations to counteract the lower metabolic rates, membrane fluidity, and protein flexibility associated with decreased temperatures (De Maayer et al., 2014). The membranes of cold-adapted microorganisms tend to be enriched in branched and unsaturated fatty acids (Hassan et al., 2020) and insert specialized proteins or molecules such as carotenoids to increase overall membrane fluidity (Chattopadhyay and Jagannadham, 2001; Jagannadham and Chowdhury, 2012; Seel et al., 2020). Synthesized proteins often have fewer salt bridges and proline residues compared to the proteins of mesophiles which decrease the energy required for conformational changes (Metpally and Reddy, 2009). The genomes of cold-adapted organisms also tend to have higher copy numbers of genes associated with translation and protein folding compared to mesophilic relatives (De Maayer et al., 2014). Additionally, subzero temperatures can lead to the formation of ice crystals inside of the cell, leading to lysis. To counteract freezing, some psychrophiles can utilize a combination of ice active proteins, which lower the eutectic temperature inside the cell to prevent internal freezing, and ice nucleation proteins, which control the formation of smaller, less hazardous ice crystals outside of the cell (Kawahara et al., 2004).

Freezing leads to the cryoconcentration of dissolved solutes, thus many psychrophilic organisms also are adapted to salt stress (Wilson et al., 2012). Salts reverse the positive turgor pressure on a cell, which is required by almost all known microorganisms for cell expansion (Kempf and Bremer, 1998; Gunde-Cimerman et al., 2018). Halophilic adaptations can generally be sorted into either “salt-in” or “compatible solute” strategies, depending on the dominant mechanism of restoring osmotic balance (Oren, 2008). Salt-in organisms rely on ion pumps (largely K^+ and Cl^-), to match their internal salt concentration with the outside concentrations of salt (DasSarma and DasSarma, 2017). Additionally, the proteins of salt-in organisms often feature more acidic residues and fewer hydrophobic amino acids, and tend to require high

concentrations of salts for proper folding and function (Oren, 1999). Organisms that rely on organic osmolytes (i.e., compatible solutes) will either synthesize or import neutral compounds such as glycine betaine or ectoine to balance the osmotic pressure between the interior and exterior of the cell (Shivanand and Mugeraya, 2011).

Desiccation and UV radiation are also prominent stressors within the MDV. Depending on the wavelength, the UV fraction of solar flux causes damage to the cell through the production of reactive oxygen species (ROS), as well as DNA damage through the formation of strand breaks and thiamine dimers (Castenholz and Garcia-Pichel, 2012). Several strategies exist to counteract the damaging effects of UV, including ROS-protective enzymes and the biosynthesis of specialized pigments that absorb damage from UV light. Pigments are diverse secondary metabolites that are found in numerous Antarctic bacteria and provide a wide variety of benefits to the cell (Marizcurrena et al., 2019). For example, carotenoids in Antarctic bacteria have been linked to protection from solar radiation as well as increased survival following multiple freeze-thaw cycles (Dieser et al., 2010). Microbial adaptations to desiccation have many similarities to cryo- and halotolerance, as evaporative properties increase solute concentration and therefore osmotic pressure on the cell (Lebre et al., 2017). In addition to the strategies discussed above, bacteria also will produce extracellular polymeric substances (EPS), which can form hydrated microenvironments through hygroscopic activity (Chang et al., 2007). Other organisms may rely on dormancy strategies, including the formation of spores with germination occurring under more favorable conditions (Jones and Lennon, 2010; Setlow and Johnson, 2012).

The MDV climate is presumed to have been relatively stable over possibly millions of years (Marchant and Head, 2007), thus evolutionary pressures and useful adaptations to extreme conditions have likely remained fairly consistent within microbial communities. However, environmental change in the MDV may lead to novel conditions and therefore unseen combinations of organisms, with unknown impacts of ecosystem function (Williams and Jackson, 2007; Fountain et al., 2014; Lee et al., 2022).

Environmental change in the MDV

The Antarctic region is experiencing unprecedented warming, especially over West Antarctica (Nicolas and Bromwich, 2014). In addition to the climatic influence of both the East and West Antarctic Ice Sheets, the coastal location of the MDV means they are influenced by sea ice and Southern Ocean dynamics, as well (Fountain et al., 2014). Thus, manifestations of climate change in the MDV are often complex and only observable through long term monitoring, such as the subtle alteration of abiotic gradients. The distribution of solutes within soils are dependent on local hydrology, as salts, nutrients, and organics are mobilized as a consequence of the melting of glacier ice, snow, and permafrost (Foreman et al., 2004; Ball et al., 2011). Wetted soil margins of glacier melt streams tend to have lower salt concentrations compared to the surrounding dry soils, as solutes within hyporheic zones have been leached from the soils and transported downstream to terminal basins, such as the ice-covered lakes (Northcott et al., 2009). Contrastingly, the melting of near surface permafrost leads to wicking processes and thus the movement of salts to the surface, where they are deposited and concentrated by evaporation (Ball et al., 2011; Ball and Virginia, 2012). While notable trends in air temperature have not been observed (Obryk et al., 2020), soil temperature monitoring stations have recorded significant decreases in winter freezing degree days (sum of days with average daily temperature below freezing) in the winter and significant increases in summer thawing degree days (sum of days with average daily temperature above freezing), leading to an increase in permafrost melt (Fowler and Levy, 2025). Thus, climate related changes in MDV hydrology can dramatically impact the distribution of salts within soils and in turn community assembly along newly formed salinity gradients.

Subtle changes within the MDV are overlaid by large scale temperature anomalies than can have long lasting impacts (i.e., observable after 10+ years; Fountain et al., 2014; Gooseff et al., 2017). For example, in the 2001-2002 austral summer, unusually high air temperature and solar flux led to unprecedented glacial melt, after which increases in microbial mat and soil invertebrate biomass were observed over several seasons following an initial lag (Gooseff et al., 2017). In March 2022, an off-season “weather

whiplash” event saw daily temperatures 30 °C above seasonal average leading to stream reactivation (Barrett et al., 2024). Multispectral satellite imaging of areas impacted by the 2022 weather anomaly showed increased photosynthetic activity during the event (as normalized differential vegetation index), and sampling during the following season identified increases in both invertebrate diversity and mortality of the dominant soil nematode, *S. lindsayae* (Barrett et al., 2024). In an ecosystem strongly shaped by abiotic factors (Andriuzzi et al., 2018; Bottos et al., 2020), even temporary climatic and hydrological shifts can lead to large impacts in the resident communities.

Field and laboratory studies have sought to understand the impact of environmental perturbation on MDV soil communities. Microbial communities show rapid response to the addition of exogenous water and nutrients, leading to transcriptional changes (Buelow et al., 2016), increases in community respiration (McKnight et al., 2007; Aanderud et al., 2018; Ball et al., 2018; Niederberger et al., 2019), and large alterations in bacterial community structure (Van Horn et al., 2014; Aanderud et al., 2018; Niederberger et al., 2019). Similarly, laboratory incubations of whole communities have been utilized to measure the impact of water and nutrient amendments on soil respiration rates (Barrett et al., 2006) and microbial community structure and abundance (Knox et al., 2017; Shaw and Wall, 2019; Monteiro et al., 2023). Together, these studies have identified potential impacts of environmental change on MDV communities, though the diversity of responses by individual microorganisms, especially to subtle manifestations of change, requires further investigation.

Terrestrial extremes and the search for life

The MDV have long been used as an analog feature for Mars, due to the similarity of its climate and geomorphology (Cameron et al., 1976; Doran et al., 2010; Salvatore and Levy, 2021). Results from the Mars *Viking* landers were specifically compared to soils collected from the MDV (Wheeler Valley) due to similarities in predicted climatic and edaphic conditions (Cameron et al., 1970; Biemann et al., 1977), and University Valley was utilized as an analog site for testing the prototype Mars Icebreaker Drill (Zacny et al., 2013). Surface morphology and mineralogy of possible lacustrine deposits

on Mars suggest the presence of paleolakes in the Noachian and Hesperian, and potentially lasting into the Amazonian (Wordsworth, 2016; Michalski et al., 2022). These ancient lakes likely bore several similarities to the modern ice-covered lakes of the MDV and thus provided a suitable habitat for early Martian life (Mikucki et al., 2010). Evaporation of surface water, including of Martian paleolakes, leads to the concentration of dissolved salts, which in turn lowers the freezing point of liquid water. Concentrated MDV brines, including that of DJP, provide an analog to investigate possible microbial oasis on the surface of Mars (Mikucki et al., 2010; Davila and Schulze-Makuch, 2016). Beyond Mars, MDV analog features can inform missions to ocean worlds such as Europa and Enceladus, which harbor liquid water oceans covered by icy shell (Hand et al., 2020). For example, Blood Falls, as one of the few known locales where subsurface brine and associated biology is transported from the subsurface to the surface (Badgeley et al., 2017), provides a useful analog feature for the study of the plumes detected on Enceladus and Europa (Hansen et al., 2006; Arnold et al., 2019). As such, studies within the MDV can provide insight into not only into life at the extremes of our own world, but the possibility of life on other worlds, as well.

Beyond analog features, microorganisms isolated from extreme environments also inform studies of adaptations required to survive on other planets (Abbott and Pearce, 2020). A psychro-halophile, *Planococcus halocryophilis* Or1, has been utilized by many studies to explore microbial adaptations associated with low temperatures and hypersaline conditions (Mykytczuk et al., 2013; Heinz et al., 2018, 2019). From the MDV, several strains of *Halomonas*, *Brevundimonas*, and *Rhodococcus* were isolated following Mars-relevant radiation of soils, and survival to both gamma-ray exposure and desiccation were assessed (Musilova et al., 2015). To facilitate studies of forward contamination of astrobiological targets of interest, Rettberg et al. (2019) proposed the use of Antarctic microorganisms to study of the forward contamination of ocean worlds. While not identified in clean rooms, these organisms were proposed specifically due to the likelihood of adaptations to space and ocean world-relevant stressors, such as low temperatures, radiation, desiccation, and high salinity. Insights gained from these studies

provide a crucial framework to how we search for life beyond our planet by defining habitable niches for life as we know it and identifying biosignatures that are detectable with astrobiological methodologies.

Summary of dissertation

This dissertation addresses how MDV bacteria respond to environmental changes. Specific adaptations and responses to life at the extremes are identified within MDV microbial isolates and communities. Each chapter leverages a cultured bacterial isolate to interrogate ecologically-relevant processes and activities utilizing techniques spanning multiple fields, including culture-based experiments, multi-omics approaches, and analytical instrumentation relevant to extraterrestrial exploration. Chapter Two utilizes a strain of *Massilia frigida* (strain DJPM01) to interrogate the impact of UV radiation on bacterial pigmentation. The genome of strain DJPM01 is bioinformatically compared to genomes of globally-isolated *Massilia* spp., and mass spectrometry is utilized to characterize produced pigments with and without UV radiation exposure. Chapter Three utilizes a naturally occurring salinity and moisture gradient within the Fryxell basin to examine the role of salinity in shaping the MDV soil microbial communities. Both amplicon and metagenomic sequencing are utilized to describe the environmental community, and a *Gillisia* sp. (strain FRX26) was isolated and grown at two salt concentrations to identify changes in both metabolite production and gene transcription. Finally, Chapter Four utilizes a strain of *Shewanella* previously isolated from Blood Falls to identify soluble, volatile, and mineral biosignatures associated with dissimilatory iron reduction. Instrumentation relevant to extraterrestrial missions was used to evaluate the potential signatures and then validated with traditional molecular microbiological methods. In summary, this dissertation describes specific ecologically and astrobiologically relevant microbial adaptations and responses in the cryosphere. These chapters each highlight the utility of cultivatable environmental bacterial isolates in interrogating questions about past, present, and future ecology of Earth and other planets.

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**CHAPTET TWO GENOMIC AND PHENOTYPIC CHARACTERIZATION OF A
RED-PIGMENTED STRAIN OF *MASSILIA FRIGIDA* ISOLATED FROM AN
ANTARCTIC MICROBIAL MAT**

Publication Note

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Experiments and data analysis were conducted by Jacob Shaffer, with the exception of the metabolite data, which were analyzed by Robert Samples and Lesley-Ann Giddings. Original samples were collected and processed by Jill Mikucki. The manuscript was drafted by Jacob Shaffer and Jill Mikucki and edited by all authors.

Abstract

The McMurdo Dry Valleys of Antarctica experience a range of selective pressures, including extreme seasonal variation in temperature, water and nutrient availability, and UV radiation. Microbial mats in this ecosystem harbor dense concentrations of biomass in an otherwise desolate environment. Microbial inhabitants must mitigate these selective pressures *via* specialized enzymes, changes to the cellular envelope, and the production of secondary metabolites, such as pigments and osmoprotectants. Here, we describe the isolation and characterization of a Gram-negative, rod-shaped, motile, red-pigmented bacterium, strain DJPM01, from a microbial mat within the Don Juan Pond Basin of Wright Valley. Analysis of strain DJPM01's genome indicates it can be classified as a member of the *Massilia frigida* species. The genome contains several genes associated with cold and salt tolerance, including multiple RNA helicases, protein chaperones, and cation/proton antiporters. In addition, we identified 17 putative secondary metabolite gene clusters, including a number of nonribosomal peptides and ribosomally synthesized and post-translationally modified peptides (RiPPs), among others, and the biosynthesis pathway for the antimicrobial pigment prodigiosin. When cultivated on complex agar, multiple prodiginines, including the antibiotic prodigiosin, 2-methyl-3-propyl-prodiginine, 2-methyl-3-butyl-prodiginine, 2-methyl-3-heptyl-prodiginine, and cycloprodigiosin, were detected by LC–MS. Genome

analyses of sequenced members of the *Massilia* genus indicates prodigiosin production is unique to Antarctic strains. UV-A radiation, an ecological stressor in the Antarctic, was found to significantly decrease the abundance of prodiginines produced by strain DJPM01. Genomic and phenotypic evidence indicates strain DJPM01 can respond to the ecological conditions of the DJP microbial mat, with prodiginines produced under a range of conditions, including extreme UV radiation.

Introduction

Upwards of 80% of Earth's environments are permanently cold ($<5^{\circ}\text{C}$), including polar, alpine, maritime, and atmospheric regions (De Maayer et al., 2014). Understanding how life survives at low temperatures is crucial to our understanding of life itself, as well as how life might exist on other planets. On the Antarctic continent, the McMurdo Dry Valleys (MDV) constitute the largest ice-free region (Levy, 2013). The MDV region is the coldest, driest desert on our planet, and experiences average annual air and absolute surficial soil temperatures ranging from -26.8 to -16.5°C and -59.8 to $+31.1^{\circ}\text{C}$, respectively (Obryk et al., 2020), and receive a maximum of ~ 50 mm of precipitation in the form of snowfall annually (Fountain et al., 2009). Additionally, the MDV experiences long periods of sustained light and dark, due to its extreme southern latitude, and high levels of UV-A and UV-B radiation from thinning in the ozone layer (Frederick and Snell, 1988).

Antarctic microbial mats provide a unique system for studying the effects of UV radiation and other adaptations due to their high biomass compared to other microbial niches within the MDV (Davey and Clarke, 1992). The formation of these communities is reliant on photosynthetic cyanobacteria, including either *Nostoc* species or *Phormidium* and *Oscillatoria* species, depending on the geomorphology and hydrology of the niche (Alger et al., 1997). Other photosynthetic organisms, such as green algae and diatoms, can be found in Antarctic mats, as well as heterotrophic bacteria and microscopic invertebrates (Andriuzzi et al., 2018). Microbial mats that occupy perennial stream channels and shallow ponds are particularly exposed to extremes of seasonal variation and experience fluctuations in meltwater flow from glaciers due to changes in

temperature and solar angle, which in turn can lead to changes in nutrient availability and community structure (Zoumplis et al., 2023). In the winter, communities are exposed to months of darkness, desiccation, and subzero temperatures. MDV mats in stream channels and surface ponds lie dormant for ~10 months of the year due to the lack of water and sunlight, followed by rapid reactivation in response to rehydration by glacial meltwater (McKnight et al., 2007). In addition to freezing and dehydration, MDV mats are exposed to intense ultraviolet (UV) radiation. This UV exposure causes DNA damage and the production of reactive oxygen species (ROS; Castenholz and Garcia-Pichel, 2012). UV radiation has been proposed as a major limiting factor on growth for communities within the MDV (Tosi et al., 2005).

Microbial mats are scattered throughout the MDV, largely concentrated in meltwater streams and lake margins (Power et al., 2020) that wax and wane in volume in response to climate (Mikucki et al., 2010). Within the South Fork of the Wright Valley is a near-saturated CaCl_2 brine pond, known as Don Juan Pond (DJP). DJP is bounded to the west by a rock glacier, which provides a source of meltwater to the basin (**Figure 2.1**; tables and figures in this chapter can be found in the **Chapter Two Appendix**). While there are conflicting reports regarding the habitability of the brine (Toner et al., 2017), the DJP basin hosts a dense microbial mat that borders the rock glacier (**Figure 2.1B**). This mat was first described as approximately 500–600 m² and 2–5 mm thick (Siegel et al., 1979). Microscopic investigation of the mat revealed an abundance of cyanobacteria phenotypically similar to *Oscillatoria*, in addition to the green algae *Chlorella* and *Dunaliella*, diatoms, invertebrates, and bacteria (Siegel et al., 1983). The extent to which the saturated brine of DJP might influence the mat is currently unknown, though it is possible that dried salts are deposited in the mat by aeolian processes (Diaz et al., 2018).

The genus *Massilia* was first described in 1998 in a report on the isolation of *Massilia timonae* from the blood sample of an immunocompromised patient (La Scola et al., 1998). The genus belongs to the *Oxalobateraceae* family and to date includes 61 validly published species (January 2023; <https://lpsn.dsmz.de/genus/massilia>). *Massilia* species have been isolated from disparate environments across the globe and have been

implicated in human disease (La Scola et al., 1998; Van Craenenbroeck et al., 2011; Kämpfer et al., 2012; Chiquet et al., 2015) and as a common member of rhizosphere communities of *Arabidopsis* (Tkacz et al., 2015) and wheat (Schlatter et al., 2020). Rhizosphere *Massilia* are of interest due to their possible role in plant development through the production of phytohormones and solubilization of phosphates, among other activities (Ofek et al., 2012; Schlatter et al., 2020). Several *Massilia* have also been isolated from the cryosphere, including from Arctic (Son et al., 2021; Dahal et al., 2021a), Antarctic (Holochová et al., 2020; Sedláček et al., 2022), and alpine regions (Shen et al., 2013, 2015; Guo et al., 2016; Gu et al., 2017; Wang et al., 2018).

While their ecological role in polar environments is not as well understood, *Massilia* species have been detected in diverse Antarctic niches. For example, *Massilia* ranged from 54 to 79% of culturable community members in both closed and open cryoconite holes in the Larsemann Hills, Dronning Maud Land, and Amery Ice Shelf regions (Sanyal et al., 2018). Sommers et al. (2019) identified *Massilia* as one of the ten most abundant sequences in cryoconite water collected from Taylor Glacier, a glacier within the MDV. These authors suggested that *Massilia* may play a role in cryoconite colonization. *Massilia* have also been isolated from microbial mats in both peninsular and continental Antarctica, including other ice-free regions such as the Schirmacher and Syowa Oasis (Peeters et al., 2012).

Here we characterize a red-pigmented *Massilia* species, strain DJPM01, isolated from the DJP microbial mat. We identified genomic evidence for environmental adaptations in strain DJPM01 and compared these findings with other Antarctic *Massilia* species as well as the taxonomic distribution of secondary metabolite biosynthetic gene clusters (BGCs) within the *Massilia* genus. We then analyzed cultures of strain DJPM01 for the presence and relative abundance of pigments when grown under ecologically relevant UV-A radiation exposure to further our understanding of microbial responses in extreme environments.

Materials and Methods

Site description and sample collection

Samples of a microbial mat were collected in the South Fork of the Wright Valley at the terminus of a rock glacier in the Don Juan Pond (DJP) basin, Victoria Land, Antarctica (77.56293 S, 161.1751 E; **Figure 2.1**). Mat material was collected on 5 December 2018 using individually wrapped sterile scoops (SP Bel-Art; Wayne, NJ) and transferred into sterile Whirl-Pak bags (Whirl-Pak; Madison, WI). *In situ* temperature, pH, and salinity were measured at the time of collection using a vented Aqua TROLL 500 Multiparameter Sonde (*in-situ*), configured with pH/ORP, conductivity, rugged dissolved oxygen (RDO), and turbidity modules. Samples were stored and shipped cool (4°C) until later processing at the University of Tennessee Knoxville, TN, United States.

Isolation and incubation conditions

Mat material was incubated in R2A broth (Difco; Becton, Dickinson and Company; Franklin, NJ) at ~10°C for 10 days, after which the broth was used to inoculate R2A agar plates (BD Difco; Franklin, NJ). Bright red-pigmented colonies appeared on the plate after 4 days of incubation at 10°C and were selected for further isolation through multiple restreaks on R2A agar. Gram staining and microscopy was performed on strain DJPM01 using the American Society of Microbiology Gram stain protocol (Smith and Hussey, 2005) and observed with an Axio Imager M2 microscope (Zeiss; Oberkochen, Germany). Motility assays were conducted by spot inoculation of strain DJPM01 onto 0.5% agar R2A plates. To assess resistance to UV-C light, R2A agar plates of strain DJPM01 were prepared and exposed UV light (254 nm) as described by Hirsch et al. (2004). Plates were exposed for 10, 20, 30, 60, 120, and 300 s and left to incubate in the dark at 10°C for 10 days. Strain DJPM01 was incubated in R2A broth under a nitrogen headspace to test for anaerobic growth. To test the effects of temperature on the growth rate of strain DJPM01, incubations were performed in 96-well plates. Briefly, 180 µL R2A broth was aliquoted into each well. Strain DJPM01 was grown to late log phase and inoculated into 48 wells of a 96-plate (10% inoculum). Plates were incubated at 4, 10, 15,

20, and 25°C, and full spectra were collected for each plate once per day for 14 days. Outer wells were removed from analysis to control for unequal levels of evaporation.

Phylogenetic analysis and whole genome comparison

For taxonomic identification, DNA was extracted from bacterial colonies using a DNeasy PowerSoil PowerLyzer Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol with the addition of a modified bead beating step as follows: 45 m sec⁻¹ for 25 sec followed by 5 min rest at 10°C (FastPrep-24 Bead Lysis System; MP Biomedical; Santa Anna, CA). The 16S ribosomal RNA (rRNA) gene was amplified from extracted DNA using the following combination of primers: bacterial 27F (5'-AGAGTTTGATCCTGGCTCAG-3'; Weisburg et al., 1991) and 806R primers (5'-GGACTACNVGGGTWTCTAAT-3'; Apprill et al., 2015) and 515F (5'-GTGCCAGCMGCCGCGGTA-3'; Caporaso et al., 2011) and 1492R primers (5'-GGTTACCTTG TTACGACTT-3'; Weisburg et al., 1991). The resulting PCR products were purified using a Wizard SV Gel and PCR Clean-Up System Kit (Promega; Madison, WI) before Sanger sequencing, performed by Eurofins Genomics (Louisville, KY). Returned sequences were aligned using Geneious v6.1.8 (for a sequence length of 1,388 bp; Kears et al., 2012) and compared to the NCBI nucleotide collection type material database (Federhen, 2012). Publicly available sequences for *Massilia* 16S rRNA genes were obtained from the RefSeq database (O'Leary et al., 2016), aligned, trimmed to the length of the shortest sequence (1,335 bp), and used to construct a maximum likelihood tree in MEGA v11.0.13 (Tamura et al., 2021). Additionally, strain DJPM01 was compared to all NCBI reference genomes within the Oxalobacteraceae family using whole genome average nucleotide identity (ANI) through the enveomics collection (Rodriguez-R and Konstantinidis, 2016). Digital DNA–DNA hybridization (DDH) for all reference genomes available in the Oxalobacteraceae family was calculated using the Genome-to-Genome Distance Calculator (GGDC) v3.0 (Meier-Kolthoff et al., 2022). A final phylogenetic tree of RefSeq reference genomes was generated using OrthoFinder v2.5.4 (Emms and Kelly, 2019) with default settings. Estimation of abundance was performed using a 16S community amplicon sequence variant (ASV) library generated

from the DJP mat (Schuler, 2022) using the Basic Local Alignment Search Toolkit (BLAST; Altschul et al., 1990). The final 16S rRNA gene sequence for strain DJPM01 was uploaded to GenBank under accession number OQ346149.

Genome sequencing, assembly, and annotation

Total genomic DNA was extracted from microbial biomass using a cetrimonium bromide (CTAB) buffer/organic phase extraction protocol (as described by Campen, 2015) for whole genome sequencing. Quality and quantity of extracted DNA was determined by a Qubit 4 fluorometer (Invitrogen Carlsbad, CA) and NanoDrop 2000 spectrophotometer (Thermo Scientific; Waltham, MA, United States). High molecular weight genomic DNA (25 ng/μL) was sequenced by MR DNA (Shallowater, TX). Library preparation was performed using the Nextera DNA Flex Library Preparation Kit (Illumina Inc.; San Diego, CA) and quality was confirmed using the Agilent 2,100 Bioanalyzer (Agilent Technologies; Santa Clara, CA). The library was diluted to 0.6 nM before sequencing on the NovoSeq platform (Illumina; San Diego, CA; paired end, 500 cycles). Sequencing adapters and low-quality reads were trimmed from using Trimmomatic v0.36 (Bolger et al., 2014) on default parameters. Trimmed reads were assembled using Unicycler v0.4.8 (Wick et al., 2017) with a maximum contig size of 500 bp and assembly quality was assessed using QUAST v5.0.2 (Gurevich et al., 2013) and checkM v1.1.3 (Parks et al., 2015). The genome draft was annotated by Prokka v1.13.7 (Seemann, 2014) and the resulting annotation was analyzed using GhostKOALA v2.2 (Kanehisa et al., 2016). Putative CRISPR-Cas sequences were identified using CRISPRCasFinder v4.2.20 (Couvin et al., 2018) and putative prophage sequences were identified by VirSorter2 v2.2.3 (Guo et al., 2021), both using the Proksee interface.² Putative secondary metabolite BGCs were identified *via* antiSMASH v6.0.0 (Blin et al., 2021) using default parameters. RefSeq *Massilia* genomes were annotated using Prokka v1.13.7, GhostKOALA v2.2, and antiSMASH v6.0.0 and all *Massilia* genomes in both NCBI and JGI databases were aligned against the *pig* operon of *Serratia* sp. strain ATCC 39006 to search for prodigiosin-specific biosynthetic genes (Harris et al., 2004). Putative prodigiosin operons were compared by pairwise alignment using SimpleSynteny v1.5

(Veltri et al., 2016). Statistical analyses of gene abundance were performed using R v4.1.2 (R Core Team, 2018). The final draft genome assembly was uploaded to GenBank under accession number JAQOZV0000000000.

Biomass irradiation and extraction of pigments produced under UV light

A lawn of strain DJPM01 was grown on R2A agar plates in quadruplicate and incubated under a UV-A light (320–400 nm, peak absorbance 395 nm; **Appendix, Figure 6.1**; SANSI Lighting; Shanghai, China). Nalgene petri dishes (Thermo Scientific; Waltham, MA) made of polymethylpentene were utilized to increase UV penetration compared to standard polystyrene petri dishes (Peng et al., 2011). Control plates were inoculated, wrapped in foil, and placed inside two black bags prior to incubation. Uninoculated media controls were also incubated under both light and dark treatments. All plates were incubated at 10°C for 11 days. Following incubation, agar was sterilely sliced into strips for storage at –80°C. Agar strips from each plate were extracted four times with 10 mL of high performance liquid chromatography (HPLC)-grade ethyl acetate (Fisher Scientific; Hampton, NH) and sonicated for 10 min. Organic phases were pooled and diluted to 50 mL with ethyl acetate. Authentic prodigiosin (1) standard (P0103; Sigma-Aldrich, St. Louis, MO) was diluted 100 µg mL⁻¹ in liquid chromatography/mass spectrometry (LC/MS)-grade methanol (Fisher Scientific; Hampton, NH) and exposed to UV-A radiation overnight to investigate potential breakdown products. UV-A exposed and unexposed prodigiosin (1) standard solutions were diluted to 10 µg mL⁻¹ prior to LC/MS analysis.

LC/MS analyses

Extracts were analyzed on a Thermo Scientific (Waltham, MA) Q-exactive HF-X Hybrid Quadrupole-Orbitrap mass spectrometer interfaced with a Vanquish Horizon Ultra high performance liquid chromatography (UHPLC) system and VH-D10-A UV detector using a Waters (Milford, MA) HSS T3 C18 column (1.8 µm, 2.1 × 100 mm) with a VanGuard FIT precolumn. Using a flow rate of 0.5 mL min⁻¹, samples (2 µL) were injected onto the column prewarmed to 40°C. UHPLC separation was achieved using the following parameters: 2% acetonitrile: 98% water with 0.1% formic acid for 1 min, 2–

40% over 4 min, 40–98% over 3 min, 98–2% over 0.2 min, and 2% for 2 min. The UV–Vis data were collected at 534 nm. The following electrospray ionization (ESI) settings were used in probe position D: 40 sheath gas flow, 8 auxiliary gas flow, 1 sweep gas flow, 3.5 kV spray voltage, 380°C capillary temperature, 50 radiofrequency (RF) funnel level, and 350°C auxiliary gas temperature. Data were evaluated using MS-DIAL (Tsugawa et al., 2015) and MPACT (Samples et al., 2022). See supplementary information for data processing methods.

A Waters Synapt G2-Si Q-ToF mass spectrometer coupled to an Acquity H-Class ultra performance liquid chromatography (UPLC) system was employed for LC/MS analysis of select samples and prodigiosin (1) standard using the previously described column and gradient. Positive mode MS^E acquisition was used, using 0.1 s MS1 survey scans from m/z 150–2000 in resolution mode, followed by 0.1 s high energy MS2 scan from m/z 50–2000. Data were acquired using the following parameters: 2 kV capillary voltage, 100°C source temperature, 20 V sampling cone, 800 L h⁻¹ desolvation gas flow, 80 V source offset, and 30 V MS2 collision energy. Real-time mass correction used a lockspray of 400 pg μL^{-1} leucine enkephalin solution (1:1 methanol to water solution with 0.1% formic acid). A 10 μL injection volume was used in the closed loop configuration.

Metabolomics data processing, filtering, and statistical analyses

The peak list and fragment database were exported from Progenesis and analyzed with MPACT (Samples et al., 2022). Data were filtered using solvent blank, mispicked peak, reproducibility, and in-source fragment filters. Blank filtering using EtOAc blanks was applied based on a relative group parsing threshold of 0.01. A minimum reproducibility threshold of 0.5 median coefficient of variation (CV) among technical replicates was applied. The presence or absence of features in a group of samples was determined using a relative abundance threshold of 0.05 compared to the sample group in which a feature was most abundant. This threshold was applied during blank filtering to remove features whose abundance in solvent blanks was greater than 5% of their

abundance in experimental samples. In-source ion filtering and mispicked peak filtering were disabled.

Results

Sample collection and isolation

At time of collection, the water within the mat was 7.7°C; collection site pH was 7.2 and salinity was 0.4 parts per thousand (ppt). Following isolation, the purity of the resulting axenic culture was confirmed by 16S rRNA gene analyses and microscopy, which revealed a single rod-shaped morphology. The resulting red-pigmented isolate was designated strain DJPM01.

Characterization and growth

Growth of strain DJPM01 on R2A yielded tall, convex, scarlet colonies with entire margins that spread outwards over time (**Figure 2.2**). Colonies were resistant to removal from the surface of the agar and remained aggregated when transferred to broth culture, unless thoroughly agitated through vortexing. Strain DJPM01 was determined to be Gram-negative using the standard Gram stain protocol (Smith and Hussey, 2005). Cells were rod-shaped and 4.65 μm in length on average (**Figure 2.2A**). Cultures exhibited growth from 4 to 25°C, but not at 30°C (**Appendix, Figure 6.2**). The fastest growth occurred at 10°C with a rate (μ) of $0.25 \pm 0.04 \text{ day}^{-1}$. Colonies were pigmented under all culture conditions, with some variation in color intensity and hue at warmer temperatures (**Appendix, Figure 6.2**). Growth was not detected under anaerobic conditions. Strain DJPM01 grew on R2A agar adjusted to pH 10.5. Abundance of colony forming units decreased following 30 s of exposure to UV-C radiation, however colonies were present on plates irradiated for up to 120 s. Evidence for motility was observed when grown on low density R2A agar. When grown in R2A broth, strain DJPM01 formed a robust pellicle at the surface and produced extracellular polymeric substances (**Figure 2.2D**).

Phylogenetic analysis

Analysis of the trimmed 16S rRNA gene sequence (1,335 bp) placed strain DJPM01 within the *Massilia* genus (**Figure 6.3**). Strain DJPM01 shared highest sequence

identity with *M. violaceinigra* strain B2 (CP024608, 99.87%) originally isolated from glacial permafrost in the Xinjiang region of China (Wang et al., 2018). Strain DJPM01 also shared high sequence identity with several other cryosphere associated *Massilia* species, including *M. aquatica* strain CCM 8693 (MN612031, 99.87%), *M. rubra* strain CCM 8692 (MN611986, 99.80%), *M. frigida* strain CCM 8695 (MN612047, 99.80%), *M. mucilaginoso* strain 8,733 (MN612043, 99.67%), and *M. atriviolacea* strain SOD (NR_171529, 99.40%). Of these six isolates, four were isolated from James Ross Island on the Antarctic Peninsula (*M. frigida*, *M. aquatica*, *M. rubra*, and *M. mucilaginoso*; Holochová et al., 2020). *M. atriviolacea*, was first isolated from agricultural soils from Hefei, China (Yang et al., 2019). Comparison of the 16S rRNA sequence to the available DJP basin mat 16S ASV library suggests that strain DJPM01 is present in low abundance with 2,110 reads or ~0.03% relative abundance (Schuler, 2022).

Whole genome comparison placed strain DJPM01 within a distinct clade ($\geq 87\%$ ANI between clade members) including all currently identified *Massilia* with either a red or purple pigmented phenotype (**Figure 2.3; Table 6.1**). This clade includes the six *Massilia* strains with highest 16S rRNA gene similarity to strain DJPM01, as well as *M. antarctica* strain P9640 (OM243919; Sedláček et al., 2022), which was also isolated from James Ross Island. All members of this clade have been shown to grow at or below 4°C, regardless of isolation locale. Strain DJPM01 shared highest ANI with *M. frigida* strain CCM8695 (97.36% identity). High sequence identity of strain DJPM01 to *M. antarctica* strain P9640 (91.92%) and *M. violaceinigra* strain B2 (91.38%) were also noted. Additionally, *M. mucilaginoso* strain CCM8733, *M. atriviolacea* strain SOD, *M. rubra* CCM 8692 (GCF 011682065), and *M. aquatica* CCM 8693 had relatively high sequence identity (87.94–88.41% similarity to strain DJPM01). Digital DNA–DNA hybridization (DDH) showed 75.6% similarity between strain DJPM01 and *M. frigida* strain CCM8695 (**Appendix, Table 6.1**).

Genomic analyses

Whole-genome sequencing resulted in 8,995,221 paired reads of which 8,693,443 (96.6%) pairs survived trimming with 0 N's. Assembly yielded a genome 7.7 Gb in size

across 236 contigs with 99.80% completeness (573 of 574 marker genes present) and 1.64% contamination (10 marker genes with two copies each; **Figure 2.4**). Following annotation, 6,626 predicted protein coding genes were identified, 3,037 of which had a non-hypothetical function (45.8% functional annotation). Of these genes, the largest functional categories within the KEGG database were protein families: signaling and cellular processes (361 genes), environmental information processing (339 genes), genetic information processing (339 genes), and carbohydrate metabolism (248 genes). The genome contains complete pathways for glycolysis and the TCA cycle, oxidative phosphorylation, flagellar motility, and denitrification. Additionally, we detected 9 putative CRISPR arrays and 4 Cas clusters (**Figure 2.4**). Both putative single- and double-stranded DNA phage sequences were found throughout the genome.

Strain DJPM01 has genomic evidence for adaptation to cold environments (**Figure 2.4; Appendix, Table 6.2**). The genome contains genes homologous to those encoding protein chaperones, including two copies of ClpB (Mogk et al., 2003) and DnaJ (Qiu et al., 2006), three copies of DnaK (Thompson et al., 2012), and one copy each of GroEL (Lenz and Ron, 2014), GroES (Takei et al., 2012), and GrpE (Wu et al., 1996). There was also evidence for genes encoding cold shock proteins involved in repairing misfolded mRNA, such as ATP-dependent RNA helicases DpbA (Tsu et al., 2001), DeaD (Prud'homme-G  n  reux et al., 2004), and three copies of RhlE (Bizebard et al., 2004). Strain DJPM01 contained two copies of genes putatively encoding the cold shock protein CspA (La Teana et al., 1991). Three genes were also associated with the formation of unsaturated fatty acids, including a $\Delta 9$ -desaturase (Li et al., 2009) and two acyl-CoA thioesterases, TesA (Cho and Cronan, 1993) and YciA (Postle and Good, 1985).

Several genes putatively involved in osmotic and oxidative stress were present as well (**Figure 2.4; Appendix, Table 6.2**). Strain DJPM01 has genomic evidence of several cation/proton antiporters in the NhaA (Vimont and Berche, 2000) and CPA1 families (Utsugi et al., 1998). Both the biosynthetic pathway and transporters for the compatible solute glutamate are present (Saum et al., 2006). Strain DJPM01 also contained genes for superoxide dismutase (Fee, 1991) and peroxiredoxin (Weber et al.,

2006), which are enzymes known to combat oxidative stress, in addition to six copies of the catalase gene (von Ossowski et al., 1991). Several genes were associated with DNA repair (**Figure 2.4; Appendix, Table 6.2**). Strain DJPM01 encodes for several copies of genes for nucleotide excision, mismatch repair, and homologous and non-homologous recombination. The genome also encodes for two proteins associated with light-dependent DNA repair, deoxyribodipyrimidine photolyase (Sancar et al., 1984) and DNA photolyase (Zhang et al., 2013).

Annotation by antiSMASH (Blin et al., 2021) revealed a total of 17 putative biosynthetic gene clusters (BGCs), including high similarity matches to characterized gene clusters for aryl polyene APE_{Ec} (36%), violacein (100%), cephamycin C (36%), myxochelin (41%), iso-migrastatin (45%), and prodigiosin (42%; **Appendix, Table 6.3**). Of these genes, aryl polyenes have been proposed to mediate oxidative stress (Cimermancic et al., 2014), myxochelins are iron-chelating siderophores (Li et al., 2008), cephamycin C is a β -lactam antimicrobial (Alexander and Jensen, 1998), and iso-migrastatin is a glutaride-containing cytotoxic agent (Ju et al., 2005). The pigments violacein (blue-violet) and prodigiosin (1; red) have both been described to have antibiotic properties and UV-protective effects (Danevčič et al., 2016; Cauz et al., 2019). Closer analysis of the iso-migrastatin BGC using PRISM v4.4.5 (Skinnider et al., 2020) revealed this cluster encodes genes which are 58–72% identical (based on amino acid sequence) to those involved in the biosynthesis of the antifungal gladiofungin, however the cluster was on the end of a contig, missing roughly 15% of the third polyketide synthase gene (**Appendix, Figure 6.4; Niehs et al., 2020**).

To understand how the diversity of BGCs in DJPM01 compares to other *Massilia* genomes, putative secondary metabolite BGCs were identified in representative *Massilia* genomes from RefSeq using antiSMASH (**Figure 2.5; O'Leary et al., 2016; Blin et al., 2021**). Antarctic *Massilia* genomes contained an average of 14.5 secondary metabolite BGCs per genome, significantly higher than both mesophilic *Massilia* (9.2 BGCs per genome; Tukey, $t = -4.95$, $p < 0.001$) and Arctic and alpine *Massilia* (7.8 BGCs per genome; Tukey, $t = -4.31$, $p < 0.001$). This trend was still significant when normalized to

genome size (ANOVA, $F = 4.27$, $p = 0.023$) and number of coding sequences per genome (ANOVA, $F = 4.38$, $p = 0.021$; **Appendix, Table 6.4**). Strain DJPM01 was the only *Massilia* to contain the putative gladiofungin polyketide gene cluster. The BGC for prodigiosin was only identified in Antarctic *Massilia* species (*M. aquatica* CCM 8693, *M. frigida* CCM 8695, *M. mucilaginoso* CCM 8733, and *M. rubra* CCM 8692).

Genes for *Serratia* sp. strain ATCC 39006 prodigiosin BGC were aligned to all *Massilia* genomes available on NCBI (Harris et al., 2004). Only five genomes were identified to contain BGCs with high sequence identity to the prodigiosin BGC, with the highest bit scores for *pigC*, *pigD*, and *pigE* (**Appendix, Figure 6.5A**). Of these five organisms, four were *Massilia* species originally isolated from James Ross Island. The fifth genome was reported as *M. violaceinigra* strain sipir (GCF 022811105.1) in NCBI; the metadata associated with this entry indicated it was isolated from freshwater. Our analyses indicated this genome had >97% ANI to the Antarctic *M. aquatica* CCM 8693, which also has genotypic and phenotypic evidence for prodigiosin production. Given the uncertainty about the phylogeny of this strain we excluded it from further analyses. The four Antarctic *Massilia* prodigiosin BGCs had high sequence identity (93.59–100.00% identity) at the hypothetical gene level to strain DJPM01 (**Appendix, Figure 6.5B**).

Gene annotations were compared between strain DJPM01 and *M. frigida* strain CCM 8695. Both strains shared 1990 genes with non-hypothetical functions (97.9%), with 41 genes unique to strain DJPM01 and 51 genes unique to *M. frigida*. Genes unique to strain DJPM01 included two genes associated with lipopolysaccharide biosynthesis (*hddA* and *hddC*), one copy of fatty acid elongation gene *fabI*, and three genes associated with toxin-antitoxin systems (*fitA*, *fitB*, and *yefM*). Both genomes contain genes associated with type IV pilin production, multifunctional fibers produced on cell surfaces, though *M. frigida* does not have a copy of *mshC* (Toma et al., 2002). An additional 91 genes had a higher copy number in strain DJPM01 and 102 genes had lower copy number in strain DJPM01. Strain DJPM01 had 2 additional copies of genes for catalase, and four fewer copies of the fatty acid biosynthesis gene *fabB*. When compared to non-Antarctic species, Antarctic *Massilia* tended to, on average, have slightly more genes associated

with fatty acid biosynthesis (especially *fab* genes; 4 more genes), protein chaperones (1.4 more genes), and catalase (1.4 more genes).

Pigment extraction for UV-Vis analysis.

Pigments were extracted from individual colonies of strain DJPM01 following mechanical cell lysis using a FastPrep-24 bead beating system (MP Bio). Cells were incubated for 10 minutes with a 10% SDS solution (5% final concentration) before the addition of water-saturated butanol. Cells were centrifuged for 20 minutes at 14,000 x g and the absorption spectrum of the organic phase was analyzed from 400-700 nm in steps of 5 nm using a Synergy 2 Plate Reader (Biotek; Winooski, VT).

Confirmation of prodigiosin in strain DJPM01

LC/MS and UV–Vis spectroscopic analyses of extracted DJPM01 agar plates resulted in the detection of prodigiosin (1), which was confirmed with an authentic standard (**Figures 2.6A,B**). Prodigiosin (1, *m/z* 324.2072; calculated for C₂₀H₂₆N₃O [M + H]⁺, 324.2076) was detected with a characteristic UV–Vis spectrum (λ_{max} = 540 nm; **Figure 2.6A,B; Appendix, Figure 6.6**; Andreyeva and Ogorodnikova, 2015). MS/MS spectra of the peak identified as prodigiosin (1) were congruent with the fragmentation pattern of the authentic standard. Furthermore, a number of distinct peaks with similar UV–Vis absorbances were detected in chromatograms with masses consistent with prodiginines based on mass defect values and even [M + H]⁺ masses. Metabolomic analyses indicated the presence of numerous features consistent with those of 2-methyl-3-propyl prodiginine (2), 2-methyl-3-butyl prodiginine (3), 2-methyl-3-heptylprodiginine (4), cycloprodigiosin (5), and other uncharacterized prodiginines (**Appendix, Figure 2.6D and Table 6.5**).

Effects of UV light on the production of prodigiosin and other secondary metabolites

Incubation under UV light yielded a less-saturated, darker-hued pigmentation compared to control plates grown in the dark, with no UV exposure (**Figure 2.6C**). LC/MS analyses of UV-treated strain DJPM01 indicated statistically significant differences between UV-treated, control, and media blank samples (**Appendix, Figure 6.7**). A statistically significant decrease was observed in prodigiosin (1; $q < 0.001$) and

other metabolites (**Appendix, Figures 2.6C and 2.7**). Mass spectral analyses suggested that these uncharacterized prodiginines may be cyclized, reduced, oxygenated, and otherwise modified variations of prodigiosin. LC/MS analysis of a UV-treated prodigiosin (1) standard indicated a conversion of the compound to a non-pigmented degradation product with an m/z of 354.2169 $[M + H]^+$ and probable molecular formula of $C_{21}H_{28}N_3O_2$ (**Appendix, Figure 6.8**). Additionally, several other features with mass shifts consistent with addition of CH_2O to major prodiginines were observed. The only compound unique to the UV-treated strain DJPM01 was glycerophosphoserine (m/z 522.2831; calculated $[M + H]^+$ for $C_{24}H_{45}NO_9P$, 522.2832;). Masses congruent to those of arylpolyenes and migrastatin (m/z 512.2617; calculated $[M + H]^+$ for $C_{24}H_{45}NO_9P$, 522.2832) were also detected. No LC/MS or UV–Vis spectroscopic evidence of other pigments was found.

Discussion

Massilia species have been identified in diverse ecological niches across Antarctica, though little is known about their function within cold systems. We isolated strain DJPM01 from a microbial mat which sits adjacent to one of the saltiest aquatic features on Earth, the hypersaline Don Juan Pond in the McMurdo Dry Valleys (Toner et al., 2017). We identified this aerobic, Gram negative bacterium as a member of the *M. frigida* species. This strain was isolated on R2A agar, which is commonly utilized to cultivate bacteria from Antarctic environments, though growth on this medium is not an indicator of abundance or importance to a given system (Pulschen et al., 2017). Strain DJPM01 is estimated to be present at low abundance (~0.03%) in the established microbial mat community; however, abundance and ecological importance are not mutually exclusive, as highlighted with the rare biosphere concept (Lynch and Neufeld, 2015). Members of the *Massilia* genus have been of interest for their agricultural and pharmaceutical applications (Lewin et al., 2021; Dahal et al., 2021b). Thus, we examined both potential adaptations to ecologically relevant conditions and production of microbial secondary metabolites, including the pigment prodigiosin, in strain DJPM01.

Phylogeny and ecophysiology of an Antarctic Massilia strain

Modern prokaryotic species concepts strive to incorporate both the advanced sequencing tools available to interrogate microbial genomes as well as phenotypic assays (Rosselló-Móra and Amann, 2015). While specifics may be debated, it is generally agreed that species exhibit a large degree of genomic and phenotypic coherence (Rosselló-Móra and Amann, 2015). Conservative estimates indicate that a DDH of >70% (Tindall et al., 2010), ANI threshold of 95–96% (e.g., Konstantinidis and Tiedje, 2005; Konstantinidis et al., 2006), and 16S rRNA gene sequence identity of >98% (Kim et al., 2014) is appropriate for delineating prokaryotic species for taxonomic purposes. While strain DJPM01 has >99% sequence identity with several *Massilia* type strains, highest ANI (97.36%) and DDH (75.6%) were found with *M. frigida* strain CCM8695. Thus, we conclude that strain DJPM01 is a strain of this species group.

Strain DJPM01 shares phenotypic similarities with *M. frigida* strain CCM 8695 and other psychrotolerant strains, including colony morphology, motility, inability to grow at temperatures at or above 30°C, and sensitivity to UV-C irradiation (Holochoová et al., 2020). Strain DJPM01 was more tolerant of alkaline growth conditions compared to reported observations of both psychrotolerant and mesophilic *Massilia*, though several *Massilia* species are also able to grow on media above pH 10, including *M. alkalitolerans*, which can survive on media adjusted to pH 12 (Xu et al., 2005). Based on 16S rRNA gene and whole-genome comparisons, strain DJPM01 forms a clade with seven named psychrotolerant *Massilia*, including the five described Antarctic species (Wang et al., 2018; Yang et al., 2019; Holochoová et al., 2020; Sedláček et al., 2022). Of the Antarctic *Massilia* with published genome sequences, four have been described as producing a pink-red pigment when grown on R2A agar (Holochoová et al., 2020) and contain the complete pathway for prodigiosin biosynthesis. The fifth, *M. antarctica* strain P9640, produces a blue-purple pigment, likely violacein (Sedláček et al., 2022). However, *Massilia* isolates beyond this clade (<82% ANI) have only been described as either unpigmented or as producing a yellow pigment, which could be carotenoids based on the putative BGCs present in their genomes (Gallego et al., 2006; Zhang et al., 2006).

Genomic adaptations to life in an Antarctic microbial mat

The genome of strain DJPM01 contains several adaptations to life in an Antarctic microbial mat. Low temperatures can negatively impact transcription and translation, both through decreases in reaction rate and through changes in the secondary structure of DNA, RNA, and proteins (De Maayer et al., 2014). Strain DJPM01 encodes multiple sets of genes associated with either preventing the formation of transcription-inhibiting RNA secondary structures (cold shock proteins) or unraveling the structures that do form (RNA helicases; Siddiqui et al., 2013). Similarly, strain DJPM01 has genes encoding for several protein chaperones to ensure correct folding of polypeptide chains. Other genes, such as *fab* genes and a putative $\Delta 9$ -desaturase, are involved in the biosynthesis of unsaturated fatty acids to increase membrane fluidity at low temperature (De Maayer et al., 2014). Cold-adapted organisms synthesize compatible solutes to prevent the formation of ice crystals within the cell and combat changes in salinity associated with freezing temperatures (Chin et al., 2010). Genes involved in the biosynthesis and transport of the compatible solute glutamate are present within the genome, as well as several cation/proton antiporters, all of which are utilized to counteract changes in osmotic pressure (Kuroda et al., 2005; Saum and Müller, 2008).

UV irradiation can lead to both DNA damage and the generation of ROS (Castenholz and Garcia-Pichel, 2012). Strain DJPM01 contained several genes for reducing ROS (e.g., catalase and peroxiredoxin) and for DNA repair, including two photolyases, which may enable photo-repair of DNA during long Antarctic days in the austral summer. UV stress can also be mediated through the production of radiation-absorbing pigments. Strain DJPM01 contains BGCs for prodigiosin and violacein, pigments associated with UV survival in other organisms, such as *Serratia*, *Streptomyces*, and *Vibrio* species (Borić et al., 2011; Stankovic et al., 2012; Cediël Becerra et al., 2022), and increases the UV-screening ability of commercial sunscreen (Suryawanshi et al., 2015). Violacein has been described in a number of *Massilia* species, including *M. antarctica* from James Ross Island and *M. violaceinigr*a from Tianshan glacier, China (Agematu et al., 2011; Myeong et al., 2016; Wang et al., 2018; Yang et al., 2019;

Sedláček et al., 2022). To date, prodigiosin has only been described in Antarctic *Massilia* species (Holochová et al., 2020). The prevalence of pigment biosynthesis genes in Antarctic *Massilia* suggests that both violacein and prodigiosin could play a role in adaptation to the environment. However, it remains unclear why only the Antarctic *Massilia* appear to possess genes for prodigiosin. Regardless, climatic change in the MDV likely involves dynamic changes in solar radiation (Obryk et al., 2018), thus the production of UV-screening pigments may not provide a universal advantage to the organisms in question.

In addition to pigments, several other putative secondary metabolite BGCs were identified in the genome, including a homoserine lactone BGC potentially involved in quorum sensing, and a putative siderophore with similarity to myxochelin, shown to play a role in iron acquisition (Kunze et al., 1989; Montgomery et al., 2013). Several RiPP clusters are also present, which produce diverse molecules with a wide variety of functions, including antibacterial or enzyme inhibiting activities (Arnison et al., 2013). Notably, strain DJPM01 contained a putative BGC involved in the biosynthesis of the antifungal polyketide gladiofungin (Niehs et al., 2020). Further analysis of PKS modules in this gene cluster supported its identification as encoding for gladiofungin biosynthetic machinery (**Figure 6.4**). This is the first report of a *Massilia* with the genetic potential to produce this antifungal. Gladiofungin has been shown to inhibit the fungus *Purpureocillium lilacinum* (Niehs et al., 2020), which has been isolated from the Antarctic peninsula (Gonçalves et al., 2015). The gladiofungin BGC in strain DJPM01 may play a role in inhibition of fungal competitors.

Strain DJPM01 has a similar abundance of genes associated with cold adaptation when compared to other psychrotolerant *Massilia* species, including genes for protein chaperones, RNA helicases, and fatty acid biosynthesis. Minor differences exist between strain DJPM01 and the closely related *M. frigida*, including the presence of specific toxin-antitoxin genes, which are believed to play a role in stress response (Fucich and Chen, 2020). Compared to non-Antarctic *Massilia*, the six *Massilia* isolated from Antarctic regions contained a significantly greater abundance of secondary metabolite

BGCs (**Appendix, Table 6.4**). The genetic potential to synthesize a wide arsenal of molecules in response to specific stressors under poly-extreme conditions would impart an ecological advantage (Giddings and Newman, 2015b; Sayed et al., 2020). Of the putatively identified BGCs, the myxochelin cluster is present across all six genomes, suggesting a strong selection pressure for iron acquisition (Kunze et al., 1989). Other gene clusters that are only present in one or two genomes, such as cephamycin and the gladiofungin we identified here, may play more specialized roles in competitive inhibition of other community members *in situ*. Secondary metabolism of Antarctic microbes remains poorly characterized and the abundance of putative BGCs that encode for yet uncharacterized molecules may be of interest in the search for novel bioactive compounds (Correa and Abreu, 2020). Extreme environments likely offer an untapped wealth of secondary metabolites that may prove useful in a number of industrial or biomedical applications (Giddings and Newman, 2015a; Sayed et al., 2020).

Prodigiosin, a molecule involved in environmental adaptation

Our work provides the first molecular characterization of prodiginines produced by a member of the *Massilia* genus. Holochová et al. (2020) noted the pigmentation and BGC for prodigiosin in four Antarctic *Massilia* species, and Woodhams et al. (2018) examined a mesophilic *Massilia* (now *Pseudoduganella*; Lu et al., 2022) for the production of prodigiosins, however none were detected. In our study, mass defect analysis (**Figure 2.8**) identified several metabolites congruent with prodiginines detected within similar retention times (5–8 min), including analogs formed from either the addition of oxygen, reduction, or cyclization. Some of these metabolites are known compounds; however, most are unknown and warrant further investigation (He et al., 2022). Due to the high sequence similarity between putative prodigiosin BGCs in *M. frigida*, *M. rubra*, *M. mucilaginoso*, and *M. aquatica*, it is likely that all five red-pigmented Antarctic *Massilia* are genetically capable of producing related pigments.

The biosynthesis of prodigiosin may provide numerous advantages to the cold-adapted *Massilia*, which may serve as pioneer species during microbial mat establishment (Sommers et al., 2019). Given prodigiosin's role as a UV-protectant in other microbial

species (Borić et al., 2011; Stankovic et al., 2012; Cedić Becerra et al., 2022), we sought to determine the effect of intense UV-A radiation on pigment production by strain DJPM01. Following irradiation, we saw a decrease in the abundance of most metabolites, including prodigiosin (1), with the exception of an increase in an oxygenated prodiginine (m/z 340.2020), which may represent a UV photolysis product of 2-methyl-3-butyl-prodiginine (3). We did not detect the pigment violacein under any growth condition tested, despite strong genomic evidence for a violacein BGC. Thus, it is likely that this molecule may be produced under yet-unknown conditions, such as in response to predation by metazoans (Choi et al., 2015). The color change (**Figure 2.6C**) of UV-treated strain DJPM01 was likely related to prodiginine degradation, as the irradiated prodigiosin standard was observed to lose color intensity following 24-h of UV-A exposure (**Appendix, Figure 6.8**). As pigment degradation is likely the result of UV-A exposure, and the rate of photolysis is independent from molecular synthesis, transcriptional or translational response in prodigiosin biosynthesis upon UV exposure should be further investigated. Alternatively, *Massilia* within the natural mat environment may also benefit from other photoprotective mechanisms. Photoprotective compounds can provide a shielding effect to the entire community within microbial mats, so that not only the producers benefit from the UV protection (Gareia-Pichel and Castenholz, 1994).

In addition to UV-protection, prodigiosin has been linked to numerous bioactivities. For example, prodigiosin has been shown to function as an antimicrobial agent against both Gram positive and Gram negative bacteria (Danevčič et al., 2016), and thus may serve as a mediator of interspecies competition within the microbial mat environment. Prodigiosin has also been reported as a nematocidal agent (Habash et al., 2020). Nematodes in the MDV function as grazers within microbial mats, and have been identified as present within the DJP mat (Siegel et al., 1979; Andriuzzi et al., 2018), thus prodigiosin may serve as a defense against overgrazing. Additionally, psychrophilic organisms increase intracellular ATP concentrations to support metabolic activity at low temperatures (Amato and Christner, 2009). In high-density cultures of *S. marcescens*, accumulation of intracellular ATP occurred at a higher rate in prodigiosin pigmented

cells, compared to non-pigmented cells (Haddix and Shanks, 2018). Thus, prodigiosin may also play an undescribed role in energetics and adaptation to low temperatures. More directed experiments are necessary to elucidate the ecological function of prodiginines in strain DJPM01 *in situ*.

In the coming decades, changes in solar radiation will impact MDV communities through both direct and indirect processes. The low albedo of MDV soils leads to increased levels of energy absorption compared to the surrounding ice (Dana et al., 1998) resulting in higher summer *in situ* temperatures (Fountain et al., 2014), which could lead to possible hydrological and geochemical changes, such as enhanced permafrost melting and salt mobilization (Ball and Virginia, 2012). Additionally, both anthropogenic and natural (i.e., volcanoes and wildfire) emissions drive variations in solar flux in the MDV, potentially resulting in a decrease UV radiation (Obryk et al., 2018). Thus, microbial community response to UV irradiation will likely vary with local climatic trends. Investigating the responses of individual community members to simulated stressors (i.e., UV radiation) can inform how natural microbial communities may respond to environmental variation associated with anthropogenic climate change.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at:

<https://www.ncbi.nlm.nih.gov/genbank/>, JAQOZV0000000000.

<https://www.ncbi.nlm.nih.gov/genbank/>, OQ346149.

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Appendix: Figures

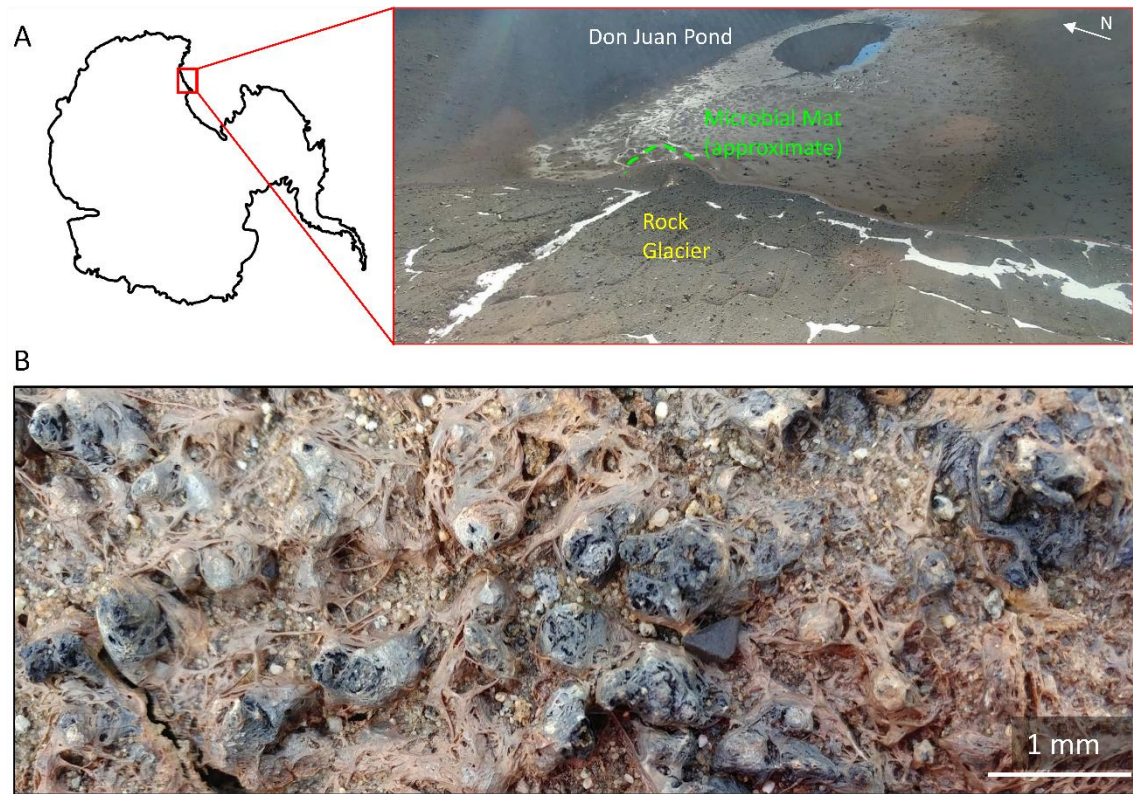


Figure 0.1. The Don Juan Pond (DJP) microbial mat. (A) Location of the microbial mat within DJP basin. Approximate location of the basin in Antarctica is inset. (B) Close up of the DJP basin microbial mat collected for enrichment cultures that yielded strain DJPM01.

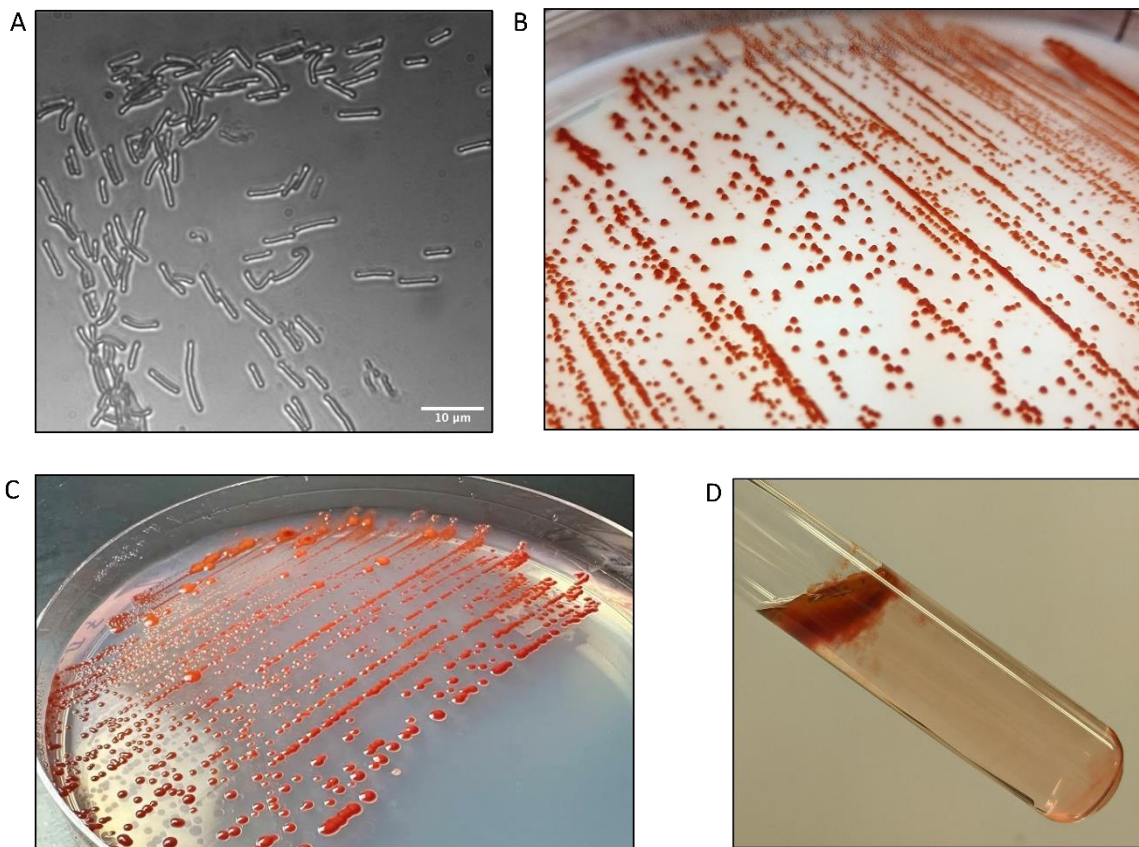


Figure 0.2 Morphology of strain DJPM01. (A) Light microscopy image of strain DJPM01 using phase shift. Cells were rods and 4.65 μm in length on average. (B,C) Strain DJPM01 grown on R2A agar at 10 (B) and 15 (C) °C. Colonies were robust and resisted removal from the agar. Bright red pigmentation was present at all temperatures. (D) Pellicle formation by strain DJPM01 in R2A broth, held at an angle.

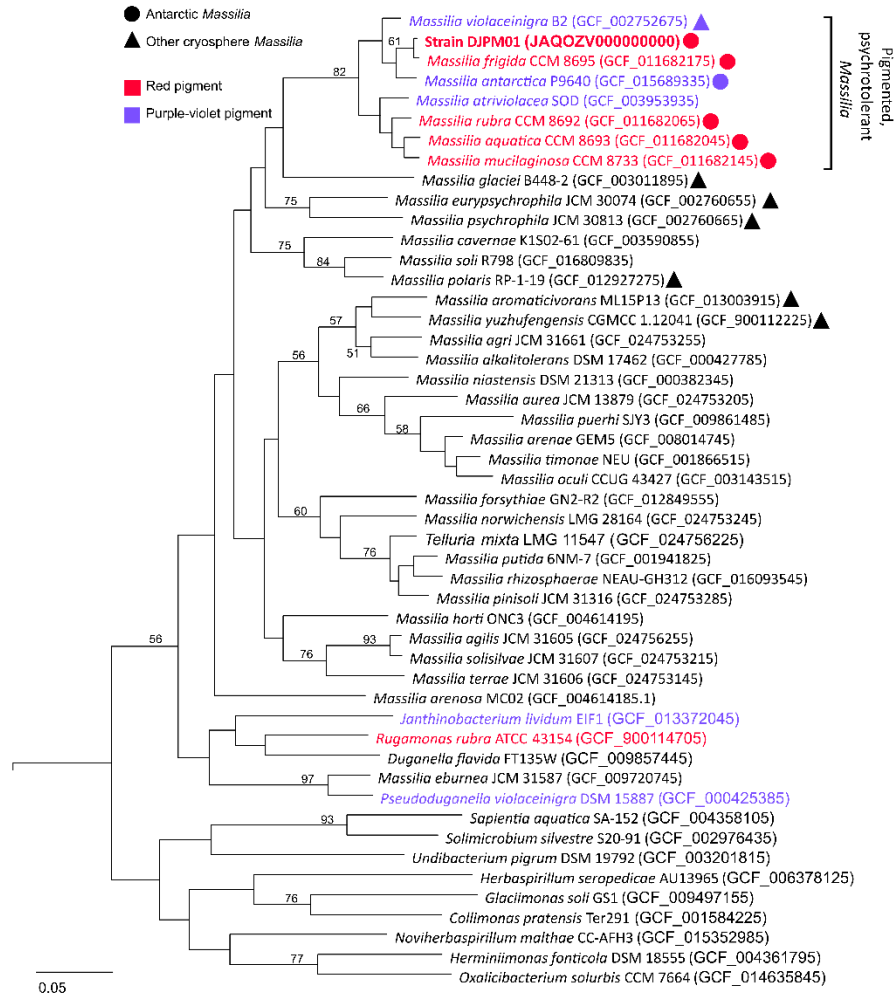
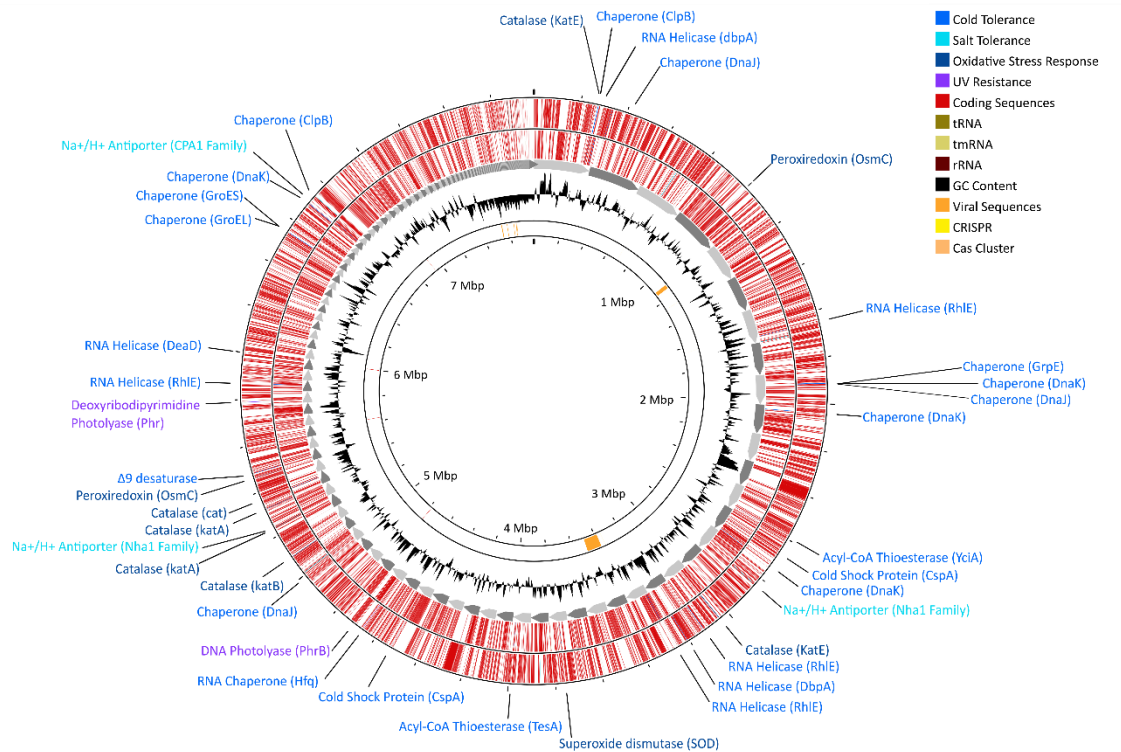


Figure 0.3 Multiple-gene tree of *Oxalobacteraceae* family members. Tree was constructed using OrthoFinder v2.5.4 (Emms and Kelly, 2019). All *Massilia* with phenotypic and genotypic evidence for red (prodigiosin) or purple-violet (violacein) pigment production form a clade with >87% ANI between any given pair. All members of this clade were isolated cryosphere environments, including all five Antarctic *Massilia*. All *Massilia* genomes cluster into a single clade, with the exception of *M. eburnea*, originally identified as a species of *Pseudoduganella* (Lu et al., 2020). Node values represent bipartition support values >50. An Antarctic *Shewanella* species (strain BF02_Schw) was used as an outgroup (DQ677870.1).



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Figure 0.4 Gene map for strain DJPM01 highlighting proposed environmentally-relevant genes. Linear contigs are sorted by length and represented by grey arrows. Outer tracks depict coding sequences as annotated by Prokka v1.13.7 (Seemann, 2014), separated by strand. Genes related to environmentally-relevant stress adaptation and response are highlighted and labeled. Results of VirSorter2 v2.2.3 (Guo et al., 2021) and CRISPRCasFinder v4.2.20 (Couvin et al., 2018) are present on the inner ring.

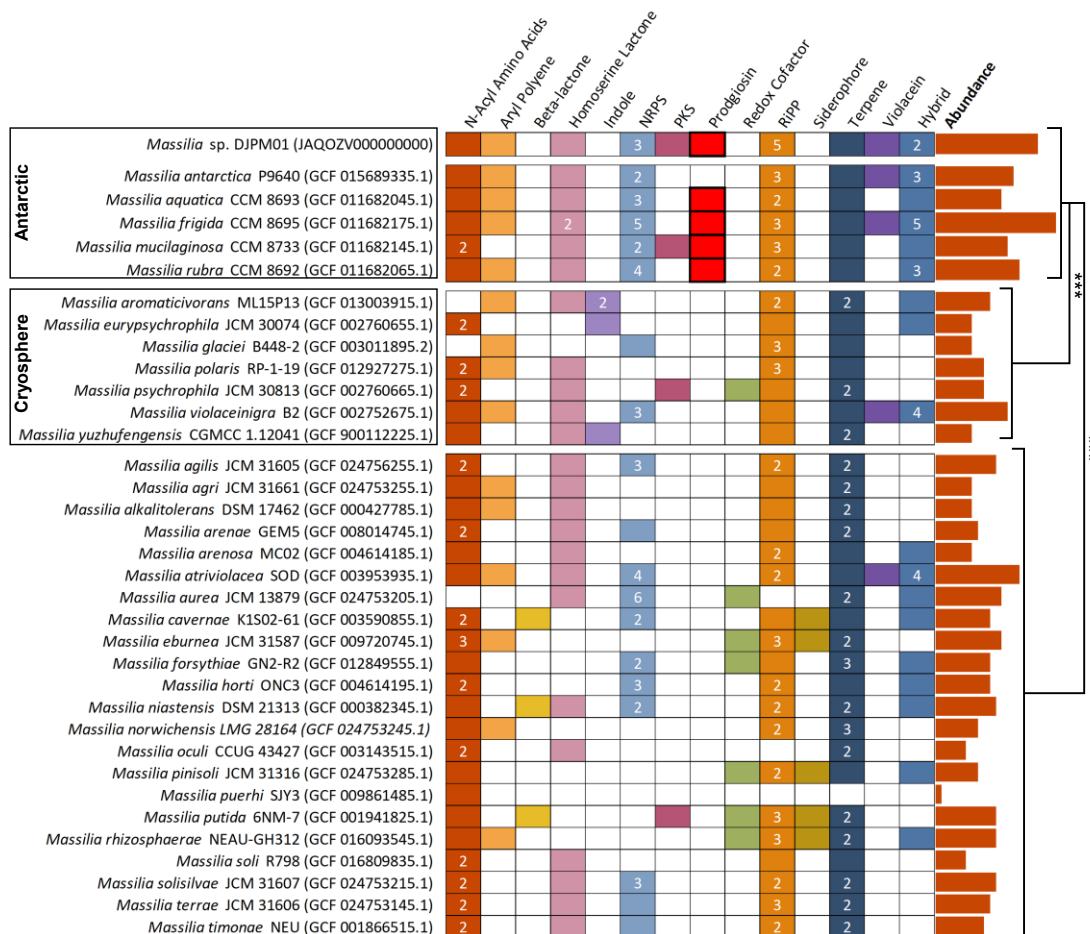
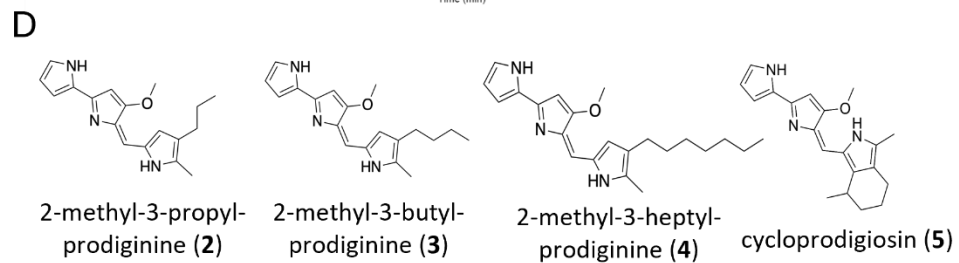
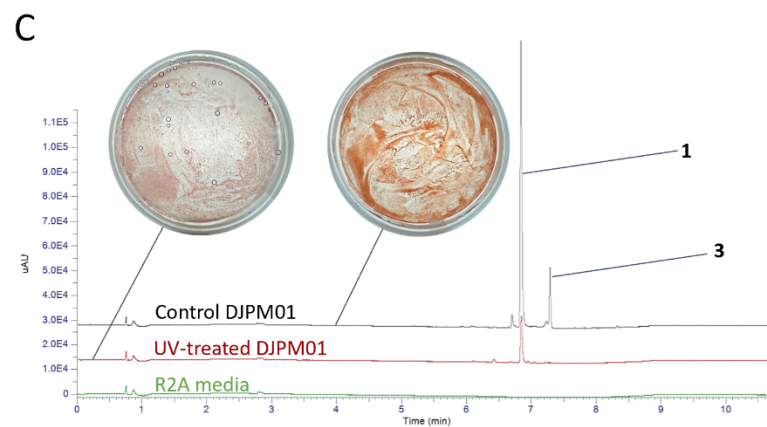
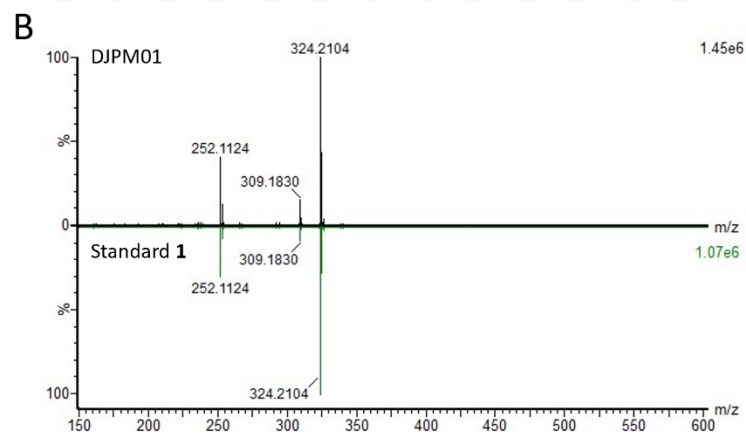
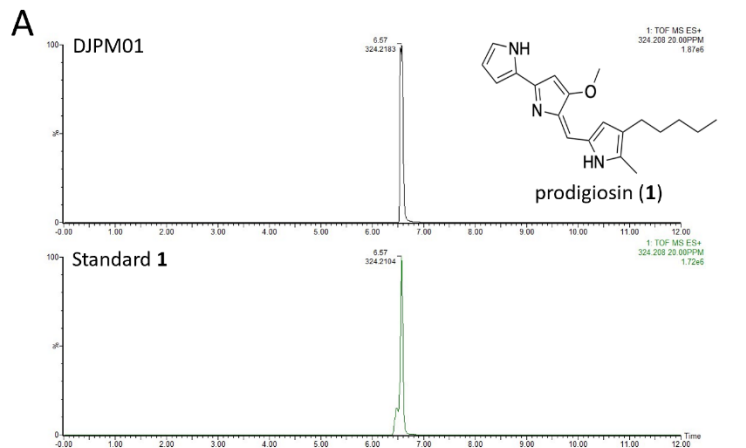


Figure 0.5 Annotated secondary metabolite BGCs in *Massilia* reference genomes, sorted by isolation environment. AntiSMASH-annotated clusters have been sorted into broad categories, with hybrid clusters containing motifs of multiple categories (i.e., PKS-NRPS). Numbers represent the total abundance of clusters when multiple are present within the genome. Prodigiosin and violacein BGCs were confirmed via BLAST. Absolute abundance of clusters are plotted to the right of the figure and are significantly different between isolation environments (Tukey test, *** = $p < 0.001$). NRPS - nonribosomal peptide synthetase; PKS - polyketide synthase; RiPP - ribosomally synthesized and post-translationally modified peptide.

Figure 0.6 Detection of prodigiosin and derivatives in strain DJPM01 extracts. (A) Extracted ion chromatograms (m/z 324.208) of prodigiosin (1) in DJPM01 R2A agar extracts (black) compared to a prodigiosin standard (green). (B) Mirrored MS/MS mass spectra of prodigiosin (1) in DJPM01 agar extracts and prodigiosin standard. UV–Vis spectra of prodigiosin (1) in DJPM01 agar extracts (black) and prodigiosin standard (green) showing identical spectra. (C) Stacked UV trace diagram for plate extracts from control and UV-treated cultures. Dominant peaks corresponding to prodigiosin (1) and 2-methyl3-butyl-prodiginine (3). Images of UV-exposed (left) and control (right) plates following 11 days of incubation. (D) Molecular structures of detected prodigiosin derivatives.



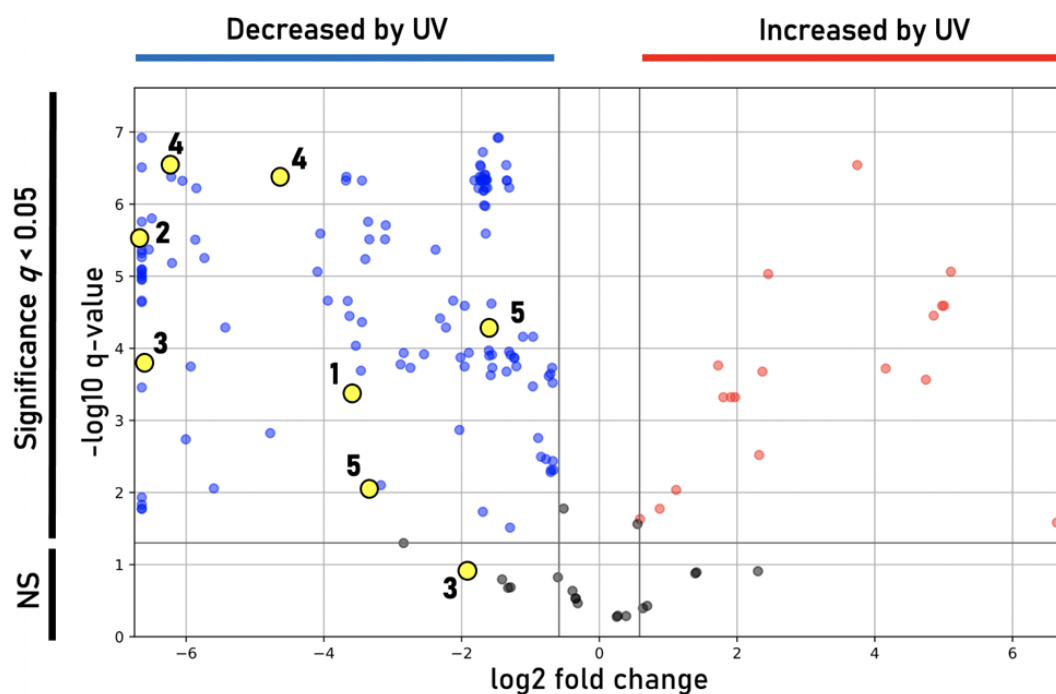


Figure 0.7 Differential metabolite features observed under UV treatment. Volcano plot showing statistically significant ($q < 0.05$) features in DJPM01 grown on R2A agar that are either increased (red) or decreased (blue) relative to control samples that were not irradiated with UV-A light. Numbered and unnumbered yellow dots correspond to known features and unknown, prodiginine-related features, respectively.

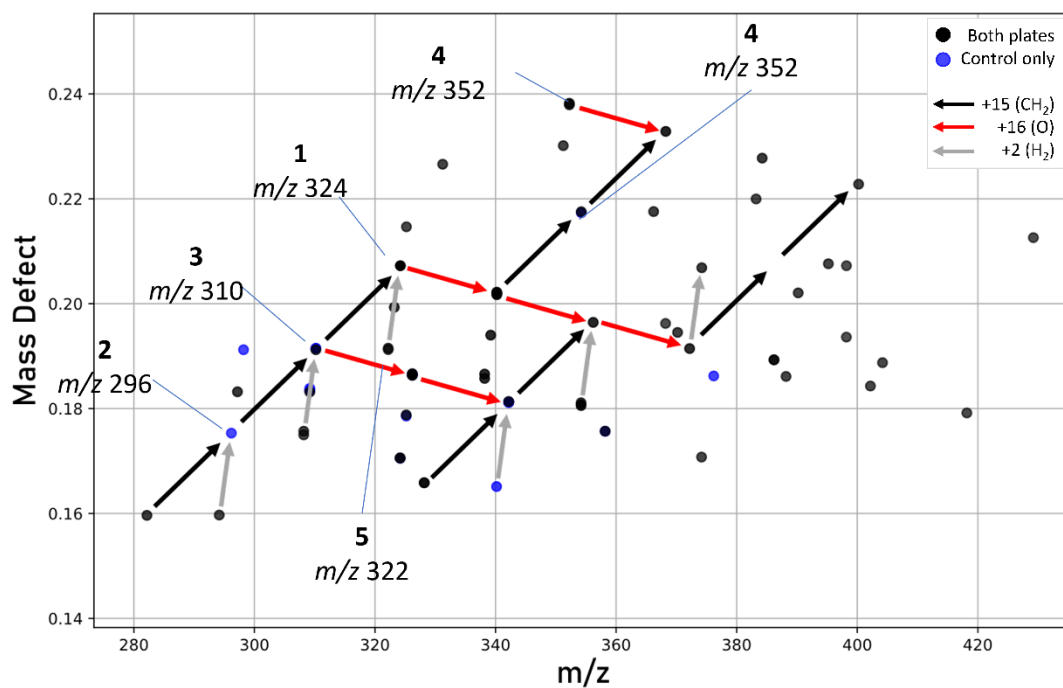


Figure 0.8 Mass defect plot showing features hypothesized to be prodiginines.

Features seen in either both treatments (black dots) or control plate extracts only (blue dots). No unique prodiginine features were seen in UV-treated plates. Arrows represent mass defect vectors, with red denoting the addition of oxygen (+16), black the addition of a methyl group (+15), and gray a loss of hydrogen possibly indicating cyclization.

**CHAPTER THREE COMMUNITY ADAPTATION AND RESPONSE OF AN
ECOLOGICALLY-RELEVANT POLAR DESERT BACTERIUM TO SUBTLE
CHANGES IN SALINITY**

Publication Note

This chapter is in preparation for submission to a peer-reviewed journal. The contributors are Jacob Shaffer, Robert Samples, Lesley-Ann Giddings, Becky Ball, Ross Virginia, and Jill Mikucki

Microbial ecology analyses including biomass description, microbial cultivation work, DNA and RNA extraction, and bioinformatic analyses were all performed by Jacob Shaffer. Metabolite extraction was performed by Lesley-Ann Giddings, and metabolite data were generated by Robert Samples, with resulting data analyses performed by Jacob Shaffer. Samples utilized in this study were originally collected by Becky Ball and Ross Virginia, who also performed the geochemical analyses. This chapter was written by Jacob Shaffer with editorial assistance from Jill Mikucki.

Abstract

Microbial communities in the polar desert soils of McMurdo Dry Valleys (MDV) of Antarctica are largely structured by abiotic factors. In the dry mineral soils, the melting of near surface permafrost leads to the formation of soil seeps, characterized by subtle increases in soil moisture and salinity. Here we describe the microbial community structure and functional potential along a MDV moisture and salinity gradient within a soil seep. This natural gradient reflects the subtle changes likely to occur under steadily warming conditions. Amplicon sequencing of 16S rRNA genes identified increases in the phyla Bacillota and Bacteroidota correlated with soil conductivity, and a higher relative abundance of salt stress related genes were present in metagenomes from moderately saline soils. A potentially novel *Gillisia* sp. (strain FRX26; phylum Bacteroidota) present in several saline MDV basins, was isolated from the seep soils and contains several genomic adaptations to elevated salinity. Using a combination of transcriptomic and metabolomic analyses, we found that the production of extracellular polymeric substances (EPS) increases in response to subtle changes in salinity (0.2 M vs 0.5 M NaCl) which may provide protection against osmotic stress in the seep environment. Together, these results highlight the impact of subtle environmental change on soil communities in a region experiencing increases in melt.

Introduction

The McMurdo Dry Valleys (MDV) are the largest ice free region of the continent (Lee et al., 2017), and receive less than 50 mm of precipitation annually (Fountain et al., 2009). Regional air temperatures range from -50 °C in the winter to > 10 °C in the summer (average -15 to -30 °C, dependent on location; Obryk et al., 2020). Communities within the MDV are microbially dominated and lack both vertebrates and vascular plants (Cary et al., 2010). Together, these factors make the MDV a unique natural laboratory for studying how microbial life both responds and adapts to environmental challenges.

The soils of the ice-free regions of Antarctic host diverse microbial communities structured largely by abiotic factors (Cary et al., 2010; Lee et al., 2012; Bottos et al., 2020). While the vast majority of water in the MDV is frozen in the form of glacier ice, lake ice, or near surface permafrost, seasonal melt (Barrett et al., 2009; Ball et al., 2011) from perennial glacial streams creating well defined microbial hotspots both in the stream channels and in the surrounding hyporheic zones (Barrett et al., 2006; Zeglin et al., 2009; Stanish et al., 2013). Hydration of the arid soils leads to a redistribution of both nutrients and salts, such that soils in the wetted margins of streams and lakes tend to have lower salinity than nearby dry soils (Gooseff et al., 2007; Northcott et al., 2009). Other hydrological processes within the MDV, such as the melting of near surface, ice cemented permafrost (< 70 cm; Bockheim et al., 2007, 2008) or deliquescence by hygroscopic salts (Levy et al., 2011; Gough et al., 2017), create transiently wetted soils in the form of water tracks and seeps. As with hyporheic zones, formation of these features lead to the redistribution of salts; however, because wet patches do not flow to a terminal location for salt deposition (e.g., a lake or pond; Foreman et al., 2004), mobilized salts become concentrated at the surface following evaporation (Ball et al., 2011; Ball and Virginia, 2012; Ball and Levy, 2015). Thus, the formation of seeps and water tracks lead to increases in geochemical heterogeneity, and therefore habitat suitability, for soil microbial communities (Ball and Virginia, 2012). As increases in ice and permafrost melt within the MDV are leading to changes in geomorphology and community dynamics (Gooseff et al., 2017; Barrett et al., 2024), these natural gradients of moisture and salinity can be utilized to investigate both abiotic controls and the impact of a changing climate on soil community diversity and function.

Soil communities in the MDV are spatially distributed along abiotic gradients, largely of moisture, salinity, and pH (Cary et al., 2010; Lee et al., 2012; Geyer et al., 2014; Van Horn et al.,

2014; Bottos et al., 2020). Phototrophic primary production occurs largely through cyanobacteria in microbial mats and biocrusts (Cary et al., 2010; Power et al., 2024), bacterial community members are grazed upon by tardigrades and nematodes (Adams et al., 2006), and nematodes of the genus *Eudorylaimus* serve as the apex predators for the region (Shaw et al., 2018). Fungi and archaea are both present, though at relatively low abundance compared to bacteria (Dreesens et al., 2014; Richter et al., 2014). Nutrient and water amendment studies within the MDV show that soil communities respond quickly to environmental changes. Following the amendment of either water or dilute organic matter sourced from cyanobacterial mats, communities within the Fryxell basin showed decreases in community diversity following 30 days, with final community structure dependent on the conductivity of the soils (Van Horn et al., 2014). Pseudomonadota and Bacteroidota increased in soils ranging from ~ 100 to $500 \mu\text{S cm}^{-1}$ following either water or organic matter amendment, and members of the Bacillota phylum increased significantly following organic matter treatment, and constituted nearly the entire community of organic-amended high salinity ($4800 \mu\text{S cm}^{-1}$) soils. In a follow-up study conducted on $100 \mu\text{S cm}^{-1}$ soils, Buelow et al. (2016) sequenced community RNA following amendments and identified differential responses within Pseudomonadota and Bacteroidota, and a similar increase in Bacillota following organic amendment. Additionally, transcripts associated with the transport and utilization of organic matter by Actinobacteriota, Bacteroidota, Bacillota, and Pseudomonadota were significantly more abundant following organic matter amendment, suggesting that rapid community response to nutrient input is dominated by members of a few phyla. In a longer-term study within the same basin, community composition was shown to be dramatically altered following 6 years of C, N, or P amendments, though treatment by water alone did not have lasting impacts on community structure (Aanderud et al., 2018). Because ice and permafrost melt redistribute both legacy organic matter and salts within soils, similar changes in community structure, and therefore function, will likely occur throughout the MDV (Fountain et al., 2014). Thus, we can utilize extant naturally occurring features, such as permafrost melt seeps, to describe the impact of environmental change on soil communities.

To assess how subtle changes in moisture and salinity impact natural soil communities in the MDV, we analyzed soil profiles from the Fryxell basin in wet seep soils and dry soils from outside of the seep boundary. Community structure of the seep was described via amplicon sequencing of the 16S rRNA gene, and genomic potential was examined via metagenomic

sequencing. We then examine the transcriptome and metabolome of an ecologically relevant isolate from seep sediments, a yellow pigmented *Gillisia* sp. (strain FRX26), to describe how an individual community member responds to salt stress. Collectively, this approach provides insight into both the current impact of seep formation on MDV soil communities, as well as how ecologically-relevant bacteria may respond to changes in its local environment as a consequence of environmental change.

Materials and Methods

Site description and sample collection.

Lake Fryxell occupies the eastern most basin within the Taylor Valley (77°37'S, 163°12–13'E; **Figure 3.1a**; tables and figures in this chapter can be found in the **Chapter Three Appendix**). Taylor Valley is glacially-carved, with relatively shallow walls in the east compared to the steep-walled basins in the western upper Valley. Not including the ice-covered lake, the Fryxell basin is approximately 61 km² (Salvatore et al., 2021) with an estimated average soil moisture of ~ 1.5% (Salvatore et al., 2023). Soils in the Fryxell basin are generally highly oligotrophic (0.1 – 0.3 mg organic C g soil⁻¹), dry (< 1-5% moisture), poorly developed (95 – 99% sand), and heterogeneously saline (Campbell and Claridge, 1987; Campbell et al., 1998; Burkins et al., 2000). The active layer within the Fryxell basin is shallow, averaging 10 – 70 cm depth to ice-cemented permafrost boundary (Bockheim et al., 2007, 2008).

Soils were collected on 13 December 2009 in triplicate both the inside and outside of a reoccurring meltwater seep (77°36.578'S 163°15.950'E; **Figure 3.1b**) previously characterized by Ball and Virginia (2012). At each sampling site, depth to permafrost was determined using a T-bar probe, and a pit was dug with samples collected at regular depths throughout the active layer (summarized in **Table 3.2**). Soil temperature was measured by inserting a thermistor probe several inches horizontally into the soil at each depth. Soils were placed into sterile Whirl-pak bags, transported to Crary Laboratory in coolers and frozen at -20 °C before shipment to Dartmouth College for storage and analysis.

Soil geochemistry and biomass.

The standard soil methods of the McMurdo Long Term Ecological Research program were followed for geochemistry (as described by Ball and Virginia, 2012). Briefly, soil moisture (as gravimetric water content) was estimated from a 20 g soil sample dried at 105 °C and recorded as g water g soil⁻¹. Soil pH from a 1:2 slurry of soil:deionized H₂O using a pH meter

(VWR) and electrical conductivity was measured using a 1:5 soil:deionized H₂O slurry and a conductivity meter (Orion Research). Soluble anions (SO₄⁻, PO₄⁻, NO₃⁻, NO₂⁻, Br⁻, and Cl⁻) were extracted from 10 g of soil in 50 mL of deionized water for 60 min on an orbital shaker, decanted, and centrifuged (25,250 × g; 10 min). Extracts were measured on a DX-120 ion chromatograph (Dionex Corporation). C and N were determined for air-dried and ground subsamples (75 mg) using a Carlo Erba 1500 elemental analyzer, and an additional subsample was acidified with 50% HCl, dried, and ground to determine organic C content.

Total microbial cells were enumerated via direct microscopic counts of stained cells as per Mikucki and Priscu (2007). Soils (~1 g) were homogenized with 1X PBS buffer (final ratio 1:10) and fixed with paraformaldehyde (final concentration 4%). Slurries were shaken horizontally at 100 rpm for 30 min (4 °C) and centrifuged at 50 × g (1 h at 4 °C) to settle larger particles. Resulting supernatants were filtered onto 0.2 µm black polycarbonate filters and stained with 25X SYBR Gold nucleic acid stain (Invitrogen) for 15 min. Filters were rinsed with 1 mL of filter-sterilized MilliQ water and enumerated by fluorescence microscopy on a Zeiss Axio Imager.M2 microscope.

Soil DNA extraction and analysis.

DNA was extracted from soils using a DNeasy Powerlyzer PowerSoil Kit (Qiagen) following manufacturer's protocol with an amended bead beating step. Approx. 0.5 g soil were added to Lysing Matrix E bead beating tubes (MP Biomedical) and homogenized twice on a FastPrep-24 Bead Beater (MP Biomedical) at 4 m s⁻¹ for 35 s followed by 5 min rest on ice. DNA was eluted in 25 µL molecular grade water and quantified via Qubit fluorometer (Invitrogen). Eluent from at least 8 extractions were pooled and concentrated via vacuum centrifugation, and shipped to Novogene Corporation Inc. for either 16S rRNA gene amplicon sequencing or metagenomic sequencing. Amplicon sequencing targeted the V3-V4 region (341F-CCTAYGGGRBGCASCAG and 806R-GGACTACNNGGTATCTAAT; Caporaso et al., 2011; Klindworth et al., 2012) using the NovaSeq 6000 platform (Illumina) with 250 bp paired-end reads at 100,000 raw reads per sample. Shotgun metagenomic sequencing was performed with 150 bp paired-end reads at ≥ 20 million raw reads per sample.

Initial amplicon read filtering was performed using an in-house Novogene pipeline. Sequencing adapters and barcodes were trimmed for all samples, and paired-end reads were merged using FLASH v1.2.11 (Magoč and Salzberg, 2011) and quality filtered using fastp

v0.23.1 (Bokulich et al., 2013). Analysis of amplicon libraries was performed in R v4.4.1 (R Core Team, 2023) using the DADA2 v1.32.0 (Callahan et al., 2016) and phyloseq v1.48.0 packages (McMurdie and Holmes, 2013). Reads were filtered to remove all sequences containing Ns and with expected errors >2. Paired ends were aligned and chimeric sequences were removed. Taxonomic identity was assigned using the Silva database v138.2 (Quast et al., 2013). Sequences belonging to chloroplast or mitochondria were removed before analyses. Amplicon sequence variants (ASVs) were clustered at 97% similarity via DECIPHER v3.0.0 (Wright, 2016) and speedyseq v0.5.3.9021 (McLaren, 2020) to generate operational taxonomic units (OTUs) for comparison to previously published MDV datasets. Diversity metrics were calculated for both ASVs and OTUs using the vegan package v2.6-10 and analyses were visualized using ggplot2 v3.5.1 (Hadley Wickham, 2016).

Shotgun metagenomic reads were trimmed and filtered using Trimmomatic v0.36 (Bolger et al., 2014) and assembled using metaSPAdes v3.11.0 (Nurk et al., 2017). Quality was assessed via QUAST v4.4 (Gurevich et al., 2013). Assembled metagenomes were annotated using metaprokka v1.15 (Seemann, 2014; Ryan Cook and Andrea Telatin, 2023), and resulting coding sequences were further analyzed via the KEGG (Kanehisa and Goto, 2000) database using GhostKOALA v3.1 (Kanehisa et al., 2016). Putative secondary metabolite biosynthetic gene clusters (BGCs) were identified using antiSMASH v 7.1.0 (Blin et al., 2021).

Isolation and genomic analyses of strain FRX26.

Strain FRX26 was isolated from a sediment slurry of sample WB1 using the methods described in Shaffer et al. (2023). Briefly, 10 mL of filtered 1X PBS was added to 1 g of soil and shaken horizontally at 10 °C for 30 min. Soil particles were settled via gentle centrifugation (50 x g, 1 hr, 4 °C) and the resulting supernatant was serially diluted and plated on either Difco Marine 2216 (BD) or R2A agar. Plates were incubated at 10 °C for up to 1 month, after which colonies of with distinct phenotypes were aseptically picked and streaked for isolation. Strain taxonomy was determined via amplification and Sanger sequencing of the 16S rRNA gene using 515F (GTGCCAGCMGCCGCGGTAA; Caporaso et al., 2011) and 1391R primers (GACGGGCGGTGTGTRCA; Turner et al., 1999), followed by comparison to the NCBI Taxonomy database (Federhen, 2012).

Genomic DNA was extracted from individual colonies using a DNeasy Powersoil PowerLyzer DNA extraction kit (Qiagen) as described above. Genomic libraries were prepared

and sequenced by MR DNA (Molecular Research LP) using the NovaSeq 6000 platform (Illumina, 250 bp paired-end reads, 500 cycles). Genomic sequences were trimmed and filtered using Trimmomatic v0.39 (Bolger et al., 2014) and assembled using Unicycler v0.5.1 (Wick et al., 2017). Assembly quality was assessed via QUAST v5.2.0 and CheckM v1.2.3 (Parks et al., 2015). The resulting assembly was annotated using Prokka v1.14.6 (Seemann, 2014), GhostKOALA v3.1 (Kanehisa et al., 2016), COG, antiSMASH v7.1.0 (Blin et al., 2021), and comparison to in-house databases via BLAST v2.12.0 (Altschul et al., 1990). Taxonomic placement was assessed via the Type Strain Genome Server (TYGS; Meier-Kolthoff et al., 2022) using 16S rRNA gene sequences and whole-genome comparisons, as well as through the enveomics Average Nucleotide Identity (ANI) server (Rodriguez-R and Konstantinidis, 2016). Relative abundance of strain FRX26 within other MDV amplicon libraries was determined by processing raw reads from available datasets using the above DADA2 pipeline (Callahan et al., 2016). The FRX26 16S rRNA gene sequence was queried against the resulting databases using BLAST v2.12.0 (Altschul et al., 1990); sequences were considered a match at $\geq 97\%$ identity.

Salt challenge experiment.

Strain FRX26 was grown in a saline complex medium (SCM) defined in **Table 3.1**. Cultures were grown in 75 mL volumes in nephalo flasks supplemented with either 0.2 M or 0.5 M NaCl and incubated at 15 °C for two weeks. Optical density measurements at 600 nm (OD₆₀₀) were performed daily, and aliquots for colony forming unit (CFU) counts were collected after one and two weeks of incubation, as well as at the time of inoculation. CFUs were determined via serial dilution and plating on SCM agar; colonies were counted following 2-weeks of incubation.

At the termination of the experiment, cultures were thoroughly homogenized and subsampled into one 40 mL aliquot and three 10 mL aliquots. All aliquots were pelleted at 3500 × g for 15 min (4 °C), the supernatant was removed, and pellets were immediately frozen in dry ice. TRIzol (Invitrogen) was added to subsamples designated for RNA extraction (9 mL TRIzol added to pellets from 40 mL subsamples) before freezing. All samples were stored at -80 °C before analysis.

Metabolite extraction and analysis.

Metabolites were extracted and analyzed via liquid chromatography mass spectrometry (LC-MS) at the Center for Mass Spectrometry at Smith College. Cell pellets were extracted with

methanol and extracts were analyzed on a Vanquish Horizon ultra high purity LC system with a Q-Exactive HF-X Hybrid Quadrupole-Orbitrap MS (Thermo Scientific). The LC instrument was configured with a HSS T3 C18 column (Waters, 1.8 μ m, 2.1 x 100 mm) using a VanGuard FIT precolumn and a VH-D10-A UV detector. Samples (2 μ L) were injected using a flow rate of 0.5 mL min⁻¹ into a prewarmed column (40 °C). UHPLC separation was performed using the following parameters: 2% acetonitrile: 98% water with 0.1% formic acid for 1 min, 2–40% over 4 min, 40–98% over 3 min, 98–2% over 0.2 min, and 2% for 2 min. UV–Vis data were collected at 534 nm. Electrospray ionization occurred in probe position D using the following conditions: 40 sheath gas flow, 8 auxiliary gas flow, 1 sweep gas flow, 3.5 kV spray voltage, 380 °C capillary temperature, 50 radiofrequency funnel level, and 350 °C auxiliary gas temperature.

Resulting data were aligned using MS-DIAL v5.5.241113 (Tsugawa et al., 2015) with a minimum peak height of 30,000 and removal of blank features below a 100 fold sample max / blank average change. Blank and control peaks were filtered using MPACT v1.00 r24.11.1 (Samples et al., 2023), and non-reproducible features were removed using a coefficient of variation (CV) filter of 0.3. Putative feature identities were assigned by alignment of MS/MS fragmentation patterns of statistically significant features to the NIST Standard Reference Database (Stein et al., 2023).

RNA extraction and analysis.

RNA was extracted from cell pellets frozen in TRIzol using a Direct-zol RNA Extraction Kit (Zymo). RNA purity was determined via PCR of the 16S rRNA gene as performed above, and quality and quantity were determined via both Qubit fluorometer (Invitrogen) and TapeStation (Agilent). Riboreduction, library preparation, and Illumina sequencing (100 bp paired end reads; 30M reads) were performed by Maryland Genomics.

Transcript sequences were aligned to the sequenced genomes using bowtie2 v2.2.5 (Langmead and Salzberg, 2012), and count files were generated using samtools v1.9 (Danecek et al., 2021) and anvi'o x5.5 (Eren et al., 2015). Reads were assembled into transcripts using StringTie v2.1.7 (Pertea et al., 2015) and transcripts were quantified via Salmon v1.10.1 (Patro et al., 2017). Differential expression of genes was assessed using the R package DESeq2 v1.34.1 (Anders and Huber, 2010), using a cut off of log₂ fold change (LFC) ≥ 1 or ≤ -1 and adjusted p-value (Benjamini-Hochberg adjusted Wald test; p_{adj}) ≤ 0.05 to determine significance.

Results

Geochemistry and biomass.

Soils collected from inside the seep had greater water content (4.99 - 8.47 % g g⁻¹) than soils outside of the seep (0.55 - 5.50% g g⁻¹), and moisture for both increased with depth (**Figure 3.1c**; **Table 3.2**). Electrical conductivity varied by an order of magnitude between soils within and outside of the seep, with the highest concentration present in the top 0 – 2 cm of the seep (Sample WB1; **Figure 3.1d**; **Table 3.2**). WB1 also had the highest concentration of Cl⁻, SO₄⁻, and NO₃⁻, while ion concentrations at lower depths (i.e., below 5 cm) were similar to dry soils. Additionally, pH differed between wet surficial soils (average pH 8.19 from 0 – 10 cm) and dry surficial soils (average pH 9.77 0 – 10 cm; **Figure 3.1e**; **Table 3.2**). Microscopic counts of DNA containing cells averaged 1.19×10^7 cells g⁻¹ within the seep, with 8.70×10^5 cell g⁻¹ present in sample WB1 (**Figure 3.1f**). In the dry profile, cells were present at an average of 5.64×10^6 cell g⁻¹.

Community structure and genomic potential.

High quality DNA was obtained from all soils except for the surficial 0 – 2 cm within the seep, despite multiple attempts and methodologies (**Table 3.3**). Sequencing of amplified 16S rRNA genes returned between 152562 – 173660 paired reads, with 99699 – 114822 merged reads passing all filters. Following removal of mitochondrial and chloroplast sequences, reads were grouped into 3072 ASVs corresponding to 1885 OTUs, with samples from within the seep containing 1902 ASVs (1243 OTUs) and external (dry) soils containing 1438 ASVs (853 OTUs). Sequences were assigned to 32 bacterial phyla and one archaeal phylum (Thermoproteota; **Figure 3.2a**). The most abundant phyla present in all samples were Actinomycetota (57.3% of total reads from seep samples, 53.7% of total reads from samples outside of seep), Gemmatimonadota (seep: 7.3%, outside: 15.7%), Chloroflexota (seep: 10.7%, outside: 10.4%), Pseudomonadota (seep: 11.2%, outside: 7.3%) and Bacteroidota (seep: 4.1%, outside: 2.2%). Three phyla had a significant correlation with conductivity measurements, including Bacteroidota (**Figure 3.2b**; $R^2 = 0.628$, $p = 0.011$), Bacillota ($R^2 = 0.487$, $p = 0.037$), and Thermodesulfobacteriota ($R^2 = 0.492$, $p = 0.034$). An additional four phyla significantly correlated with moisture: Deinococcota ($R^2 = 0.469$, $p = 0.042$), Gemmatimonadota ($R^2 = 0.469$, $p = 0.042$), Pseudomonadota ($R^2 = 0.614$, $p = 0.013$), and Synergistota ($R^2 = 0.614$, $p = 0.012$).

Alpha diversity differed between the wet and dry soil profiles, and in general, samples clustered as OTUs had lower diversity than when clustered as ASVs (**Figure 3.3**). Observed species richness outside of the seep was fairly consistent with increasing depth, and both Shannon and Inverse Simpson indices increased to a maximum value within the 10 – 25 cm samples. The lowest diversity for all metrics was observed at 2 – 5 cm depth. Both species richness and the Shannon index were higher in seep compared to outside soils. Additionally, alpha diversity correlated strongly with conductivity for seep soils, though only the Shannon index was significant when sequences were clustered as OTUs (**Table 3.4**).

Metagenomic sequencing resulted in an average of ~ 103 million reads per sample, which assembled into an average of 1.98 million contigs per sample (**Table 3.5**). Reading frame identification identified an average of 2403016 putative coding sequences per metagenome, with an average of 25.6% non-hypothetical annotations by Prokka and 24.03% non-hypothetical annotations by GhostKOALA. Of annotated genes, the most abundant KEGG Level 2 categories were shared between all metagenomes: carbohydrate metabolism (average abundance of 12.9% of annotated genes), protein families: genetic information processing (11.4%), amino acid metabolism (8.2%), protein families: signaling and cellular processes (7.9%), metabolism of cofactors and vitamins (5.1%), and translation (5.0%). The majority of annotated genes were present in all metagenomes (7725 Prokka-annotated and 4883 GhostKOALA-annotated genes).

Genes associated with salt tolerance are present in all five metagenomes (**Figure 3.4**). Genes associated with the biosynthesis or transport of compatible solutes include those for glycine betaine (*betB*, *gbcA*, *gbcB*, *proW*, *opuD*), choline (*betA*, *pcs*), ectoine (*doeA*, *ectC*), proline (*prdB*, *laaA*, *proC*, *lhpA*) and trehalose (*glgE*, *ostAB*, *thuE*, *thuF*, *thuG*, *treAYZ*). Of these, *betA*, *doeA*, *lhpA*, and *ostAB* were positively correlated with conductivity. Several putative Na⁺ transporters were also annotated within the metagenomes, including those in the *mnh*, *nha*, and *nrq*, with only *nrqA* correlating with conductivity. Five putative genes encoding mechanosensitive channels were also annotated (*mscK*, *mscL*, *mscM*, *mscMJ*, *mscS*), and all were positively correlated with conductivity.

Isolation and taxonomic placement of Gillisia sp. FRX26.

Plating of Sample WB1 on Marine Agar (Difco 2216) yielded 5.89×10^4 CFUs g sediment⁻¹, and isolation of unique colony phenotypes resulted in a total of 10 bacterial strains. A yellow-pigmented strain was designated strain FRX26 upon isolation, and was identified as a

member of the family Flavobacteriaceae (Bacteroidota) via Sanger sequencing of the partial 16S rRNA gene, with greatest sequence similarity to *Gillisia limnaea* DSM 15749 (NR_042141.1; 98.90% identity). High similarity was also present to *G. hiemivivida* IC154 (OQ619006.1; 98.05%), *G. lutea* (MZ994595.1; 97.93%), *G. myxillae* (NR_043635.1; 96.71%), *G. mitskevichiae* (NR_025822.1; 96.35%). Comparison of both whole-genome nucleic acid and amino acid sequences via TYGS confirmed taxonomic placement of strain FRX26 within the *Gillisia* genus (**Figure 3.5; Table 3.6**), and identified *G. limnaea* DSM 15749 as the closest type strain to strain FRX26. However, both digital DNA-DNA hybridization (dDDH; 22.6%) and ANI (81%) were below proposed species thresholds (dDDH $\geq 70\%$ and ANI $\geq 95 - 96\%$ (Konstantinidis and Tiedje, 2005; Konstantinidis et al., 2006; Auch et al., 2010), suggesting that strain FRX26 likely represents a novel species.

Comparison of the strain FRX26 16S rRNA to the Fryxell seep amplicon libraries reveals that reads with $\geq 97\%$ identity are present in all samples between 0.04% and 5.79% of total sequences per library (**Figure 3.2b**). Reads matching strain FRX26 are most abundant within the seep, and are highest in soil depths 2 – 5 cm (5.79%), 15 – 25 cm (1.81%), and 5 – 10 cm (0.76%) within the wet profile. *Gillisia* sequences are most abundant in the dry profile at the 2 – 5 cm depth (0.25%). Reads with $\geq 97\%$ identity to *Gillisia* sp. FRX26 are also present in 51 of 376 publicly available 16S rRNA amplicon libraries, ranging from 0.003% to 9.01% relative abundance in amended samples (**Figure 3.6; Table 3.7**). Matching sequences were in highest abundance in higher salinity environments, including pond margins near Lake Brownworth ($\sim 1000 \mu\text{S cm}^{-1}$; Wright Valley; Geyer et al., 2013, 2014), and associated with brine outflow at Blood Falls (up to $\sim 2 \text{ M Cl}^-$; Taylor Valley; Campen et al., 2019). Additionally, OTUs with $\geq 97\%$ similarity to *Gillisia* sp. FRX26 increased from 0.03% to up to 32% relative abundance in moderately saline soils ($\sim 500 \mu\text{S cm}^{-1}$) following the addition of cyanobacteria-derived carbon (Van Horn et al., 2014).

Genomic analysis of strain FRX26.

Genomic sequencing resulted in a total of 27247130 reads, which assembled into 307 contigs totaling 4.2 Mbp. Reading frame identification resulted in a total of 3705 putative coding regions. Of these, 1599 had non-hypothetical annotations via Prokka (43.2%), 1386 via GhostKOALA (37.4%), and 2253 via eggNOG (60.8%). The most abundant Level 2 categories via KEGG annotation were protein families: genetic information processing (168 genes),

carbohydrate metabolism (151), protein families: signaling and cellular processes (141), amino acid metabolism (118), and unclassified: metabolism (85; **Figure 3.7**). The most abundant COG categories were cell wall/membrane/envelope biogenesis (273 genes), amino acid transport and metabolism (270), replication, recombination and repair (203), translation, ribosomal structure and biogenesis (165), and transcription (160). A total of 642 putative genes did not have functional annotation via any utilized pipeline.

Several putative genes within the strain FRX26 genome may play a role in response to osmotic stress (**Figure 3.8**). Complete or nearly complete biosynthesis pathways are present for proline (*proABC*) and trehalose (*glgAB. treYZ*), as well as a transporter for glycine betaine (*opuD*), all of which are utilized as compatible solutes for osmotic balance in other bacterium (Kempf and Bremer, 1998). The strain FRX26 genome also contained several putative genes for Na⁺ antiporters in the *mrp*, *nap*, *nha*, and *nrq* families, and *trkAH* K⁺ importers. A total of five mechanosensitive channels were annotated from the mini, small, large, and K⁺-mediated families. Additionally, several genes are present which may play a role in the production of extracellular polymeric substances (EPS), including *wecA*, *algI*, and *rfbABCD*, which may play a role in desiccation tolerance (Chang et al., 2007; Lebre et al., 2017).

Of the 1088 KEGG Orthologies (KO) assigned by GhostKOALA, 1037 were present in at least one *Gillisia* reference genome, and 51 were unique to strain FRX26, including hydrogenase genes *hypCDEF* and thiamine biosynthesis genes *thiCDG*. Several KEGG categories included genes that were either in higher or lower abundance in strain FRX26 compared to reference *Gillisia* genomes. Compared to reference genomes, the strain FRX26 genome had a higher copy number genes encoding for antiporters, DNA repair, restriction enzymes, and fatty acid desaturases. Putative genes present at a lower abundance include dehydrogenases associated with amino acid and fatty acid metabolism. All reference *Gillisia* genomes contained putative BGCs encoding for a terpene cluster with low similarity to characterized carotenoid BGCs, an aryl polyene with low similarity (30%) to flexirubin, and either a type III PKS or a type III PKS-aryl polyene hybrid cluster. Comparison of the PKS BGC between strain FRX26 and genomes of reference *Gillisia* and an *Antarcticibacterium* revealed that this BGC appears highly conserved within this clade (**Figure 3.9**).

Metabolic and transcriptomic response to subtle increase in salt concentration.

When *Gillisia* sp. FRX26 was grown in SCM, fastest growth (via OD₆₀₀) was observed between 38 and 86 hours of incubation, with growth rates of $\mu = 0.82 \pm 0.12$ (average $\pm$ standard deviation) for cultures grown in 0.5 M NaCl and $\mu = 0.71 \pm 0.11$ for 0.2 M cultures (**Figure 3.10a**). Following 14 days of incubation, 0.2 M cultures had an average cell density of 5.01×10^8 CFU mL⁻¹ and 0.5 M cultures had an average density of 4.93×10^8 CFU mL⁻¹ (**Figure 3.10b**). Optical density varied between replicates (0.2 M culture range: 0.92 – 1.14; 0.5 M culture range: 0.81 – 1.01).

Metabolomics resulted in a total of 788 features passing all filters, with 1 feature unique to the 0.2 M treatment and 4 features unique to the 0.5 M treatment. Hierarchical Clustering Analysis of normalized metabolite features resulted in clustering of salt treatments together (**Figure 3.11**), though differences in overall profile were not statistically significant based on multivariate analysis via nonmetric multidimensional scaling (**Figure 3.12**). Using a peak size cutoff of 1×10^6 , 66 features were identified significantly more abundant in the 0.5 M treatment, and 24 features were identified as being significantly more abundant in the 0.2 M treatment. (**Figure 3.13**). Of the 49 significant features with fragmentation patterns, 10 were consistent with peptidic molecules and 16 were consistent with lipid-based metabolites (**Figure 3.14**).

Sequencing of mRNA resulted in an average of 12136560 reads per library, with 81% alignment to the draft *Gillisia* sp. FRX26 genome; one replicate of the 0.5 M NaCl treatment failed library preparation, resulting in five biological replicates for the 0.2 M treatment and four replicates for the 0.5 M treatment. Following differential expression analysis, a total of 49 transcripts were significantly more abundant in the 0.5 M treatment ($\text{LCF} \geq 1$, $p_{\text{adj}} \leq 0.05$) and 56 transcripts were significantly less abundant in the 0.5 M treatment ($\text{LCF} \leq -1$, $p_{\text{adj}} \leq 0.05$; **Figure 3.15; Table 3.8**). Of significantly different transcripts, 82 had putative functional annotations from at least one of the three utilized pipelines (32 more abundance in 0.5 M; 50 less abundant in 0.5 M). Several transcripts that were significantly more abundant putatively encode genes associated with EPS or capsule production, including *espK* (LFC = 2.03), *algA* (LFC = 1.70), and *wzi* (LFC = 1.50), or general polysaccharide biosynthesis, including three genes with putative glycosyltransferase-like function (LFC = 2.15, 1.57, and 1.44). Transcripts significantly less abundant in the 0.5 M treatment included three putative catalase genes (*ydbD_1*, LFC = -1.51; *ydbD_2* = -1.49; *katE*, LFC = -1.24), as well as a putative Na⁺/H⁺ antiporter *NhaG* (LFC = -

1.16). While not differentially abundant, several genes with putative roles in salt tolerance were present in transcripts for both treatments, including three mechanosensitive channels, five Na⁺ antiporters, and biosynthesis genes for trehalose.

Discussion

Microbiology of an Antarctic meltwater seep.

Water availability is a limiting factor in desert systems, and as such, areas of increased moisture can provide an oases for life (Lebre et al., 2017). This holds true in the MDV deserts where perennial melt supports microbial communities (Cowan et al., 2023). However, in the MDV changes in moisture also drive salt redistribution that can lead to stressful conditions for microorganisms and thus altering community structure and function (Bottos et al., 2020). Soils throughout the MDV have a considerable range in salinity, from freshwater equivalent to several times the salinity of seawater (Bockheim, 1997; Mikucki et al., 2010; Ball and Levy, 2015). The Fryxell seep profile had higher soil moisture than average soils in the basin (Salvatore et al., 2023), though was drier than measurements taken from the same seep the previous season (Patch #4 in Ball and Virginia, 2012). Surface conductivity was higher than average basin soils ($\sim 500 \mu\text{S cm}^{-1}$; Barrett et al., 2004) and on the higher end of previous seep measurements (average $\sim 4300 \mu\text{S cm}^{-1}$; maximum $> 8000 \mu\text{S cm}^{-1}$; Ball and Virginia, 2012). The seep profile was within moisture and conductivity ranges observed in water tracks throughout Taylor Valley (Ball and Levy, 2015), though was an order of magnitude more saline than was observed by George et al. (2021). Additionally, soil moisture was generally lower than what is seen in the wetted hyporheic zones and lake shores, where water content up to 60% have been observed (Lee et al., 2018). Conductivity was also considerably higher than observed in wetted margins, which have been observed up to $\sim 1600 \mu\text{S cm}^{-1}$ in the streams (Coyne et al., 2020; Monteiro et al., 2022) and $\sim 3000 \mu\text{S cm}^{-1}$ for Lake Fryxell (Northcott et al., 2009).

Salinity is a major driver in global soil community diversity (Lozupone and Knight, 2007). This holds true in the MDV; as with other investigations in MDV soils (Geyer et al., 2013, 2014; Van Horn et al., 2014; Feeser et al., 2018; Bottos et al., 2020; George et al., 2021), community diversity within the Fryxell seep was negatively correlated with conductivity (**Figure 3.3; Table 3.4**). Where comparable methods were utilized, alpha diversity metrics for seep soils were generally higher than other non-riparian Taylor Valley wet patches, including water tracks (George et al., 2021). However, as water track soils were only collected to 10 cm depth and the

greatest diversity in the seep was observed in the 10 – 15 cm depth, it is possible a similar high diversity trend in water tracks would be observed. Diversity within the seep was also higher than wet patches associated with surface snow melt (Van Horn et al., 2013), where Chao1 values ranges from ~ 30 – 300 in the Fryxell basin. Shannon diversity was similar to communities in the wetted margin of a Fryxell basin pond, though Chao indices were much lower in both the wet and dry seep soils (Lee et al., 2018). Thus, it is possible that while accumulation of salts at the surface of the seep results in lower community diversity, the elevated moisture content and lower salinity found below 10 cm in the seep provide a unique microbial niche associated with greater diversity.

While previous studies in the MDV have identified negative correlations between soil moisture and the phyla Acidobacteriota and Actinobacteriota (Van Horn et al., 2014; Lee et al., 2018; George et al., 2021; Jiang et al., 2022; Monteiro et al., 2022), neither trend was observed in the seep. The phylum Actinobacteriota did not correlate to moisture nor conductivity, and Acidobacteriota, which makes up a large proportion of other MDV soil communities (Geyer et al., 2014; Van Horn et al., 2014; Lee et al., 2018; Monteiro et al., 2022), were in < 2% abundance in all wet and dry amplicon libraries. Low abundance or absence of Acidobacteriota has been described in several MDV regions, including Upper Wright, Miers, and Beacon Valleys. Other studies have reporting contrasting abundances of Acidobacteria within the same basin (Brownworth Pond, Wright Valley; Jackson et al., 2022; Monteiro et al., 2022), potentially as a result of small scale patterns in community distribution (Feaser et al., 2018). In the Taylor Valley water tracks, Pseudomonadota, Gemmatimonadota, and Bacteroidota were in higher abundance in wetted soils compared to dry soils. In seep samples, Pseudomonadota and Gemmatimonadota both correlated with moisture, while Bacteroidota significantly correlated with conductivity. The phylum Bacillota has been observed to correlate with elevated salinity in both the Wright (Geyer et al., 2014) and Taylor Valleys (Van Horn et al., 2014); in identifying differences in Fryxell basin soils communities between ~100 and ~4800 $\mu\text{S cm}^{-1}$, Van Horn et al. (2014) identified an increase of Bacillota sequences from < 2% in low salinity samples to ~ 44% in high salinity samples. While only reaching a maximum of ~ 6% in the seep, we did see a significant correlation between Bacillota abundance and conductivity. Bacillota have been identified in several saline and hypersaline environments (Oren, 2008) and their abundance throughout the MDV has been hypothesized to be due to endospore formation, although not all

members of this phylum have that capability (Setlow and Johnson, 2012; Ramos et al., 2019; Severgnini et al., 2021). Thus, the abundance of this phylum with MDV amplicon surveys may be due to the persistence of inactive cells.

Several taxa identified as “rapid responders” to nutrient input during soil amendment studies are differentially abundant within the Fryxell seep. In addition to correlation with salt, Bacillota was identified by both Van Horn et al. (2014) and Buelow et al. (2016) as increasing dramatically in abundance following the addition of organic matter, especially in highly saline soil. The response of Bacteroidota was more complicated in amendment studies; 16S rRNA gene sequence abundance for Bacteroidota (mostly corresponding to the class Flavobacteriia) increased from ~2% to 26% at “moderate” salinity ($\sim 500 \mu\text{S cm}^{-1}$; Van Horn et al., 2014). However, in a $100 \mu\text{S cm}^{-1}$ soil plot, Bacteroidota transcripts decreased following both water and organic matter treatments, though reads associated with the class Flavobacteriia did significantly increase following organic amendment (Buelow et al., 2016). While water amendment alone was not enough to create a long-lasting impact on community structure (Aanderud et al., 2018), the increased abundance of Flavobacteriia following organic matter treatments, especially at moderately elevated salinity, and the elevated abundance of Flavobacteriia in both seeps and water tracks in Taylor Valley suggest that this class will become an increasingly abundant soil community member resulting from increased melt, and therefore salt and nutrient transportation, in the MDV.

Adaptations to dynamic conditions in polar desert soils.

Microorganisms adapted to saline and hypersaline conditions can employ a variety of mechanisms to counteract osmotic stress. Despite the comparably subtle differences in conductivity between samples with metagenomic sequencing (maximum of $636 \mu\text{S cm}^{-1}$ in sample WB3), we identified several putative coding sequences for salt-stress related genes that correlated in abundance with salinity (**Figure 3.4**). Specifically, putative genes associated with the biosynthesis or transport of key compatible solutes, including ectoine, proline, and trehalose (Kempf and Bremer, 1998). Compatible solutes are uncharged organic molecules that are utilized by the cell to reduce osmotic pressure on the cell (Shivanand and Mugeraya, 2011). While genes associated with glycine betaine are present in all five metagenomes, they are most abundant in the DA1 metagenome and are (weakly) negatively correlated with conductivity. As desiccation is associated with the concentration of soluble ions and molecules and glycine betaine has been

shown to provide a survival advantage during evaporative stress (Bonaterra et al., 2005; Dreux et al., 2008; Sheen et al., 2013), it is possible that the enrichment in DA1, the driest sample, is an adaptation to desiccation, opposed to salinity. Several other metagenome sequences putatively encode various Na⁺ transporters, which are utilized by the cell to exclude cations and reduce ion toxicity, and K⁺ importers, which can be used to balance internal osmotic pressure (Kraegeloh et al., 2005; Kuroda et al., 2005; Steuber et al., 2014; Tsai et al., 2014; Rasmussen, 2023). The relative enrichment for putative salt stress genes in the WB3 genome suggests that, while soil conductivity in this sample is only slightly above average for the Fryxell basin, the community within the 5 – 10 cm depth of the seep appears to reflect the mobilization of salts within the seep. Combined with the decrease in alpha diversity from the 10 – 15 cm depth (WB4), these results support the importance of salinity in shaping communities within MDV soils, even under relatively small increases.

Distribution and adaptations of a potentially novel Gillisia sp., strain FRX26.

To better understand the response of cells to subtle salt changes, we selected a seep isolate, *Gillisia* sp. FRX26, for further experimentation. *Gillisia* spp. have been observed throughout Antarctica, and on average correspond to ~ 0.1% of reads in soil amplicon surveys (Dragone et al., 2025). Isolates have been cultured from a number of Antarctic sites, including high salinity soils from Shackleton Glacier (> 7000 $\mu\text{S cm}^{-1}$; Dragone et al., 2025), a low salinity littoral microbial mat in Lake Fryxell (Van Trappen et al., 2004), basal sea ice-algae assemblages in the Vestfold Hills region (Bowman and Nichols, 2005), and within an Antarctic sea sponge from Terra Nova Bay (Papaleo et al., 2012; Maida et al., 2014). Additionally, a 16S rRNA gene amplicon survey of salt deposits near Boulder Clay Glacier (Victoria Land) identified *Gillisia* as a major community member, with an average of 23% relative abundance (Azzaro et al., 2022). In water track communities, George et al. (2021) identified an increased abundance of Bacteroidota in in wet soil amplicon libraries compared to dry soils, with the majority of reads corresponding to *Gillisia*. Similar to seeps, water tracks are characterized by higher moisture and salinity compared to surrounding regions, although their formation is due to deliquesce by hygroscopic salts (Ball and Levy, 2015). While not seen in high salinity Fryxell basin soils by Van Horn et al. (2014), *Gillisia* sequences increased following the addition of exogenous carbon to moderately saline soils. Combined with the survey of the Fryxell basin seep, the distribution of *Gillisia* spp. throughout the MDV supports the importance of *Gillisia*

within saline Antarctic environments. In our seep study, the strongest phylum-level correlation to conductivity was observed with phylum Bacteroidota (**Figure 3.2b**), with a high abundance of *Gillisia* sequences present in the 2-5 cm depth of the seep ($\sim 2000 \mu\text{S cm}^{-1}$). While the abundance of *Gillisia* is unknown in the highest salinity sample, we were readily able to isolate *Gillisia* strains with distinct yellow pigmentation and identical 16S rRNA sequences from this sample, suggesting that regardless of abundance, *Gillisia* sp. FRX26 is present and is able to quickly grow with increased availability of carbon substrates. These results complement the findings of soil amendment studies, which suggested that members of the Flavobacteriia class are poised to respond to nutrient input (Van Horn et al., 2014; Buelow et al., 2016).

Gillisia spp. in general are adapted to saline conditions, and have been shown to grown in up to 2.5 M NaCl (Bowman and Nichols, 2005). The Antarctic sea ice isolates *G. hiemivivida* and *G. sandarakina* both have a minimum salt requirement of 0.2 M NaCl in culture and were unable to grow without the addition of artificial sea salts, suggesting a requirement for other ions in the medium (Bowman and Nichols, 2005). Similarly, strain FRX26 was unable to grow in either lower nutrient complex media utilized to culture freshwater bacteria (R2A broth) or R2A broth supplemented with only NaCl (up to 5%) suggesting a requirement for other ions that are in low abundance in R2A alone. Several putative salt-tolerance genes were identified within the strain FRX26 genome, with the majority encoding mechanosensitive channels and ion transporters. In halotolerant microorganisms, transport of ions is used to balance osmotic pressure and are utilized either in conjunction with or instead of compatible solutes (Oren, 2015). Generally, K^+ ions are imported into the cell via active transporters in the Trk, Ktr, and Kdp (Kraegeloh et al., 2005), while Na^+ ions are excluded via proton- or NADH-powered Na^+ pumps (Steuber et al., 2014; Tsai et al., 2014). Mechanosensitive ion channels, including the MscS, MscM, MscL, and MscK families, function as osmotic pressure release valves (Wilson et al., 2013). These channels open in response to hypoosmotic shock and quickly and non-specifically release internal solutes to prevent cell lysis. The presence of coding sequences for Na^+ antiporters are shared across the analyzed *Gillisia* genomes, suggesting that this strategy for halotolerance is conserved regardless of isolation source. Genes associated with compatible solute biosynthesis were less abundant than seen in other halophilic bacteria: we were unable to identify genes for glycine betaine, choline, or ectoine in any *Gillisia* genome, and the number of genes associated with trehalose biosynthesis were ranged from five in strain FRX26 and *G. limnaea* DSM 15749 to none in *G.*

marina CBA3202. Biosynthesis of these compounds is energetically expensive, thus in saline conditions, many organisms will opt to transport compatible solutes if they are available in the environment (Oren, 1999). Strain FRX26, as well as *G. hiemivivida* IC154, *G. lutea* M10.2A, and *G. mitskevichiae* DSM 19839 all have putative glycine betaine transporters, suggesting that *Gillisia* spp. may also rely on other community members for the production of osmolytes. Considering that several species of *Gillisia* were isolated from complex communities (e.g., associated with algal biomass), it is possible that members of these genus benefit from the production of solutes by other organisms.

Secondary metabolites are diverse molecules that serve a number of functions for the cell, including mediating synergistic and competitive interactions (Tyc et al., 2017). The genome of *Gillisia* sp. FRX26 also contains three putative secondary metabolite BGCs that may serve a variety of functions. To date, all isolated *Gillisia* produce a yellow (Van Trappen et al., 2004; Bowman and Nichols, 2005; Nedashkovskaya et al., 2005; Roh et al., 2013; Maida et al., 2014) or orange pigment (Bowman and Nichols, 2005; Vidal-Verdú et al., 2023). In *G. limnaea* DSM 15749, this pigment was identified as a combination of mono-and dicyclic carotenoids and are likely produced by a terpene BGC (Riedel et al., 2012; Takatani et al., 2015), which has been identified in other sequenced *Gillisia* genomes, as well. Carotenoids in bacteria have been linked to many activities, including photoprotection and modulation of membrane fluidity (Alcaíno et al., 2016). In Antarctic bacteria, they have been linked to protection against both freeze-thaw cycles and prolonged solar radiation (Dieser et al., 2010). Similarly, polyketides are a broad class of secondary metabolite with diverse functionality for the cell, including functioning as antimicrobial agents. The shared type III PKS BGC appears to be highly conserved amongst not just *Gillisia*, but other closely related Flavobacteriaceae (**Figure 3.9**). Antimicrobial activity in *Gillisia* spp. has been reported, though bioactivity was linked to the production of volatile compounds, rather than polyketides (Maida et al., 2014). As the production of these molecules may provide a competitive advantage to *Gillisia*, further investigation to elucidate any possible bioactivities will provide additional insights into how the rapid response of this genus to environmental change in MDV soils.

Response of an ecologically-relevant bacterium to subtle changes in salinity.

To investigate how subtle changes in salinity impact a community member, we cultured *Gillisia* sp. FRX26 under in situ seep formation salinities including minimum salt concentration

(0.2 M NaCl) and an elevated concentration (0.5 M NaCl). Growth in 0.2 M and 0.5 M NaCl altered both gene transcription and metabolite production by strain FRX26. Several transcripts putatively encoding for EPS-related polysaccharides biosynthesis genes were significantly more abundant in the 0.5 M treatment compared to the 0.2 M treatment (**Table 3.8**). EPS are a general class of secreted polymer that include a wide range of polysaccharides, proteins, exonucleotides, and lipids, and are an important element of the biofilm matrix (Flemming et al., 2023). While we were unable to conclusively identify any metabolites, we were able to link several features to either lipid or peptidic origin, thus it is possible that these metabolites were components of the secreted EPS, as well. The ability to form biofilms provide a number of advantages to both individual organisms and entire communities, including close physical association with other community members to facilitate cooperation (Flemming et al., 2016) and protection from desiccation (Mortel et al., 2004), UV radiation (Frösler et al., 2017), and diffusion of antimicrobial agents (Algburi et al., 2017). In saline environments, charged EPS particles can limit the diffusion of ions, thus protecting cells from osmotic pressure (Nunkaew et al., 2015; Kim and Chong, 2017; Haque et al., 2022). Biofilm formation has also been specifically linked to desiccation tolerance in desert soils; EPS production increases the retention of water in soils, thus prolonging water availability for the community in times of drought. In the context of the seep environment, the production of EPS by specific community members would provide a protective advantage for the entire community as a shield against the abundance of ion present in the soils closer to the surface. In a sea ice bacterial isolate, the production of EPS by *Colwellia psychrerythraea* 34H has been associated with both ice nucleating and ice associating activity (Huston et al., 2004), which, combined with increased survival after ultra-low temperature freezing (Marx et al., 2009) and elevated production of EPS at lower temperatures (Marx et al., 2009), provides an advantage for any community members associated with it in and near sea ice. Future experiments with *Gillisia* sp. FRX26 may very well unveil a similar community-supporting phenotype, where the production of EPS under relatively small changes in salinity provide protection to the entire soil community within the seep environment. This phenotype may lead to an observable increase in biocrust-like phenotypes as already observed in the MDV soils (Power et al., 2024), with unknown consequences for the total flux of nutrients and biogeochemical cycling within a region experiencing the impacts of a changing climate.

Conclusion

The consequences of environmental change in the MDV are already observable through alterations in hydrology, nutrient distribution, erosion, and community shifts (Gooseff et al., 2017; Barrett et al., 2024). Because salt, nutrient, and water availability will all be significantly impacted under changes in basin climate regimes, efforts to describe the impact of even relatively small changes in soils should be investigated. We identified differences in community structure and the abundance of salt response genes within the Fryxell seep that correlated with conductivity, even at $< 1000 \mu\text{S cm}^{-1}$. Additionally, the increase from 0.2 to 0.5 M NaCl in bacterial culture led to transcriptional and metabolomic changes in *Gillisia* sp. FRX26 that were likely associated with the production of EPS. While no metabolites were conclusively identified, our study provides initial findings to guide future studies of both bacterial cultures and environmental material. For example, as biofilm formation may play a crucial role in response to elevated salinity, future work conducted on Fryxell basin seeps should include attempts to identify possible biofilms, such as through fluorescent microscopy (Li et al., 2004). Additionally, collections of bacterial isolates collected across the continent (such as Dragone et al., 2025) can be leveraged to explore functional diversity in closely related organisms. For example, *G. limnaea* DSM 15749 is a close relative of *Gillisia* sp. FRX26 and was isolated from within the same MDV basin yet have low genome similarity. However, as genomic potential does not equate to activity, direct comparison of these two organisms under similar incubation conditions can reveal both shared and unique responses to relevant environmental challenges, and thus provide insights into expected changes in microbial communities within a changing ecosystem.

Subtle environmental changes in the MDV can lead to large impacts on the biology of soil communities. The mobilization and concentration of salts by permafrost melt is a cumulative effect (Ball et al., 2011), thus successive melt seasons and increases seep size and depth will lead to greater accumulation of salts over time. As soils become more and more saline, the pressure on extant communities will increase, likely leading to yet unknown changes in ecosystem function. By defining how both communities and individuals respond to relatively subtle changes in salinity, our findings can inform current models of soil ecology and thus improve predictions of ecosystem responses to a changing climate.

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Appendix: Tables and Figures

Table 0.1. Components of Saline Complex Medium (SCM)

Carbon Sources	Concentration (g L⁻¹)
Proteose Peptone	0.5
Yeast Extract	0.5
Dextrose	0.5
Casamino Acids	0.5
Soluble Starch	0.5
Sodium Pyruvate	0.3
Marine Salts	Concentration (mM)
Sodium Chloride	200 <i>or</i> 500
Magnesium Chloride	92.43
Magnesium Sulfate	22.81
Calcium Chloride	16.22
Potassium Chloride	7.38
Sodium Bicarbonate	1.91
	Concentration (μM)
Potassium Bromide	672.3
Strontium Chloride	127.5
Boric Acid	355.8
Sodium Monosilicate	14.1
Sodium Fluoride	57.2
Ammonium Chloride	20.0
Potassium Diphosphate	29.8
Additional Supplements	
ATCC Trace Mineral Solution	1 mL L ⁻¹

Table 0.2. Geochemistry of Fryxell Basin Seep

Sample ID	Wet Profile					Dry Profile				
	WB1	WB2	WB3	WB4	WB5	DA1	DA2	DA3	DA4	DA5
Sample Depth (cm)	0 – 2	2 – 5	5 – 10	10 – 15	15 – 25	0 – 2	2 – 5	5 – 10	10 – 25	25 – 35
Soil moisture (% g g ⁻¹)	4.99	6.09	7.4	8.14	8.47	0.55	1.45	1.63	3.34	5.5
C _{total} (%)	0.17	0.17	0.09	0.11	0.15	0.18	0.18	0.12	0.13	0.11
C _{organic} (%)	0.04	0.04	0.04	0.04	0.04	0.02	0.03	0.04	0.05	0.04
N (%)	0.01	0.01	0	0	0	0	0	0	0.01	0
pH	8	8.17	8.4	8.75	8.14	10.06	9.84	9.42	8.18	8.98
Eh (mV)	431.6	429.8	419.1	395.2	419.2	340	376.7	427.9	487.7	415.3
Conductivity (μS cm ⁻¹)	7,650.00	1,939.00	636	210.8	72.6	79.5	211.4	214.7	603	83.2
Cl ⁻ (μg g ⁻¹)	11,973.99	2,249.88	751.37	177.74	853.14	1.91	83.11	239.67	665.62	52.53
SO ₄ ⁻ (μg g ⁻¹)	430.35	151.21	44.96	28.07	85.19	9.25	27.68	44.47	44.63	12.26
PO ₄ ⁻ (μg g ⁻¹)	15.82	16.04	16.26	17.35	101.67	16.29	16.59	15.39	15.65	16
NO ₃ ⁻ (μg g ⁻¹)	470.91	95.16	0.78	7.59	33.63	1.36	4.5	10.62	20.79	0.77
NO ₂ ⁻ (μg g ⁻¹)	1.14	1.16	1.17	1.18	0.63	1.09	1.1	1.11	1.13	1.15
Br ⁻ (μg g ⁻¹)	2.41	1.03	1.05	1.06	1.05	0.97	0.98	0.99	1.01	1.03

Table 0.3. Summary of DNA extraction methods attempted on sample WB1. LOD – limit of detection; CTAB – Cetrimonium bromide; EtOH – Ethanol; MB – Difco Marine Broth 2216; MeOH – Methanol; PBS – Phosphate buffered saline.

Extraction Method	Starting material	DNA Concentration Method	Total g extracted	Yield (ng DNA)
DNeasy PowerLyzer PowerSoil Kit (Qiagen)	Soil	Lyophilizer	3.37	2.70
	Soil	SpeedVac	3.87	< LOD
	Soil	DNA Clean and Concentrator Kit (Zymo)	1.62	6.20
	Soil extract: PBS		3.20	< LOD
	Soil extract: PBS, filter concentrated		15.73	< LOD
	Soil extract: PBS, lyophilized		3.89	< LOD
	Soil extract: PBS, pelleted		7.93	3.00
	Soil extract: PBS + MB, pelleted		2.20	2.58
	Soil extract: PBS + MB, lyophilized		6.61	< LOD
	Soil extract: PBS + 10% MeOH ¹		3.54	< LOD
	Soil extract: PBS + 10% MeOH ¹ , lyophilized		8.24	< LOD
DNeasy PowerSoil Pro (Qiagen)	Soil		1.92	6.95
FastDNA spin Kit for 10 g Soil (MP Bio)	Soil	EtOH Precipitation	9.15	1.65
CTAB (10%) + Chloroform ²	Soil	EtOH Precipitation	3.00	< LOD
CTAB (1%) + Phenol:Chloroform ³	Soil	EtOH Precipitation	4.12	12.6
Total:			80.03 g	35.68 ng

¹Purcell et al. (2014); ²Campen (2015); ³Mitchell and Takacs-Vesbach (2008)

Table 0.4. Correlations between alpha diversity and conductivity.

		Inside Seep		Outside Seep	
Clustering		ASV	OTU	ASV	OTU
Richness	R ²	-0.948	-0.898	-0.166	-0.202
	p-value	0.0264*	0.0524	0.496	0.4477
Shannon	R ²	-0.952	-0.903	0.392	0.497
	p-value	0.0246*	0.0497*	0.259	0.183
Inverse Simpson	R ²	-0.926	-0.883	0.587	0.543
	p-value	0.0380*	0.0604	0.131	0.1557
Chao1	R ²	-0.948	-0.898	-0.15	-0.204
	p-value	0.0265*	0.5238	0.519	0.4454

Table 0.5. Summary of seep metagenomes.

Sample	WB3	WB5	DA1	DA3	DA5
Raw Reads	137755858	59256510	82118836	86774794	151624792
Assembly Statistics					
Contigs	2067989	2093273	883786	2384080	2454894
Total length	1162116085	979132320	430357049	1141340702	1332500421
N50	2134	1797	1902	1792	2288
L50	56804	50462	24189	59865	60785
Annotation Statistics					
CDS	2600565	2518443	1016430	2831713	3047930
Genes	2600828	2518681	1016521	2831954	3048243
rRNA	263	238	91	241	313

Table 0.6. Strains utilized in whole-genome comparisons. Digital DNA-DNA hybridization (dDDH) is relative to the *Gillisia* sp. FRX26 genome. *CDS* – Coding sequences.

Strain	Isolation Site	Genome Size (Gb)	#CDS	dDDH (%)
<i>Gillisia limnaea</i> DSM 15749 (GCA_000243235)	Microbial mat, Lake Fryxell, Antarctica	3.9	3569	22.6
<i>Gillisia limnaea</i> CIP108418T (GCA_965139515)	Not Provided	3.9	3445	22.5
<i>Gillisia</i> sp. JM1 (GCA_000424645)	Not Provided	4.1	3759	21.7
<i>Gillisia hiemivivida</i> IC154 (GCA_007997295)	Sea-ice, Vestfold hills, Antarctica	3.7	3166	20.7
<i>Gillisia hiemivivida</i> CIP108528T (GCA_965139505)	Not Provided	3.7	3296	20.7
<i>Gillisia</i> sp. Q332 (GCA_046298265)	Sediment , China	3.8	3417	19.9
<i>Gillisia</i> sp. CAL575 (GCA_000403785)	Antarctic Sponge	3.6	3270	18.7
<i>Gillisia</i> sp. Hel_I_86 (GCA_007827275)	North Sea	4.2	3895	18.1
<i>Gillisia marina</i> CBA3202 (GCA_000258765)	Beach sand, Korea	3.0	3041	17.9
<i>Gillisia</i> sp. Hel I 29 (GCA_000744275)	North Sea	4.0	3567	17.8
<i>Gillisia</i> sp. Hel1_33_143 (GCA_900104765)	North Sea	3.5	3182	17.8
<i>Gillisia lutea</i> M10.2A (GCA_021568705)	Aluminum can from beach, Spain	3.2	2831	17.6
<i>Gillisia mitskevichiae</i> DSM 19839 (GCA_003634585)	Sea water, Sea of Japan	3.6	3264	17.5
<i>Aequorivita aquimaris</i> D-24 (GCA_001573155)	Sea Water, Malaysia	3.1	2917	18.8
<i>Antarcticibacterium arcticum</i> KCCM 43316 (GCA_007993795)	Marine sediment, Beaufort Sea, Canada	3.4	2942	18.3

Table 3.6 (Continued)

Strain	Isolation Site	Genome Size (Gb)	#CDS	dDDH (%)
<i>Antarcticibacterium flavum</i> KCTC 52984 (GCA_006159205)	Marine sediment, Ross Sea, Antarctica	4.4	3535	18
<i>Aquimarina latercula</i> DSM 2041 (GCA_000430645)	Seawater aquarium	6.2	5543	17.9
<i>Christiangramia antarctica</i> KCTC 52925 (GCA_042685965)	Not Provided	4.6	3920	17.8
<i>Christiangramia forsetii</i> KT0803 (GCA_000060345)	North Sea	3.8	3584	17.6
<i>Christiangramia forsetii</i> CGMCC 1.15422 (GCA_014642775)	Not Provided	3.7	3349	17.2
<i>Flavobacterium rhamnosiphilum</i> CGMCC 1.11446 (GCA_004349195)	Glacier, China	3.8	3196	18.3
<i>Gelidibacter algens</i> DSM 12408 (GCA_003259265)	Not Provided	4.5	3842	21.4
<i>Halomarinibacterium sedimenti</i> CAU 1614T (GCA_019312585)	Sea sediment, Korea	2.9	2701	17.9
<i>Joostella marina</i> DSM 19592 (GCA_000260115)	Sea water, Korea	4.5	3944	20.3
<i>Leeuwenhoekiella marinoflava</i> DSM 3653 (GCA_900129005)	Not Provided	4.7	4056	18.6
<i>Leeuwenhoekiella polynyae</i> LMG 29608 (GCA_004104075)	Sea Water, Amundson Sea, Antarctica	4.8	4064	17.9
<i>Leeuwenhoekiella aequorea</i> LMG 22550 (GCA_004104375)	Sea water, Dronning Maud Land, Antarctica	3.5	3019	17.8
<i>Salegentibacter maritimus</i> F63223T (GCA_016236915)	Sediment , China	3.9	3348	18.9
<i>Salegentibacter holothuriorum</i> DSM 23405 (GCA_900168045)	Not Provided	3.6	3113	18.7

Table 3.6 (Continued)

Strain	Isolation Site	Genome Size (Gb)	#CDS	dDDH (%)
<i>Salegentibacter salegens</i> DSM 5424 (GCA_003001895)	Not Provided	3.9	3478	18.7
<i>Salegentibacter salinarum</i> DSM 23400 (GCA_900168115)	Not Provided	4.3	3725	18.6
<i>Salegentibacter agarivorans</i> DSM 23515 (GCA_900113135)	Not Provided	4.3	3768	18
<i>Salegentibacter flavus</i> DSM 17794 (GCA_900115145)	Not Provided	3.7	3327	18
<i>Salegentibacter salarii</i> DSM 23401 (GCA_900168265)	Not Provided	3.3	3046	17.7
<i>Salegentibacter mishustinae</i> KCTC 12263 (GCA_014651335)	Not Provided	3.8	3303	17.4
<i>Salegentibacter mishustinae</i> DSM 23404 (GCA_003254095)	Not Provided	3.8	3358	17.3
<i>Salinimicrobium marinum</i> KCTC12719 (GCA_014651535)	Not Provided	4.2	3566	19.3
<i>Salinimicrobium flavum</i> KCTC 42585 (GCA_042684505)	Not Provided	3.3	3006	19
<i>Salinimicrobium oceani</i> J15B91 (GCA_012037645)	Marine sediment, China	4.0	4646	19
<i>Salinimicrobium tongyeongense</i> HN-2-9-2T (GCA_026109735)	Seawater, Korea	3.5	2984	18.7
<i>Salinimicrobium catena</i> DSM 23553 (GCA_900107495)	Not Provided	3.3	2977	17.7
<i>Salinimicrobium gaetbulicola</i> CCUG 60898 (GCA_042676025)	Not Provided	3.1	2867	17.6
<i>Winogradskyella epiphytica</i> KCTC 12220 (GCA_014651315)	Not Provided	3.4	2974	18.9
<i>Zunongwangia profunda</i> SM-A87 (GCA_000023465)	Deep sea sediment, East China Sea	5.1	4653	20.1

Table 0.7. Libraries utilized in MDV amplicon survey.

Study	Sampling Region	Libraries	Libraries with <i>Gillisia</i> hits
Campan et al., 2019	Blood Falls (Taylor Valley)	12	10
Doyle and Christner, 2022	Taylor Glacier (Taylor)	6	3
Geyer et al., 2014	Taylor and Wright Valleys	10	4
Goordial et al., 2016	University Valley	12	0
Jackson et al., 2022	Whole-MDV survey	7	4
Jiang et al., 2022	Bull Pass	101	2
Kleinteich et al., 2017	Miers and Koettlitz	21	0
Mikucki et al., <i>in prep</i>	Don Juan Pond (Wright Valley)	4	1
Solon et al., 2021	Canada Peak (Taylor Valley)	19	0
Van Horn et al., 2013	Taylor and Wright Valleys	44	2
Van Horn et al., 2014	Fryxell basin (Taylor Valley) – amendment study	88	15
Vick-Majors et al., 2014	Bonney and Fryxell lake columns (Taylor Valley)	36	0

Table 0.8. Transcripts significantly more or less abundant strain FRX26 cultures grown in 0.5 M NaCl. Fold change is relative to 0.2 M cultures. Transcripts were annotated using Prokka (P), KEGG (K), or COG (C).

Gene ID	log ₂ Fold Change	p _{adj}	Annotated by:	Annotation
01536	2.236	4.61E-04		
02917	2.152	2.40E-11	C	Glycosyltransferase like family 2
02918	2.027	5.22E-11	P, C	Putative membrane protein EpsK
00591	1.960	9.74E-05		
03693	1.958	1.92E-02		
02396	1.949	2.56E-02	C	Predicted membrane protein
02916	1.874	1.07E-07		
00590	1.863	2.62E-05	K, C	<i>rpoE</i> ; R polymerase sigma-70 factor, ECF subfamily
02919	1.850	4.58E-05	P, K, C	GDP-L-fucose synthase
02550	1.804	2.56E-02		
02912	1.731	3.10E-11		
03695	1.727	1.92E-02	P, K, C	<i>uvrC</i> ; excinuclease ABC subunit C
02922	1.702	2.16E-05	P, K, C	Alginate biosynthesis protein AlgA
00956	1.673	6.48E-03	P, K, C	<i>rpoE</i> ; R polymerase sigma-70 factor, ECF subfamily
02920	1.646	2.11E-04	C	PD-(D/E)XK nuclease superfamily
00932	1.637	7.40E-04		
02910	1.621	1.07E-04		
02921	1.591	2.26E-04	P, K, C	GDP-mannose 4,6-dehydratase
00955	1.587	9.62E-03		
02489	1.580	7.57E-03	C	L,D-transpeptidase catalytic domain
02911	1.572	2.05E-04	P, C	PGL/p-HBAD biosynthesis glycosyltransferase
03717	1.548	1.84E-02	P, C	IS21 family transposase ISPpu7
03324	1.536	2.72E-02	P, C	Flavastacin
00930	1.531	3.57E-02	P, K, C	<i>rpoE</i> ; R polymerase sigma-70 factor, ECF subfamily
02909	1.525	4.73E-02	P, K, C	Decaprenyl-phosphate N-acetylglucosaminephosphotransferase <i>wecA</i>
02369	1.502	1.15E-02		
03621	1.499	3.17E-03	C	Capsule assembly protein Wzi
00575	1.472	9.21E-08	P, K, C	<i>hemA</i> ; glutamyl-tR reductase
00544	1.443	3.72E-06	P, C	2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase

Table 3.8 (Continued)

Gene ID	log₂Fold Change	p_{adj}	Annotated by:	Annotation
02914	1.437	4.73E-08	P, C	Galc-alpha-(1->4)-Galc-alpha-(1->3)-dicBac-PP-undecaprenol alpha-1,4-N-acetyl-D-galactosaminyltransferase
00592	1.434	6.09E-03		
00326	1.432	3.80E-02		
01054	1.427	6.39E-03	P, K, C	<i>rpoE</i> ; R polymerase sigma-70 factor, ECF subfamily
02913	1.418	1.26E-12	P, C	Maltose O-acetyltransferase
02915	1.414	2.79E-04		
00996	1.413	4.11E-02		
01418	1.409	1.74E-03	P, K, C	N-acetylglucosamine repressor
00954	1.365	1.15E-02		
00475	1.322	3.80E-02		
03622	1.293	9.70E-03	P, K, C	Tyrosine-protein kinase Wzc
00574	1.290	1.12E-02	C	helix_turn_helix, arabinose operon control protein
02371	1.271	1.67E-08	C	Calcineurin-like phosphoesterase
02743	1.245	2.37E-02		
01055	1.185	1.29E-02		
03560	1.153	4.72E-03	C	Glycosyltransferase like family 2
01747	1.138	3.80E-02	P, K, C	Endoribonuclease MazF9
00031	1.109	6.59E-03	P, C	Adaptive-response sensory-kinase SasA
02048	1.050	3.20E-02	K	<i>trpCF</i> ; indole-3-glycerol phosphate synthase / phosphoribosylanthranilate isomerase
01282	1.047	9.31E-03	K, C	<i>yafQ</i> ; mR interferase YafQ
00078	0.835	4.11E-02	P, K, C	Threonylcarbamoyladenosine tR methylthiotransferase MtaB
03297	-0.938	9.89E-03	C	Lysine biosynthetic process via aminoadipic acid
01864	-0.995	4.59E-02	P, C	Protoheme IX farnesyltransferase
01105	-0.995	4.47E-02	P, C	3-isopropylmalate dehydratase large subunit
02240	-0.996	2.72E-02	P, C	Alcohol dehydrogenase
02908	-1.002	9.62E-03	P, K, C	<i>fabH</i> ; 3-oxoacyl-[acyl-carrier-protein] synthase III
00157	-1.010	7.06E-03		
01641	-1.038	2.56E-02	P, C	Adaptive-response sensory-kinase SasA
03476	-1.097	4.31E-02	C	Fungalysin metalloproteinase (M36)
00158	-1.103	1.84E-02	P, C	Thioredoxin reductase

Table 3.8 (Continued)

Gene ID	log₂Fold Change	p_{adj}	Annotated by:	Annotation
02073	-1.117	1.68E-02	C	Ferritin-like domain
02907	-1.121	2.67E-03	C	X-Pro dipeptidyl-peptidase (S15 family)
01865	-1.133	5.90E-03	K, C	TatD related DNase
02085	-1.152	4.52E-04	P, C	6-hydroxy-D-nicotine oxidase
01458	-1.160	1.54E-02	P, C	Na ⁺ /H ⁺ antiporter NhaG
00858	-1.174	9.81E-03	P, K, C	Formamidopyrimidine-D glycosylase
01823	-1.182	2.18E-03	C	GDSL-like Lipase/Acylhydrolase family
02406	-1.188	1.08E-02	P, K, C	Acyl-CoA dehydrogenase
03291	-1.188	9.62E-03	K, C	<i>topA</i> ; D topoisomerase I
02465	-1.212	9.31E-03	K, C	Putative neutral zinc metallopeptidase
02349	-1.213	2.94E-05	P, K, C	Cyanophycin synthetase
03507	-1.214	4.73E-02	P	Protein YciF
02570	-1.233	4.98E-02	C	Flavin containing amine oxidoreductase
02422	-1.243	5.70E-05	P, K, C	Catalase HP11
02152	-1.248	2.56E-02	C	Four repeated domains in the Fasciclin I family of proteins, present in many other contexts.
02404	-1.270	2.64E-02	C	Polyketide cyclase / dehydrase and lipid transport
02552	-1.270	1.92E-02	P, C	Cytochrome b6-f complex iron-sulfur subunit
02553	-1.284	2.37E-02		
03728	-1.297	3.01E-03	K	K19802 <i>ycjG</i> , <i>ykfB</i> , <i>dgcA</i> ; L-Ala-D/L-Glu epimerase / N-acetyl-D-glutamate racemase
02405	-1.298	1.03E-02	C	Polyketide cyclase / dehydrase and lipid transport
01609	-1.300	1.83E-02	P, K, C	Chaperone protein ClpB 1
00324	-1.314	3.08E-02	P, K, C	Leucine dehydrogenase
03588	-1.325	3.10E-02		
03125	-1.345	1.51E-03	P, C	Tyrosine recombinase XerC
01605	-1.347	1.17E-03	C	Tat (Twin-arginine translocation) pathway signal sequence containing protein
00861	-1.358	1.03E-02		
00092	-1.397	1.54E-08		
03188	-1.406	2.79E-04	C	Las17-binding protein actin regulator
01859	-1.416	1.25E-04	C	Pyrroloquinoline quinone binding
03131	-1.446	1.92E-02		
03630	-1.464	8.91E-04		
02571	-1.476	4.76E-02	C	Sensors of blue-light using FAD

Table 3.8 (Continued)

Gene ID	log₂Fold Change	p_{adj}	Annotated by:	Annotation
00199	-1.485	3.53E-02	K, C	<i>ydbD</i> ; manganese catalase
01701	-1.500	5.99E-03		
00198	-1.508	2.64E-02	K, C	<i>ydbD</i> ; manganese catalase
00196	-1.526	3.17E-02	P, K, C	<i>groEL</i> , HSPD1, HSP60, CPN60; chaperonin
03631	-1.561	2.16E-03	C	Membrane protein
01782	-1.582	3.39E-03	P, K, C	IS256 family transposase ISAb42
02243	-1.603	2.28E-02	P, K, C	Chaperone protein DnaK
00200	-1.609	4.73E-02	C	Dithiol-disulfide isomerase
02469	-1.621	4.75E-02	C	small multidrug export protein
00723	-1.632	3.53E-04	P, C	D ligase
00669	-1.646	1.15E-02	C	Amine oxidase
00289	-1.648	3.16E-05		
02780	-1.680	9.21E-08	C	Pyrroloquinoline quinone binding
00859	-1.697	1.14E-04	P, K, C	Multifunctional non-homologous end joining protein LigD
02896	-1.805	6.59E-03	P, K, C	Chaperone protein HtpG
00290	-1.918	4.50E-12	C	Glucose / Sorbosone dehydrogenase
02301	-1.920	6.59E-03	P, C	Putative zinc-binding alcohol dehydrogenase
00278	-2.204	1.02E-03		
03013	-2.300	2.07E-15	P, K, C	1,4-alpha-glucan branching enzyme GlgB

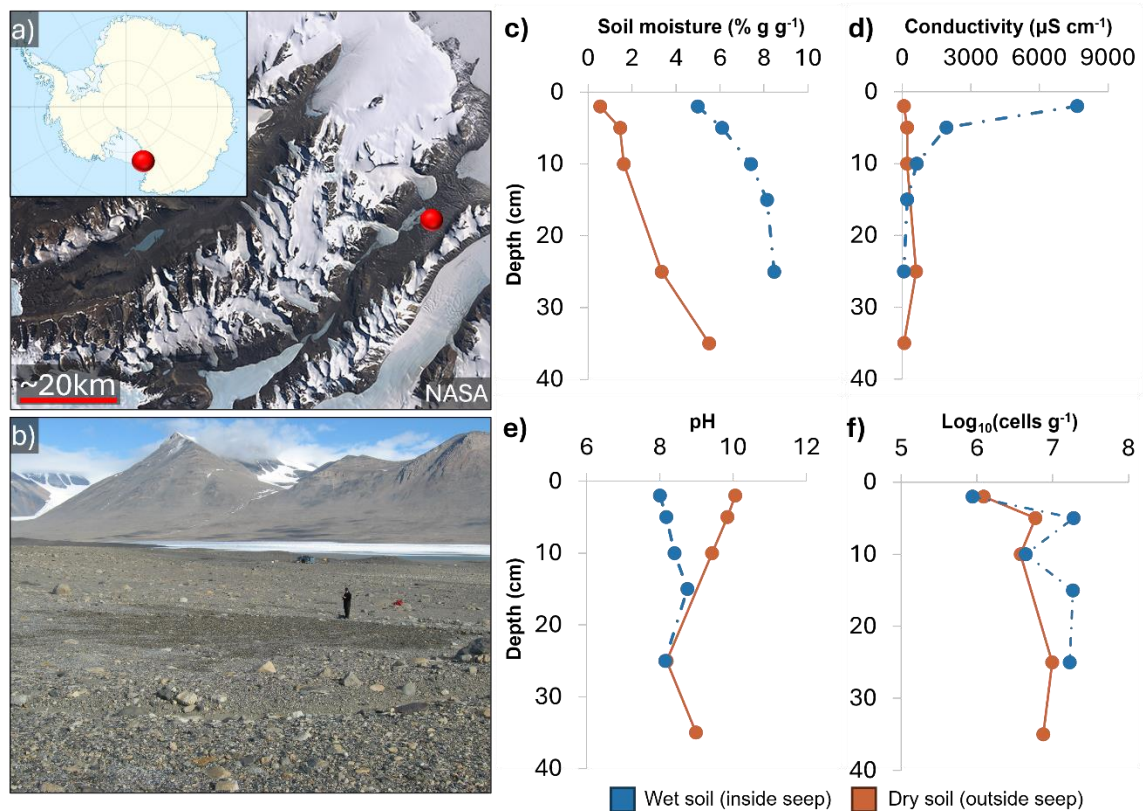


Figure 0.1. Location and geochemistry of Fryxell basin meltwater seeps. (a) Geographic of Fryxell basin within Taylor Valley, Antarctica. Satellite image by NASA. (b) Image of seep used in this study. Darker sediments are higher in moisture than surrounding areas, and surficial salts are visible as a white precipitate at the edges of the seep. (c) Measurements of soil moisture (as gravimetric water content), (d) electrical conductivity (proxy for salinity), (e) pH, and (f) microscopic counts of DNA containing cells in both wet and dry soils, by sample depth.

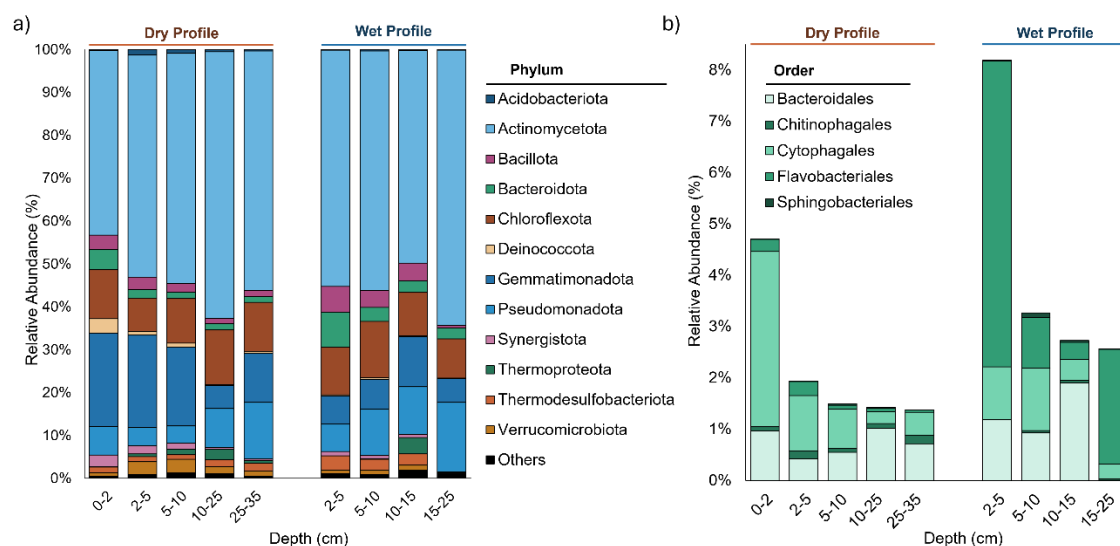


Figure 0.2. Community composition of wet and dry soil profiles. Composition of based on 16S rRNA gene amplicon sequencing and comparison of OTUs (97% similarity) to the silva database v138.2. (a) Composition of all sequences at the Phylum level. (b) Composition of phylum Bacteroidota at the Order level. Reads matching *Gillisia* sp. FRX26 correspond to between 76.5 – 100% of Flavobacteriales OTUs.

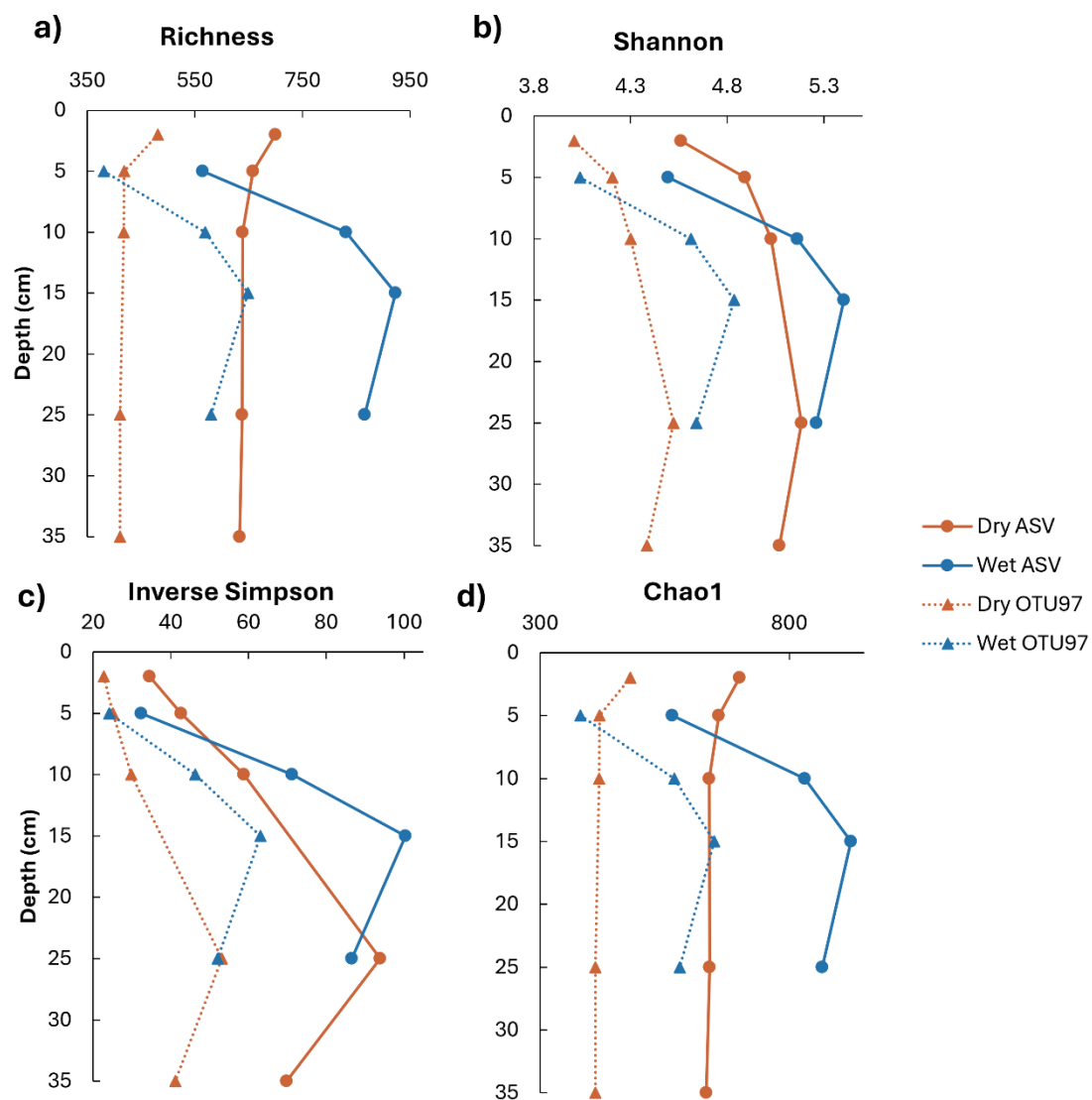


Figure 0.3. Diversity metrics of wet and dry soil profiles. Diversity assessments were performed on both operational taxonomic units grouped at 97% similarity (OTU97) and on amplicon sequence variants (ASV).

		Wet Profile			Dry profile		Description
Compatible Solutes		5-10	15-25	0-2	5-10	25-25	
Betaine biosynthesis	<i>betA</i>	5.23	4.73	4.82	4.66	4.3	K00108 Choline dehydrogenase
	<i>betB</i>	10.31	10.09	10.72	8.16	9.06	K00130 Betaine-aldehyde dehydrogenase
Betaine catabolism	<i>gbcA</i>	1.42	1.47	2.46	1.24	1.18	K00479 Glycine betaine monooxygenase A
	<i>gbcB</i>	0.19	0.16	0.3	0.11	0.2	K21832 Glycine betaine catabolism B
Betaine transport	<i>opuD</i>	0.31	0.28	0.59	0.39	0.23	K05020 Glycine betaine transporter
	<i>proW</i>	4.61	4.65	5.9	4.2	4.63	K02001 Glycine betaine/proline transport system permease protein
Ectoine biosynthesis	<i>ectC</i>	0.46	0.56	0.49	0.46	0.59	K06720 L-ectoine synthase
Ectoine catabolism	<i>doeA</i>	1.65	0.95	1.08	1.34	0.85	K15783 Ectoine hydrolase
Proline biosynthesis	<i>proC</i>	14.77	13.3	10.72	13.24	14.37	K00286 Pyrroline-5-carboxylate reductase
Trehalose biosynthesis	<i>otsA</i>	18.46	17.71	16.82	15.47	16.11	K00697 Trehalose 6-phosphate synthase
	<i>otsB</i>	11.96	9.17	8.95	9.04	9.22	K01087 Trehalose 6-phosphate phosphatase
	<i>treY</i>	22.61	22.43	25.78	24.05	19.75	K06044 (1->4)-alpha-D-glucan 1-alpha-D-glucosylmutase
	<i>treZ</i>	18.84	17.07	18.1	18.75	14.73	K01236 Maltotriose/trehalose trehalohydrolase
Trehalose catabolism	<i>treA</i>	1.35	1.15	1.28	0.78	1.05	K01194 Alpha,alpha-trehalase
Trehalose transporter	<i>thuE</i>	5.46	4.73	7.18	5.33	5.48	K10236 Trehalose/maltose transport system substrate-binding protein
	<i>thuF</i>	4.77	4.84	4.92	4.41	5.12	K10237 Trehalose/maltose transport system substrate-binding protein
	<i>thuG</i>	5.54	4.84	5.31	4.94	6.1	K10238 Trehalose/maltose transport system substrate-binding protein
Ion transport							
Cl ⁻ efflux	<i>clcA</i>	4.42	2.58	3.74	3.88	2.17	K03281 Cl ⁻ channel protein
K ⁺ efflux	<i>ybaL</i>	12.34	10.32	8.07	11.02	9.74	K26735 K ⁺ :H ⁺ antiporter
K ⁺ uptake	<i>trkA</i>	24.49	20.77	18.1	17.34	22.41	K03499 K ⁺ uptake protein
	<i>trkH</i>	17.11	14.14	20.07	16.21	12.89	K03498 K ⁺ uptake protein
	<i>kdpA</i>	0.27	0.6	0.59	0.67	1.02	K01546 K ⁺ -transporting ATPase
	<i>kdpB</i>	1	1.35	1.08	1.27	1.61	K01547 K ⁺ -transporting ATPase
	<i>kdpC</i>	0.12	0.2	0	0.25	0.1	K01548 K ⁺ -transporting ATPase
	<i>kup</i>	7.92	9.77	4.43	8.51	8.14	K03549 K ⁺ uptake protein
Na ⁺ efflux	<i>mrpA</i>	4.46	3.65	10.72	5.76	4.13	K05565 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>mrpB</i>	0.65	0.4	0.89	0.57	0.23	K05566 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>mrpC</i>	4.15	1.91	5.9	3.57	1.94	K05567 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>mrpD</i>	7.69	4.09	11.31	7.77	5.54	K05568 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>mrpE</i>	2.92	1.47	5.21	2.61	1.64	K05569 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>mrpF</i>	0.81	0.44	2.07	0.78	0.2	K05570 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>mrpG</i>	2.27	1.19	4.03	2.05	1.77	K05571 Multicomponent Na ⁺ :H ⁺ antiporter
	<i>napA</i>	4.92	4.88	2.56	4.45	4.82	K26730 Na ⁺ :H ⁺ antiporter
	<i>nhaA</i>	16.96	17.99	11.61	16.03	15.55	K03313 Na ⁺ :H ⁺ antiporter
	<i>nhaC</i>	0.85	0.83	0.49	1.09	0.52	K03315 Na ⁺ :H ⁺ antiporter
	<i>nhaP</i>	3.61	2.22	3.64	1.62	1.41	K03316 Na ⁺ :H ⁺ antiporter
	<i>nqrA</i>	0.54	0.36	0.39	0.57	0.39	K00346 Na ⁺ -transporting NADH:ubiquinone oxidoreductase
	<i>nqrB</i>	0.69	0.56	1.28	0.6	0.59	K00347 Na ⁺ -transporting NADH:ubiquinone oxidoreductase
	<i>nqrC</i>	0.23	0.24	0.2	0.39	0.2	K00348 Na ⁺ -transporting NADH:ubiquinone oxidoreductase
	<i>nqrD</i>	0.31	0.44	0.39	0.39	0.23	K00349 Na ⁺ -transporting NADH:ubiquinone oxidoreductase
	<i>nqrE</i>	0.35	0.44	0.49	0.35	0.16	K00350 Na ⁺ -transporting NADH:ubiquinone oxidoreductase
	<i>nqrF</i>	0.81	0.79	0.79	0.6	0.39	K00351 Na ⁺ -transporting NADH:ubiquinone oxidoreductase
	<i>hpaA</i>	20.73	22.28	15.05	19	21.06	K15987 K ⁺ -stimulated pyrophosphate-energized sodium pump
Mechanosensitive Channels							
	<i>mscK</i>	1.77	1.35	1.77	1.52	1.38	K05802 K ⁺ -dependent mechanosensitive channel
	<i>mscL</i>	8.46	7.03	6.2	7.17	6.56	K03282 Large conductance mechanosensitive channel
	<i>mscM</i>	0.27	0.2	0.1	0.21	0.16	K16053 Miniconductance mechanosensitive channel
	<i>mscMJ</i>	0.12	0.04	0.1	0.11	0	K16052 Small conductance mechanosensitive channel
	<i>mscS</i>	2.42	1.43	1.97	1.31	0.82	K03442 Small conductance mechanosensitive channel

-2 row z-score 2

Figure 0.4. Predicted genes associated with salt tolerance in Fryxell seep metagenomes. Values represent number of annotations present per 100000 genes. Rows are shaded by the abundance z-score for each gene, with orange indicating more abundant and blue indicating less abundant compared to the average of all metagenomes. Gene names, KEGG Orthology (KO) numbers, and putative description were determined via GhostKOALA annotation.

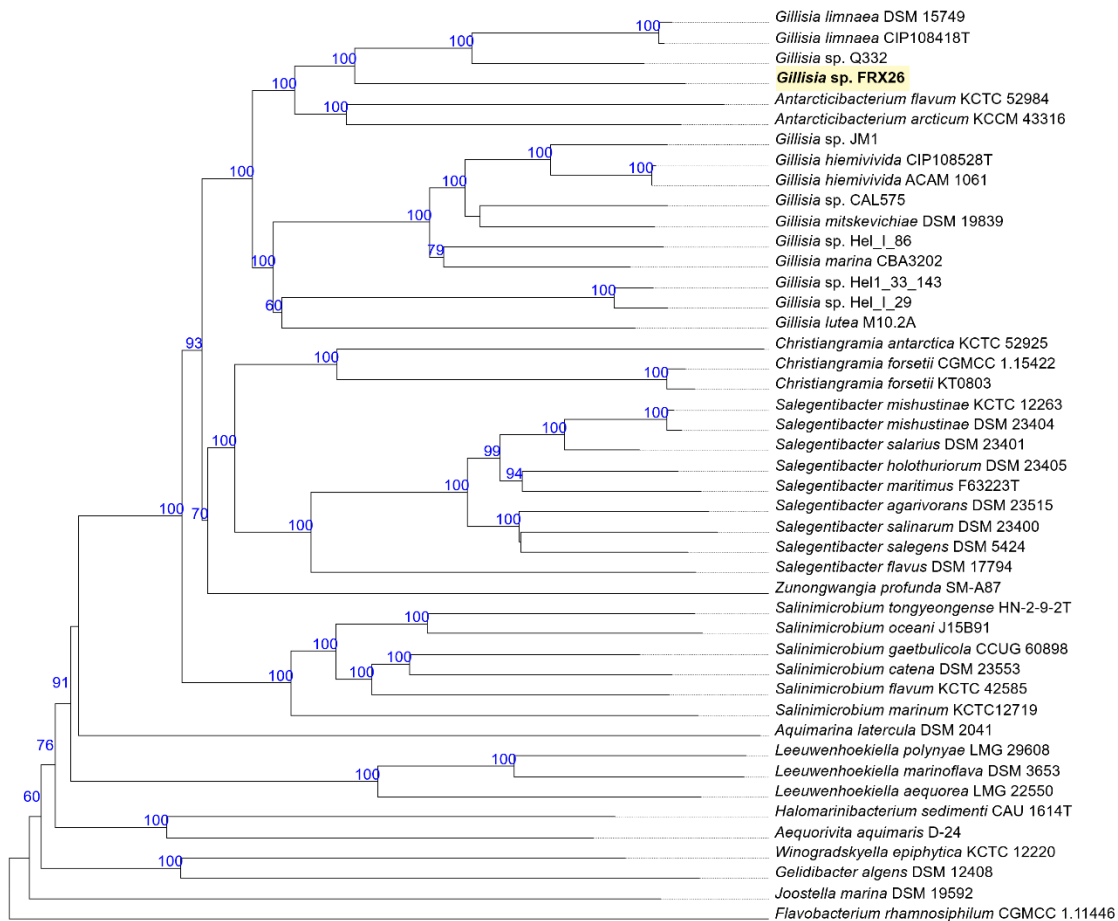


Figure 0.5. Taxonomic placement of strain FRX26 within the *Gillisia* genus.

Phylogeny is based on whole-genome comparison of translated coding sequences from genomes within the TYGS database, as well as all *Gillisia* genomes present in the NCBI RefSeq database. Accession numbers, sequence similarity, and isolation material information are available in Table 5. Node numbers represent bootstrap values. Tree was generated by TYGS.

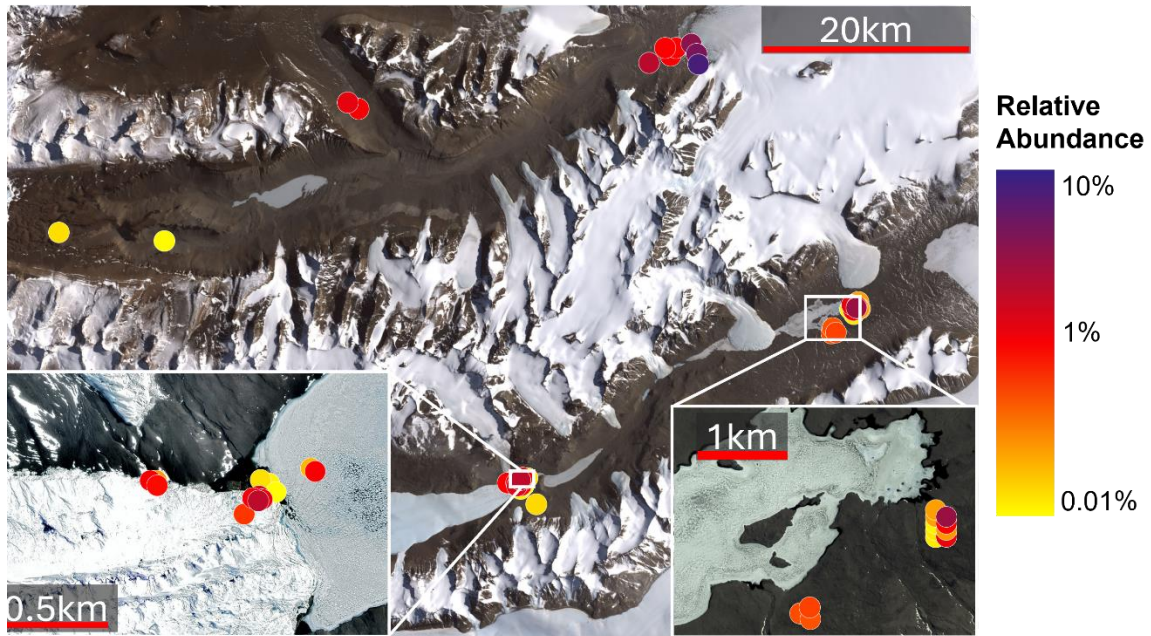


Figure 0.6. Relative abundance of *Gillisia* sp. matching FRX26 in Wright and Taylor Valley 16S rRNA gene amplicon libraries. Sequences were assigned to the same species as *Gillisia* sp. FRX26 based on 97% similarity between amplicon sequence and the FRX26 16S rRNA gene. Each circle represents an individual amplicon library (**Table 3.7**) and is colored based on the relative abundance of similar sequences within that library.

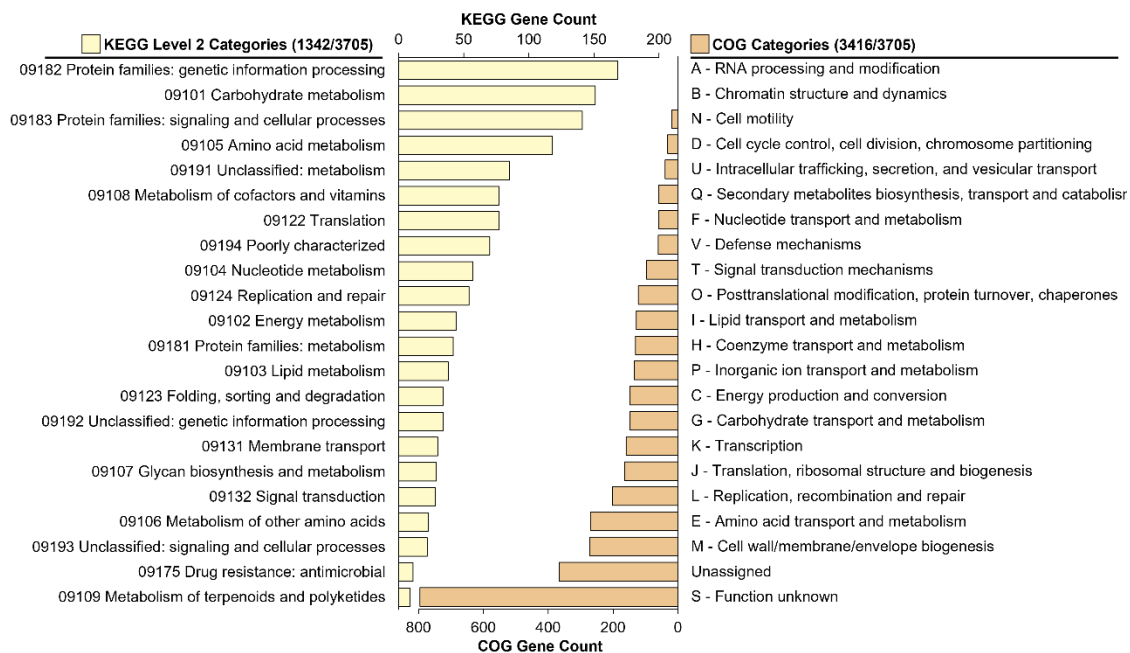


Figure 0.7 Functional categories of annotated genes in the *Gillisia* sp. FRX26 genome associated with either KEGG (left) or COG (right) databases.

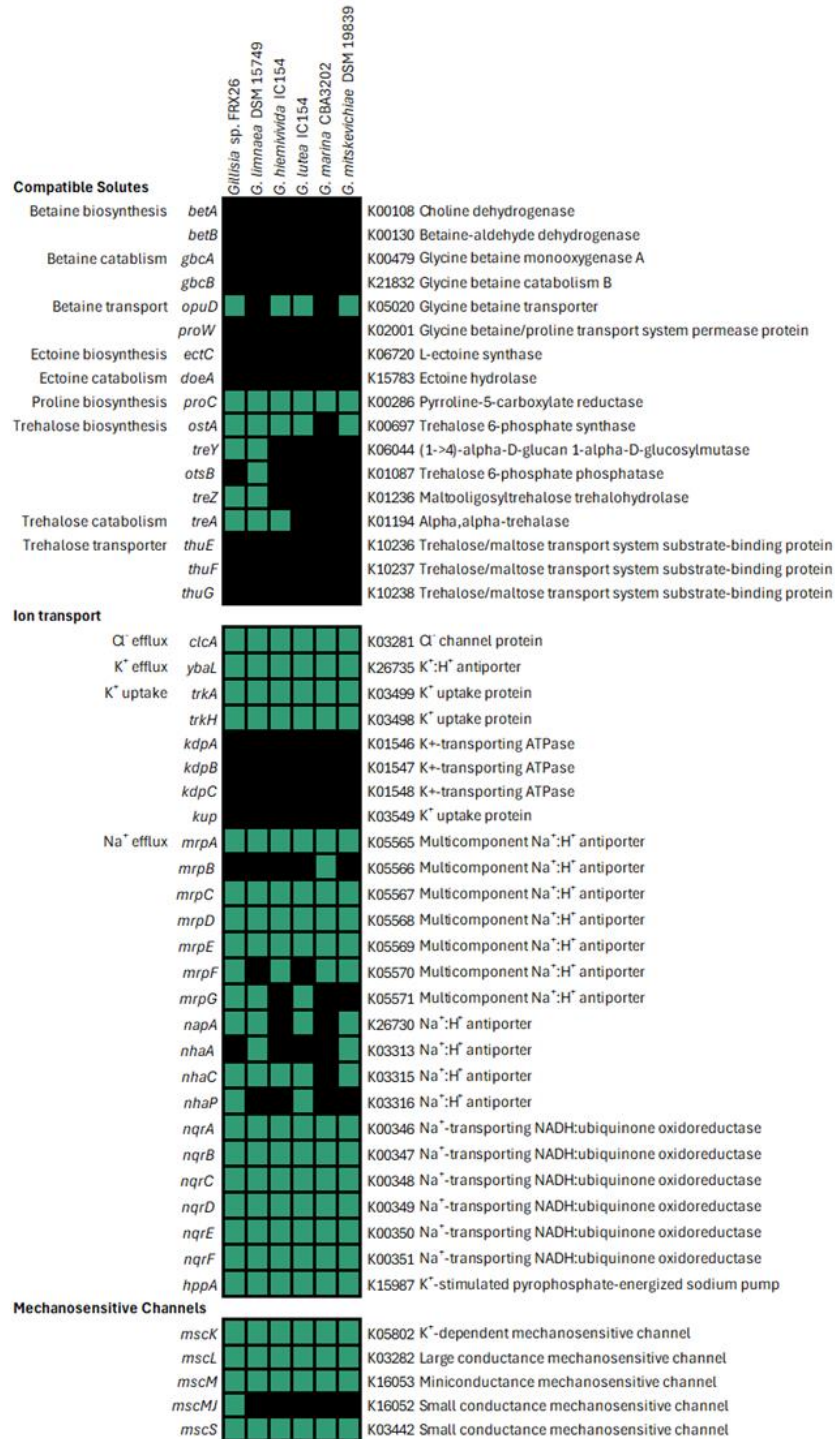


Figure 0.8. Presence of annotated genes related to salt response in *Gillisia* genomes. Gene names, KEGG Orthology (KO) numbers, and putative description were determined via GhostKOALA annotation. Green boxes represent genes with at least one copy.

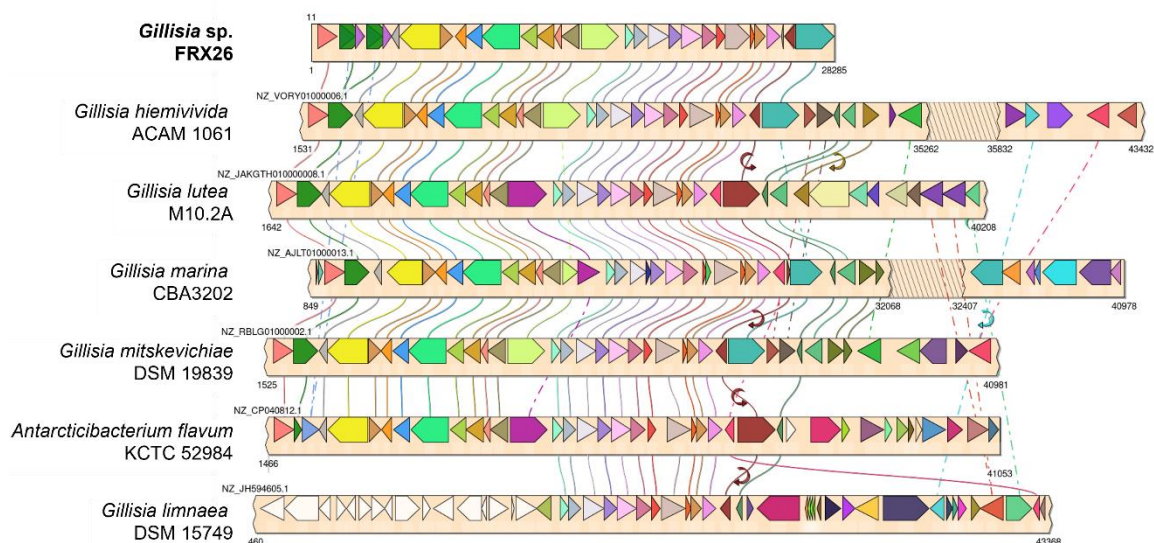


Figure 0.9. Comparison of type III PKS gene cluster between FRX26 and other *Gillisia* spp. Genes associated with biosynthesis of the unknown PKS product appear highly conserved between all sequenced members of the genus, as well to another closely-related genus (*Antarcticibacterium*).

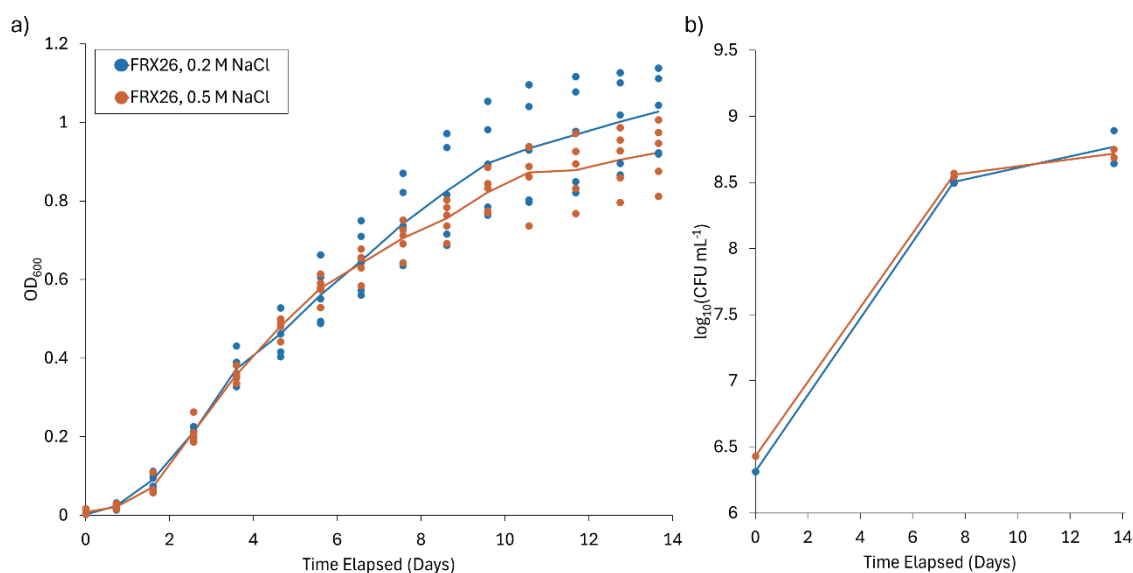


Figure 0.10. Growth of *Gillisia* sp. FRX26 in either 0.2 M or 0.5 M NaCl. (a) Optical density measurements taken via spectrophotometer. (b) Number of colony forming units present following one and two weeks of incubation. Plate counts were performed on the hybrid medium at 0.2 M NaCl.

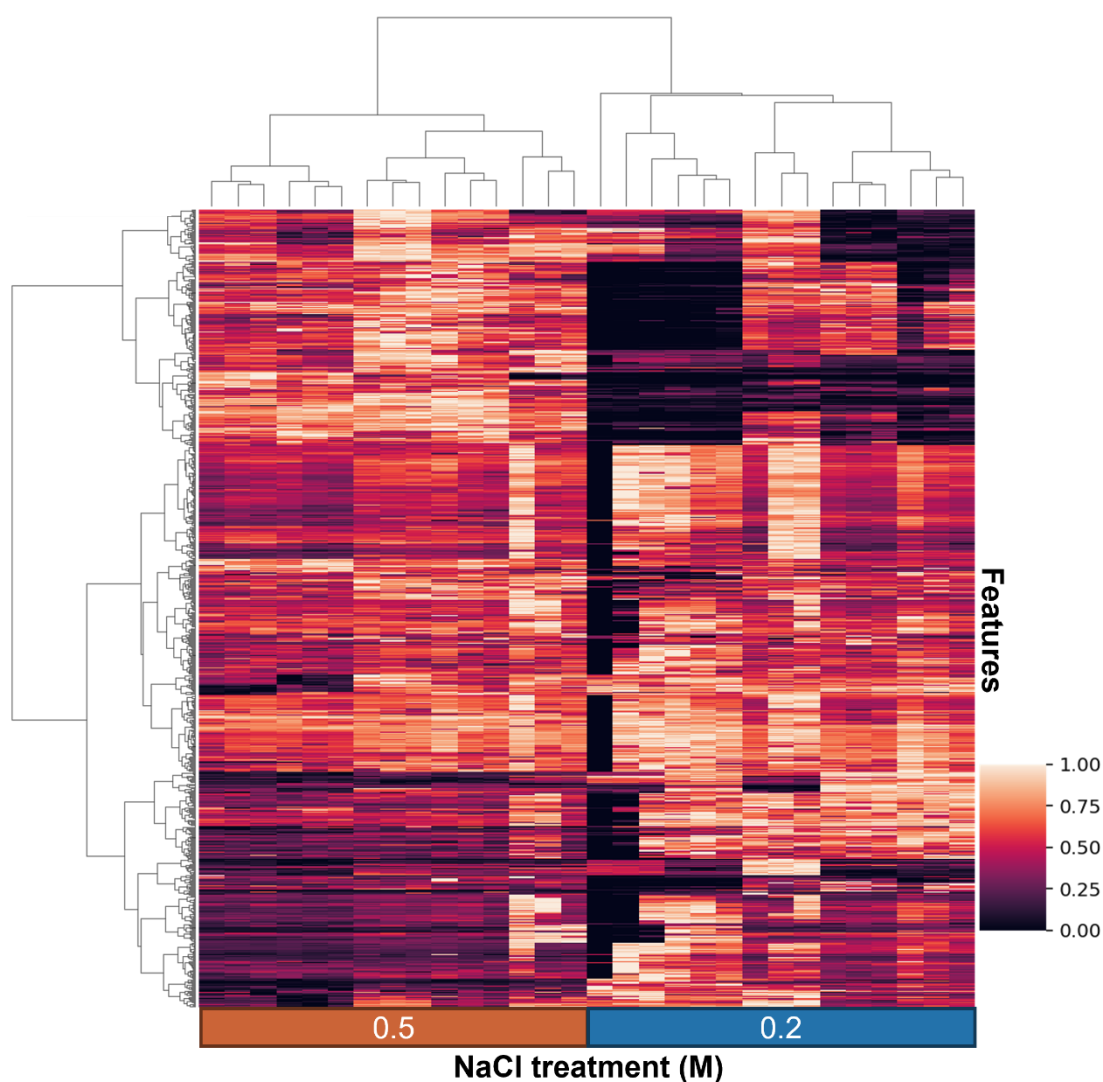


Figure 0.11. Heatmap of metabolite features passing all filters in FRX26 cultures. Columns correspond to biological and instrument injection replicates sorted by hierarchical clustering analysis based on similarity of normalized metabolite profile. Rows represent individual metabolite features passing all filters, sorted by similarity of normalized abundance profile. Each cell is colored by normalized abundance of a given metabolite signature in a given injection replicate.

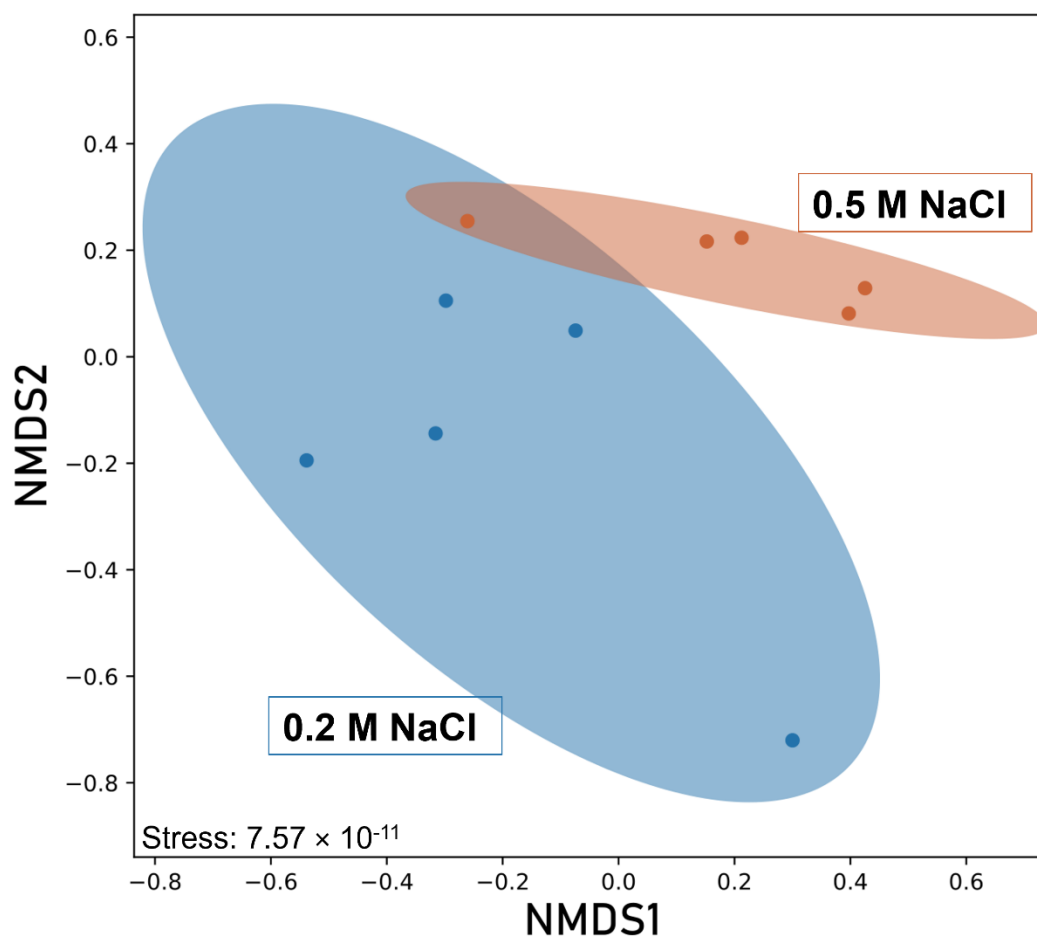


Figure 0.12. NMDS plot of *Gillisia* sp. FRX26 metabolite profiles grown under 0.2 M or 0.5 M NaCl. Each point corresponds to a single biological replicate, grouped by treatment. Overlap of 95% confidence intervals indicates that metabolite profiles for salt treatments are not significantly different from each other.

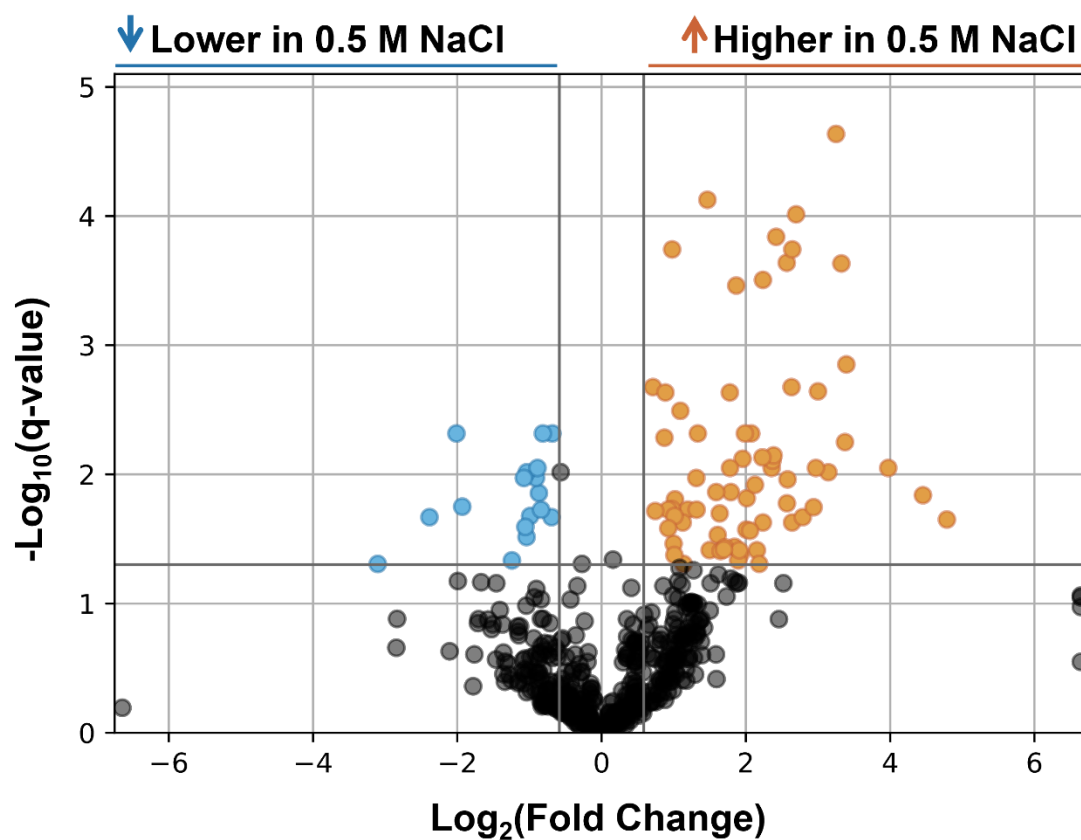


Figure 0.13. Volcano plot of differentially abundant metabolite features between FRX26 salt treatments. Blue points represent metabolites significantly more abundant in 0.2 M cultures, and orange points represent metabolites significantly more abundant in 0.5 M treatments.

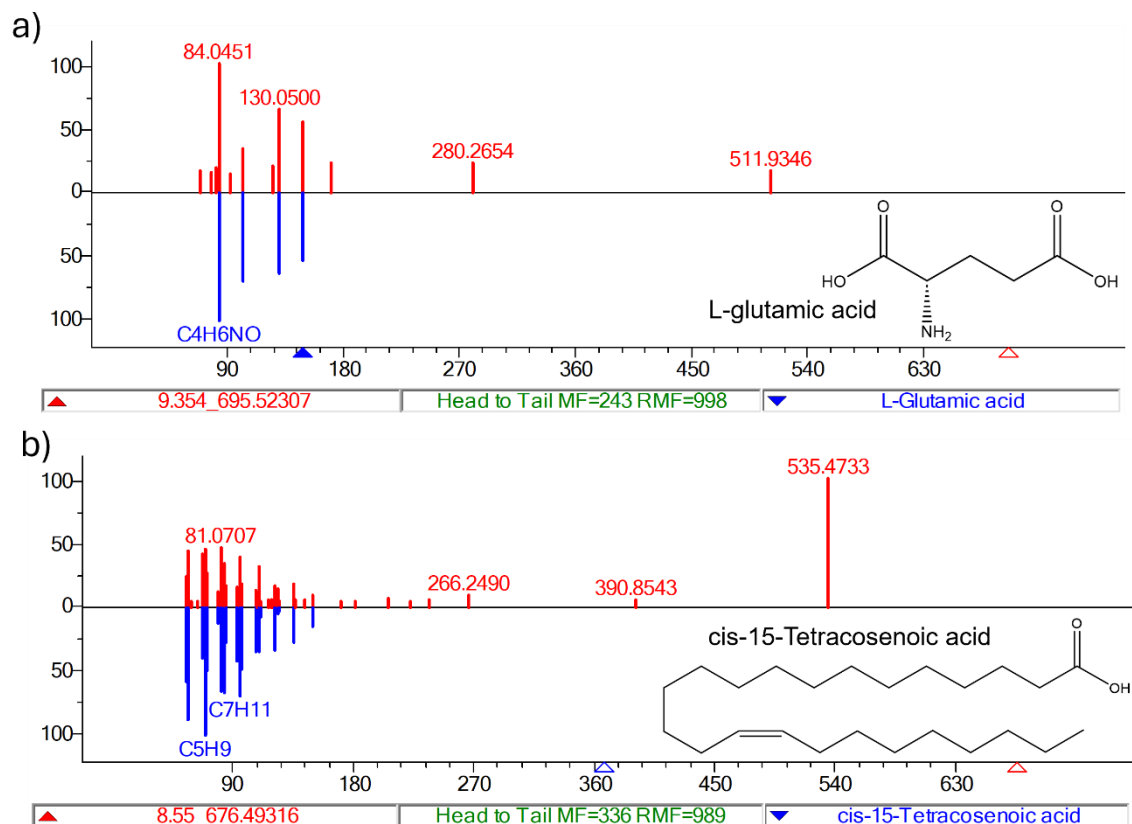


Figure 0.14. Example fragmentation patterns for metabolite features consistent with (a) peptidic or (b) lipid-based metabolites.

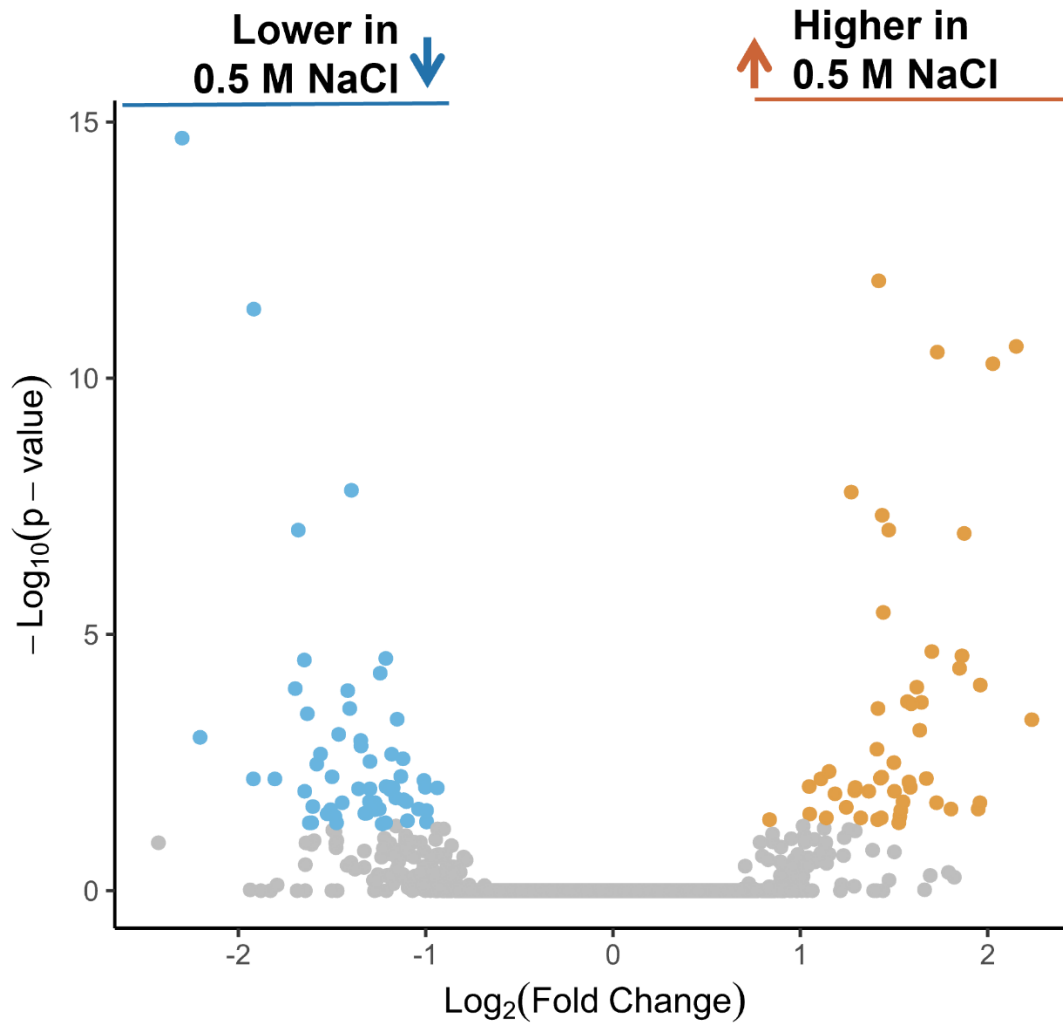


Figure 0.15. Volcano plot of differentially abundant *Gillisia* sp. FRX26 transcripts. Orange points are significantly more abundant in 0.5 M treatments, and blue points are significantly less abundant in 0.5 M treatments.

**CHAPTER FOUR MULTI-TECHNIQUE CHARACTERIZATION OF IRON
REDUCTION BY AN ANTARCTIC *SHEWANELLA*: AN ANALOG SYSTEM
FOR PUTATIVE MARTIAN BIOSIGNATURE IDENTIFICATION**

Publication Note

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Abstract

Microbes from terrestrial extreme environments offer an opportunity to test biosignature production in conditions relevant to astrobiological targets, such as Mars, which was likely more conducive to life during early warmer and wetter epochs. Missions to Mars search for signs of early life in the surficial rock record, including mineral or organic biosignatures. On Earth, microbial iron reduction is a common and ancient metabolism that may have also operated on other rocky celestial bodies. To investigate the production of biosignatures during iron reduction, a *Shewanella* sp. (strain BF02_Schw) isolated from a subglacial discharge known as Blood Falls, Antarctica, was incubated with ferrihydrite (Fh) as an electron acceptor. Biosignatures associated with Fh reduction were identified using a suite of instrumentation currently utilized or proposed for Mars missions. The biotic origin of identified features was validated by comparison to

killed controls and transcriptional changes between treatments with and without Fh. In live treatments, Fh was reduced to magnetite and goethite, both of which have been detected in Martian lacustrine basins. Several soluble and volatile metabolites were also detected, including riboflavin and dimethyl sulfide (DMS), which could be astrobiological indicators of active microbial processes. While none of these biosignatures individually would serve as definitive proof of life (past or present), detection of concomitant features associated with known terrestrial biotic processes would provide compelling rationale for more targeted life detection missions. Terrestrial extremophiles can support the exploration of astrobiologically relevant microbial processes, validation of life detection instrumentation, and potentially the discovery of new biomarkers.

Importance

Culture-based experiments with terrestrial extremophiles can elucidate biosignatures that may be analogous to those produced under extraterrestrial conditions, and thus inform sampling and technology strategies for future missions. Here we demonstrate the production of several biosignatures under iron-reducing conditions by *Shewanella* sp. BF02_Schw, originally isolated from an Antarctic analog feature. These biosignatures could be detectable using flight-ready instrumentation. Growth experiments with terrestrial extremophiles can identify biosignatures measurable by current methodologies and inform the development and optimization of techniques for the detection of extant or extinct life on other worlds.

Introduction

Any life that emerged on Mars was likely during the warmer, wetter climates of its early history (i.e., the Noachian or Hesperian eons; Clark et al., 2021). Decades of Martian orbital and surface missions revealed evidence of historic liquid water at the surface (Wordsworth, 2016; Ramirez and Craddock, 2018; Clark et al., 2021) associated with possible redox gradients which would be available for biological exploitation (Rampe et al., 2020). While habitability on Mars may have only been transient (Cockell, 2014), it is possible that life emerged independently of that on Earth, or that life emerged

first on Mars and then seeded early Earth in a panspermia event (Clark et al., 2021) or vice versa. However, the Martian surface today is cold, dry, and likely uninhabitable with the possible exception of localized Special Regions (Rummel et al., 2014).

Recent missions to Mars have sought to identify evidence of ancient habitability (Mellon et al., 2024). Because water is crucial to life as we know it, both the *Curiosity* and *Perseverance* rovers landed at sites with evidence of ancient fluvial and lacustrine settings (Grotzinger et al., 2012; Farley et al., 2020). On Earth, depositional environments also supported the oldest generally accepted microbial fossils, stromatolites, layered sedimentary features originating from the interactions between sedimentary particles and biofilm communities (Djokic et al., 2017; Rodriguez et al., 2024). Such environments may also lead to preservation of diverse biosignatures, including microbially produced secondary minerals, micro- or macro-scale textures, and preserved structural components of biomolecules such as lipids (McMahon et al., 2018). While the identification of a singular biosignature within regions hypothesized to have been habitable would be intriguing, concomitant biosignatures would be significantly more compelling.

Attempts to detect evidence of life from Noachian or Hesperian Mars can be informed by investigations of early terrestrial life. Evidence for life in Earth's Precambrian oceans exists largely as chemical (e.g., complex organics, isotopic composition) or morphological biosignatures within sedimentary basins (Holland, 2016; Javaux, 2019). Accurate interpretation of these records is challenging due to the rare and incomplete nature of fossilization, abiotic mineral alterations, and the inherent ambiguity of mineral biosignatures, which often have both biotic and abiotic routes of formation. As such, claims of evidence of early Earth life rely on the use of multiple observations to rule out abiotic formation, leaving biogenesis as the most probable origin (Javaux, 2019). These lines of evidence take many forms, including both macro- and microscale morphological characteristics (Djokic et al., 2017), isotopic composition (Homann et al., 2018), the presence of microfossils (Javaux et al., 2010), and secondary mineral abundance and composition (Li et al., 2011). Similar concerns are applicable to the search for ancient life on Mars (McMahon and Cosmidis, 2022); regional morphology,

possible microfossils, and mineral structures have been proposed as lines of evidence for, albeit controversial, claims of Martian life (e.g., McKay et al., 1996; Noffke, 2015). Methods that can better elucidate complex biosignatures or more clearly confirm their biogenic origin would strengthen claims of both ancient and extant life, especially those associated with the production of biogenic mineral biosignatures.

Planetary science missions to Mars utilize payloads with a wide array of instruments to assess present and past habitability. The earliest attempts at measuring life *in situ* were conducted by the *Viking* missions, which included experiments designed to detect active metabolic processes in Martian soils through respiration or pyrolytic release of labeled and unlabeled compounds (Klein, 1979). Of the three deployed biological experiments, only the Labeled Release experiments, in which soils were enriched with ^{14}C -labeled organics and monitored for radioactive gas production, presented a possible positive result (i.e., gas evolution following injection that was quenched by sample heating; Levin and Straat, 1976b, 1976a), though interpretation of these results remains highly controversial (Klein, 1999; Levin and Straat, 2016). Since the *Viking* missions, surface landers and rovers have focused on morphological and geochemical characterization, with an emphasis on determining if Mars was previously habitable. To this end, several instruments have been deployed to describe mineral and organic features, including possible biosignatures such as chemistry or morphology indicative of ancient metabolic processes. Infrared spectrometers, spanning visible and near-infrared (VNIR) to mid-far infrared ranges, have been deployed on orbital and surficial missions, including on the Mars Exploration Rovers (MERs) *Spirit* and *Opportunity* (Christensen et al., 2003; Squyres et al., 2003). The MERs were also equipped with Mössbauer spectrometers specifically for identification of Fe-bearing minerals (Klingelhöfer et al., 2003). The Mars Science Laboratory (MSL) rover *Curiosity* features both X-ray diffraction (XRD) and limited fluorescence (XRF) capabilities as a part of the ChemMin instrument (Blake et al., 2012), and XRF was also deployed on the Mars 2020 PIXL (Planetary Instrument for X-ray Lithochemistry) instrument (Allwood et al., 2020). Both the ChemMin and PIXL instruments are designed for identification of surface mineral

elemental chemistry. *Curiosity* also features the SAM (Sample Analysis at Mars) instrument suite for characterization of organic components and isotopic states of surface materials including a gas chromatograph, laser spectrometer, and quadrupole mass spectrometer (Mahaffy et al., 2012), in addition to the SHERLOC (Scanning Habitable Environments with Raman & Luminescence for Organics and Chemicals) Raman and fluorescence spectrometer (Bhartia et al., 2021). While none of the recently deployed instruments alone are likely to detect biosignatures that preclude an abiotic origin, when utilized in conjunction, the possibility remains that concomitant chemical complexities and morphological features may provide compelling, though likely not definitive, evidence of ancient life.

Similar to how terrestrial analog features are utilized to test astrobiological instrumentation and hypotheses, terrestrial microorganisms isolated from analog environments are beneficial for exploring possible adaptations and metabolic processes associated with early Earth and Mars. *Shewanella* spp. have long been utilized as model organisms for studying bacterial Fe transformation (Fredrickson et al., 2008). Under anaerobic conditions, *Shewanella* spp. can utilize a wide variety of terminal electron acceptors, including a number of Fe(III) species like amorphous ferrihydrite (Fh; Fredrickson et al., 2008). Dissimilatory iron reduction (DIR) is believed to be an ancient metabolic pathway on Earth (Vargas et al., 1998) that mediates the reduction of Fe(III) to Fe(II) via external electron transfer (Myers and Nealson, 1990). Because Fh and other Fe(III) (hydr)oxides are unable to diffuse or be transported through cellular membranes, DIR by *Shewanella* spp. relies on membrane-bound redox machinery and soluble electron carriers (Shi et al., 2012). Electrons are transferred from the inner membrane electron transport chain to insoluble Fe(III) oxides through the Mtr complex, a series of heme-bearing proteins localized in the inner and outer membrane. Electrons may also be passed to soluble electron shuttles, including flavins, that discharge electrons to external sources (Marsili et al., 2008). *Shewanella* spp. have also been employed as model organisms for astrobiology; Malas et al. (Malas et al., 2024) investigated transcriptional changes in *Shewanella oneidensis* MR-1 when exposed to Titan-relevant pressure to understand

survivability and possible adaptations for living in sub-ice oceans. *S. oneidensis* MR-1 was also shown to reduce Fe(III) present in a simulated Martian regolith (Castelein et al., 2021), supporting the hypothesis that DIR is a plausible metabolic strategy for Martian life (Nixon et al., 2012).

To further investigate the production of mineral and chemical biosignatures during bacterial DIR, we utilized a psychro- and halotolerant *Shewanella* strain (BF02_Schw), isolated from an Fe-rich subglacial brine feature in Antarctica known as Blood Falls (Mikucki and Priscu, 2007; Boles et al., 2021). Biogenic mineral transformations and the production of associated soluble and volatile organic compounds (VOCs) were measured using mission-relevant techniques (Sklute et al., 2022). To link observed “biosignatures” to biotic processes, the transcriptome of BF02_Schw grown with and without Fh was sequenced to identify genes associated with the production of observed mineral and chemical features.

Materials and methods

Culture Conditions.

Shewanella sp. strain BF02_Schw was isolated from Blood Falls, Antarctica in 2002 (described by Mikucki and Priscu, 2007). For all experiments, strain BF02_Schw was first grown in Difco Marine Broth 2216 (BD) at 15 °C to stationary phase before transfer to a marine minimal medium. Marine minimal medium broth contained the following chemicals per 1 L of Milli-Q water: sodium chloride (333.0 mM), magnesium chloride (92.43 mM), sodium sulfate (22.81 mM), calcium chloride (16.22 mM), potassium chloride (7.377 mM), sodium bicarbonate (1.905 mM) potassium bromide (672.3 µM), strontium chloride (127.5 µM), boric acid (355.8 µM), sodium metasilicate (14.1 µM), sodium fluoride (57.2 µM), and ammonium chloride (20.0 µM). The medium was supplemented with 1 mL L⁻¹ of MD-TMS Trace Minerals Supplement (ATCC) and 2 g L⁻¹ of SC Amino Acids Mixture (MP Biomedicals) and dispensed into serum vials. Vials were thoroughly flushed with nitrogen gas (Ultra High Purity Grade; Airgas), capped with butyl stoppers, and crimp-sealed before autoclaving at 121 °C for 20 minutes. Following autoclaving, each container was supplemented with a filter-sterilized

disodium phosphate solution (29.8 μ M). Sterile solutions of sodium lactate (5 M) and sodium thiosulfate (2.5 M) prepared using Milli-Q water and autoclaved for 20 min at 121 °C. Fh (1M) were prepared after Lovley and Phillips (1986) and sterilized by heating to 90 °C for 20 minutes and cooling to 4 °C twice. Fh sterility was confirmed via plating on Difco Marine Agar 2216 (BD).

Media contained combinations of Fh (100 mM), lactate (30 mM), and thiosulfate (10mM; e.g., Fh + lactate + thiosulfate, Fh + lactate, Fh only; **Table 4.1**; tables and figures in this chapter can be found in the **Chapter Four Appendix**). Unless otherwise noted, marine minimal medium + lactate + thiosulfate was considered the “No Fh” medium, and marine minimal medium + lactate + thiosulfate + Fh is designated as the “+Fh” treatment.

BF02_Schw cultures were pelleted and washed three times with the basal salt solution (i.e., no Fh, thiosulfate, lactate, or amino acids) before inoculation in serum vials with at a 1:50 dilution. “Dead” inocula were prepared by autoclaving an aliquot of washed cells for 20 minutes at 121 °C. All vials were wrapped in aluminum foil to prevent photodegradation of Fh and incubated at 4 °C. For each time point, cultures were thoroughly mixed before 2 mL aliquots were drawn via syringe, with 1 mL aliquoted for plate counts and insoluble Fe concentration, and 1 mL passed through a 0.2 μ m syringe filter for soluble Fe concentration, as described below. Unfiltered aliquots were diluted using marine minimal medium basal salts, plated on Difco Marine Agar 2216 (BD), and incubated at 15 °C for one week for CFU counts. Both filtered and unfiltered aliquots designated for Fe were diluted 10-fold in 0.5 N HCl in acid-washed glass vials and stored at 4 °C until analysis.

Colorimetric Iron Concentration

Fe concentration was determined via colorimetric assay using FerroZine™ Iron Reagent (Thermo Scientific). Briefly, a 0.02% FerroZine™ solution was prepared in 1.2% HEPES buffer. A reducing buffer was prepared by adding hydroxylamine to FerroZine™ solution (final concentration 1%). Unfiltered samples were diluted 1:10 and aliquoted into two 96-well plates in triplicate, followed by the addition of either

FerroZine™ solution or the reducing buffer, and incubated for 15 minutes, then absorbance was measured at 562 nm using a Synergy HT plate reader (BioTek) and compared to a standard curve of ferrous ammonium sulfate and statistically compared in R v4.3.2 (R Core Team, 2023) using linear mixed-effect models to account for biological and technical replicates through the lmerTest package v3.1-3 (Kuznetsova et al., 2017). Models were assessed via ANOVA and Tukey's all-pair comparison using the car v3.1-2 (Fox and Weisburg, 2019) and multcomp v1.4-25 (Hothorn et al., 2008) packages, respectively. Features were considered statistically significant if $p \leq 0.05$.

Mineralogy

Incubations designated for mineral analyses were performed in 50 mL volumes as described above for Fh containing treatments (Fh + lactate + thiosulfate, Fh + lactate; **Table 4.1**). At each time point, cultures were heat-killed at 40 °C for three hours to avoid abiotic transformation of Fe species and shipped at 4 °C for analysis.

X-ray diffraction (XRD). XRD analyses used a Rigaku Smartlab II SE XRD with Cu K α radiation in Bragg-Brentano geometry. Scans were performed as 5-80° (0.01° step size) or 2-80° (0.02° step size) 2 θ scans at a rate of 2° min⁻¹. Both baseline correction and mineral identification were performed using Smartlab Studio II software (Rigaku) with a licensed International Centre for Diffraction Data PDF 2 database (2019 version; Fawcett et al., 2017). Both profile and peak data were used to identify and quantify mineral phases via the Smartlab Studio II XRD plugin. Each pattern was adjusted to a tight B-spline baseline as well as an adjusted baseline to account for amorphous baseline swell.

Fourier transform infrared (FTIR) spectroscopy. FTIR spectra were collected using a Bruker ALPHA FTIR spectrometer with a platinum diamond ATR attachment. Spectra were collected at a 4 or 8 cm⁻¹ resolution from 360-400 cm⁻¹ and final spectra were the result of averaging 128 scans per sample. Bruker's OPUS software 7.3 was utilized for continuum removal via concave rubberband correction. Reference spectra include previously collected spectra (Sklute et al., 2018) and spectra from the USGS Spectral Library (Kokaly et al., 2017).

Visual and near-infrared (VNIR) spectroscopy. VNIR spectra were obtained on an ASD Fieldspec 4 Max spectrometer in bidirectional geometry. The spectrometer was equipped with an Oceans Optics HL-2000 light source directed down a 1000 μm Si optical fiber at 30° and an 8° foreoptic for perpendicular collection. Prior to measurement, samples were leveled without packing in matte-black painted samples cups and spectra were collected without ambient illumination. Spectra were obtained as 3×240 136 ms integrations and referred against Spectralon standards, USGS references (Kokaly et al., 2017), and previously collected spectra (Sklute et al., 2018).

Raman spectroscopy. Raman spectra were obtained using a Bruker BRAVO spectrometer with excitation lasers at 758 and 852 nm and a 300-3200 cm^{-1} range. Samples were analyzed through the bottom of one dram glass vials and resulting spectra were averaged over 100 1000 ms integrations. Baseline correction was performed using the rubber band algorithm as implemented at <http://nemo.mtholyoke.edu>. Spectra were compared to the RRUFF database (Lafuente et al., 2015) and previously collected spectra (Sklute et al., 2018).

Mössbauer spectroscopy. Mössbauer spectroscopy was performed using a Web Research (now See-Co) W302 Mössbauer spectrometer at 295, 220, 150, 80, and 4K employing a Janus closed cycle He compressor at < 295 K. Samples were dried and ground lightly with sugar in secured plastic washers backed with Kapton tape. Each 1024-channel spectrum was folded about the midpoint and an α -Fe foil was used for calibration via the WMOSS4 program (Prisecaru, 2009). Spectra fitting was performed using the Mexfield program (University of Ghent).

Transmission electron microscopy (TEM). TEM images with selected area electron diffraction (SAED) data were collected on the Johns Hopkins University Materials Characterization and Processing Center's Thermo Fisher TF30 instrument operating at 300 kV with an EDAX windowless silicon drift energy dispersive X-ray (EDX) detector. Conventional imaging, high-resolution lattice imaging (HRTEM), SAED, scanning transmission electron microscopy (STEM), bright-field (BF), and high-angle annular dark-field (HAADF) imaging, and STEM EDX maps were collected for

selected samples. Images of the *Shewanella* cells were acquired at Mount Holyoke College using a Phillips CM 100 TEM equipped with a 40-100KV tungsten filament, single-tilt goniometer stage, and an AMT digital camera.

RNA Extraction and Sequencing

Cultures for RNA sequencing were incubated in 50 mL volumes as described for the mineralogical analysis, with No Fh and +Fh treatments. At each time point, cultures were pelleted ($2500 \times g$ for 30 min) and frozen at -80°C until extraction. RNA extractions were performed using the Invitrogen TRIzol reagent following the manufacturer's protocol with the following amendments: pellets from +Fh microcosms were thawed in 3.75 mL PB buffer ($112.87 \text{ mM Na}_2\text{HPO}_4 + 712 \text{ mM NaH}_2\text{PO}_4$) and 1.25 mL TNS buffer ($500 \text{ mM Tris-HCl} + 100 \text{ mM NaCl} + 10\% \text{ wt/vol SDS}$) before centrifuging at $2500 \times g$ (1 min) to pellet insoluble Fe, as described by Huang et al. (2021). This step was repeated twice more, until no visible Fe pellet remained, followed by the addition of TRIzol reagent. Pellets without Fh were thawed directly in TRIzol. Following extraction, remaining genomic DNA was removed via DNase I treatment (Invitrogen) and samples were concentrated via ethanol precipitation. Removal of DNA was confirmed via PCR, and RNA was quantified via both fluorometry and automated electrophoresis before shipment to the University of Maryland Genomics Core for sequencing via Illumina NovaSeq 6000 ($2 \times 100\text{bp}$).

DNA and RNA Sequence Analysis

Returned RNA sequences were aligned to the published strain BF02_Schw genome using bowtie2 v2.2.5 (Langmead and Salzberg, 2012) using default parameters. Count files were generated using samtools v1.9 (Danecek et al., 2021) and aniv'o v5.5 (Eren et al., 2015). Reads were assembled into transcripts using StringTie v2.1.7 (Pertea et al., 2015) and transcripts were quantified using Salmon v1.10.1 (Patro et al., 2017). Differential expression was analyzed using DESeq2 v1.34.1 (Anders and Huber, 2010) in R v4.3.2 (R Core Team, 2023). Transcripts were considered significantly more or less abundant if $\log_2$ fold change (LFC) was ≥ 1 or ≤ -1 and the adjusted p value (Benjamini-Hochberg adjusted Wald test; p_{adj}) was ≤ 0.05 .

Both genes and transcripts were annotated using three methods. Initial open reading frame identification and sequence annotation was performed using Prokka v1.14.5 (Seemann, 2014). Open reading frames were placed within pathways using the KEGG database (Kanehisa and Goto, 2000) through GhostKOALA v2.0 (Kanehisa et al., 2016). Specific gene targets were identified using BLAST v2.15.0 (Altschul et al., 1990) against both in-house curated databases and the UniProt database (The UniProt Consortium, 2023). Only sequences with evidence at the protein level were considered for database inclusion. Further investigation of putative function for halogenase genes involved alignment of BF02_Schw sequences to characterized flavin-dependent tryptophan halogenase sequences to compare known conserved binding domains using Geneious v6.1.8 (Kearse et al., 2012). Proteins with hypothetical binding domains were computationally folded using ColabFold v1.5.5 (Mirdita et al., 2022) and compared to databases of characterized protein structures using the Dali web server (Holm, 2022). The alignment figure was generated with ESPrint v3.0 (Robert and Gouet, 2014).

Soluble Metabolites

Soluble metabolites were identified in pooled supernatant collected from the transcriptomics experiment via UHPLC-MS. Supernatant from the eight-week control microcosms were not collected. Supernatant was extracted in an equal volume of methanol (LC-MS grade) via sonicating water bath (10 min) and centrifugation. Extracts were analyzed on Vanquish Horizon UHPLC system and Q-Exactive HF-X Hybrid Quadrupole-Orbitrap MS (Thermo Scientific). The LC instrument was configured with a HSS T3 C18 column (Waters, 1.8 μm , 2.1 x 100 mm) using a VanGuard FIT precolumn and a VH-D10-A UV detector. Samples (2 μL) were injected using a flow rate of 0.5 mL min^{-1} into a prewarmed column (40 $^{\circ}\text{C}$). UHPLC separation was performed using the following parameters: 2% acetonitrile: 98% water with 0.1% formic acid for 1 min, 2–40% over 4 min, 40–98% over 3 min, 98–2% over 0.2 min, and 2% for 2 min. UV–Vis data were collected at 534 nm. Electrospray ionization occurred in probe position D using the following conditions: 40 sheath gas flow, 8 auxiliary gas flow, 1 sweep gas flow, 3.5 kV spray voltage, 380 $^{\circ}\text{C}$ capillary temperature, 50 radiofrequency funnel level, and 350

°C auxiliary gas temperature. Resulting spectra were aligned using MS-DIAL v5.2.240218.2 (Tsugawa et al., 2015) and peak filtering and analysis were performed using MPACT v23.05.15 (Samples et al., 2023). Solvent blank filtering was applied using a group parsing threshold of 0.01 and a minimum reproducibility threshold of 0.5 median coefficient of variation among technical replicates was utilized. Presence or absence of features was determined using a 0.05 relative abundance threshold. Putative riboflavin peaks were compared to a known riboflavin standard (Sigma-Aldrich).

Volatile Organic Compounds (VOCs)

Two different techniques were used to assess the VOCs produced by BF02_Schw: an Ionicon PTR-TOF-1000 Mass Spectrometer (PTR-MS) operating in the hydronium ion mode to survey the suite of VOCs present with m/z ratios ranging from 20 to 250, and a Bay Instrument Membrane Inlet Mass Spectrometry (MIMS) system to examine the production of specific compounds from precursors of known masses.

Incubations designated for VOC surveys were performed on 100 mL aliquots of experimental treatments in acid-cleaned 1L Schott media bottles and sealed with a GL45 screw cap incorporating a butyl rubber septum. This ratio of sample-to-container size was chosen to maximize headspace volume for sampling. Sterile bottles thoroughly flushed with Ultra High Purity nitrogen were utilized as additional headspace controls.

Upon instrumental analysis using the PTR-MS, samples were connected to the mass spectrometer via a sampling manifold with 2 gas lines. Each gas line was fitted with syringe needles that were inserted through the septum for sampling. The needle from the inflow line was placed close to the surface of the sample and the outflow needle positioned near the top of the bottle close to the septum. Sample headspace was then flushed past the mass spectrometer's inlet with zero-grade nitrogen gas at a rate of 80 mL min⁻¹, with the mass spectrometer subsampling the gas stream at a rate of 50 mL min⁻¹. Mass spectra were acquired every second for 10 minutes and averaged to provide a single mass spectrum for each sample. Subsequent baseline and peak detection was carried out using Progenesis MALDI (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). Differential abundance was determined using ANOVA in Progenesis

MALDI, and identified features were statistically compared in R via ANOVA (car v3.1-2; 123) and Tukey's all-pair comparisons (multcomp v1.4-25; Hothorn et al., 2008) using a 0.05 cut-off for significance.

Production of volatile sulfur compounds by BF02_Schw from specific precursors was investigated using MIMS as per Schanke et al. (Schanke et al., 2021). BF02_Schw cultures were grown in minimal media with the addition of Fh + lactate + thiosulfate. Labeled dimethyl sulfoxide (D₆-DMSO, 118.6 μ M) was added to the medium and the production of both isotopically and non-isotopically labeled dimethyl sulfide (DMS) was assessed over two weeks.

Results

Evidence of Bacterial Growth.

Following incubation, all Live treatments (**Table 4.1**) displayed an increase in total CFUs (**Appendix, Figure 6.9**). At the time of inoculation, Live BF02_Schw microcosms contained an average of 10^6 CFU mL⁻¹ across treatments. Both two- and eight-week Live incubations contained $\sim 10^8$ CFU mL⁻¹. Fastest growth in minimal medium was observed in Fh + lactate + thiosulfate (“+Fh”) treatments (average $\mu = 0.49$ days⁻¹). No Fh incubations (i.e., lactate + thiosulfate) had an average growth rate of 0.46 days⁻¹. Viable cells were not observed in No Cell and Dead Cell controls.

Live +Fh treatments showed an increase in Fe(II)_{Total} following both two- and eight-week incubations (**Appendix, Figures 6.10 and 6.11**). All Live incubations in +Fh medium showed a visual transformation from reddish-brown to black after two weeks (**Appendix, Figure 6.10a**). Insoluble black mineral products were magnetic (**Appendix, Figure 6.10b**). Aliquots from +Fh incubations showed an increase in total Fe(II) relative abundance from Day 0 to Day 60 ($p < 0.001$ for all pairwise differences). A transient increase in both soluble Fe(II) and Fe(III) was observed in Live treatments at Day 13, but not Day 60 (**Figure 6.11a**). Additionally, +Fh Live incubations showed increased Fe reduction compared to Live incubations without thiosulfate (i.e., Fh + lactate; **Appendix, Figure 6.11b**).

Mineral Transformations

Coordinated diffraction and spectroscopic techniques were used to determine changes in mineralogy following incubation. Because each technique employs a different energy of impending radiation to query the sample, each discerns a different type of information. Taken collectively, the results document changes before and after reactions (**Figure 4.1**). An additional treatment, Fh + lactate, was utilized as a control; No Fh treatments were not utilized (**Table 4.1**).

X-ray diffraction (XRD). XRD data from all samples showed the characteristic two broad peaks of the starting Fh, and did not provide clarity on mineral transformations (**Figure 4.1c** and **Appendix, Figure 6.12**). Because XRD is a bulk technique and the bio-reduced transformations are likely surface phenomena, this result indicates that, volumetrically, changes were minor.

Fourier transform infrared (FTIR) spectroscopy. FTIR results (**Figure 4.1d**) show that both No Cell and Dead Cell control spectra were similar to reference Fh but contained organic signatures resulting from the medium. Live cultures containing Fh + lactate + thiosulfate (+Fh medium) showed significant transformation into a combination of goethite and magnetite after two weeks of incubation, with a larger relative proportion of magnetite after eight weeks (**Appendix, Figures 6.13** and **6.14**). For these products, the main magnetite peak was shifted to longer wavenumbers with respect to pure, synthetic magnetite, and an additional peak at 475 cm^{-1} not directly attributable to either (hydr)oxide was apparent. In Live cultures with Fh + lactate, magnetite and goethite were only present following eight weeks of incubation with a primary absorption at 560 cm^{-1} , consistent with the position and appearance to synthetic magnetite.

Visual and near-infrared (VNIR) spectroscopy. VNIR spectra of the media aged under incubation conditions showed minor spectral alteration of starting Fh due to media and aging conditions alone (**Figures 4.1b** and **Appendix, Figure 6.15**). Products from Fh + lactate treatments showed the least spectral change with only slight muting of overall spectral contrast, whereas products from Fh + lactate + thiosulfate treatments were significantly more muted. For all three conditions, peak positions remained consistent

except for the absorption at $\sim 1.403\ \mu\text{m}$ in pure Fh spectra, which shifted to $\sim 1.444\ \mu\text{m}$ in all aged samples. Products from Dead Cell incubations lost significant spectral contrast, flattening at wavelengths $> \sim 1\ \mu\text{m}$. All Fh-like absorptions near $0.92\ \mu\text{m}$, $1.444\ \mu\text{m}$, and $1.937\ \mu\text{m}$, along with the VIS maximum at $0.791\ \mu\text{m}$, remained visible. The loss of spectral contrast would make the $\sim 0.92\ \mu\text{m}$ absorption appear to shift in continuum-removed spectra. All products from growth experiments are spectrally dark ($< \sim 0.1$ reflectivity with respect to Spectralon) with a loss of Fh-like features between the VIS maximum and a small NIR maximum $\sim 2.142\ \mu\text{m}$, which is replaced with a long flat (Fh + lactate) to convex (Fh + lactate + thiosulfate) appearance likely due to multiple overlapping absorptions, including those for Fe^{2+} . One replicate of the Fh + lactate + thiosulfate live cell experiment was completely absorbing throughout the VNIR and was spectrally consistent with magnetite.

Raman spectroscopy. Bulk Raman spectra were overwhelmed by fluorescence from the Fe-bearing starting material (**Appendix, Figure 6.16**). Spectra of the experimental samples were compared to Raman spectra of sulfates, sulfides, carbonates, and (hydr)oxides (**Appendix, Figure 6.16a**), but no phase was immediately identifiable as a reaction product. Prominent features at ca. 428 and $492\ \text{cm}^{-1}$ appeared in all samples except for a single analysis of the Live Fh + lactate treatment (**Appendix, Figure 6.16b**). These peaks cannot be assigned to a specific mineral phase, and are assumed to be due to elements of the media. In the Live Fh + lactate incubation, there was a peak at $336\ \text{cm}^{-1}$ common to most sulfides but could not be assigned to a specific phase. Sulfides with partial matches include greigite (peak at $350\ \text{cm}^{-1}$), pyrite (doublet at 348 and $384\ \text{cm}^{-1}$), and pyrrhotite (348 and $380\ \text{cm}^{-1}$). An additional prominent peak in that sample at $524\ \text{cm}^{-1}$ was not a match to any of the reference phases analyzed. Of the (hydr)oxides identified by other techniques used in this paper, none of the main features arising from hematite (26 , 294 , and $408\ \text{cm}^{-1}$), akaganéite (307 and $387\ \text{cm}^{-1}$), goethite (305 and $397\ \text{cm}^{-1}$), magnetite ($670\ \text{cm}^{-1}$), maghemite (673 and $709\ \text{cm}^{-1}$) and Fh ($716\ \text{cm}^{-1}$) were apparent.

Mössbauer spectroscopy. Mössbauer spectra provided specific details on the nature of the Fe transformation in live samples and confirmed the minimal transformation in control samples (**Appendix, Tables 6.6 and 6.7**). Evidence for Fe²⁺ was only seen in Live Fh + lactate + thiosulfate treatments (**Figures 4.1a and Appendix, Figure 6.17**).

Transmission electron microscopy (TEM). TEM of both two- and eight-week incubations showed evidence of Fe transformation (**Figure 4.2**) and visible association between cells and Fe particles (**Appendix, Figure 6.18**). Signatures of Fh were present in all Live samples. Although visually similar, selected area electron diffraction (SAED) ring diffraction of the Live +Fh culture showed predominately magnetite with some Fh after two weeks with the addition of goethite, seen as a shoulder at 0.23 1/Å, after eight weeks (**Figure 4.2d**). Moreover, there are subtle differences between treatments at two weeks. Fh, magnetite, and akaganéite were present in the Fh + lactate sample (**Appendix, Figure 6.19**).

Transcriptional Changes

Transcript sequencing yielded 248789368 total reads (average of 33696840 reads for three +Fh replicates and 36924712 reads for four No Fh replicates). Of these, an average of 79.45% of reads aligned with the published BF02_Schw genome. Read processing yielded an average of 18627177.99 normalized reads for No Fh treatments and 26213974.72 normalized reads for +Fh treatments associated with 4637 genes in the BF02_Schw genome. Of these, 460 genes had significantly different transcript abundance (as log₂ fold change; LFC ≥ 1 or ≤ -1, p_{adj} ≤ 0.05), with 318 transcripts more abundant in +Fh treatments (LFC ≥ 1) and 142 transcripts less abundant in + Fh treatments (LFC ≤ -1). An additional 974 transcripts were considered non-significantly differentially abundant (LFC ≥ 1 or ≤ -1, p_{adj} > 0.05).

Of significant different transcripts, 287 were annotated as non-hypothetical. Of the 202 transcripts with greater abundance, the most represented pathway categories were carbohydrate metabolism (38 genes), protein families: signaling and cellular processes (20), unclassified: metabolism (20), environmental information processing (18), and energy metabolism (16). Only 78 of the significantly less abundant transcripts were

annotated, with the largest categories belonging to protein families: genetic information processing (13), genetic information processing (8), environmental information processing (7), amino acid metabolism (7), and unclassified: metabolism (7).

Transcripts associated with anaerobic Fh metabolism were significantly higher in +Fh treatments (**Figure 4.3; Appendix, Table 6.8**). Genes for the canonical *Shewanella* metal reduction complex (*mtr*) were identified in the BF02_Schw genome via BLAST, and included *omcA*, *mtrC*, *mtrA*, *mtrB*, and *cymA*. Transcripts for the second half of the heme biosynthesis pathway (identified via KEGG map M00121) were significantly higher in +Fh incubations, with the exception of *hemB*, *hemD*, and *hemH*, which were higher but not significant (**Figure 4.3; Appendix, Figure 6.20a and Table 6.8**). Transcripts with significantly higher abundance in the pathway included *hemC*, *hemE*, *hemN*, *hemG_1*, and *hemG_2*.

Genes associated with *psr* thiosulfate reductase had some of the highest LFC observed in +Fh treatments (**Figure 4.3; Appendix, Table 6.8**) and included *psrA*, *psrB*, and *psrC*. While included in the medium, transcripts associated with DMSO reduction were more highly transcribed. Significantly more abundant genes included *dmsE*, *dmsF*, *dmsA*, *dmsB*. Transcripts for *dmsG* and *dmsH* were more abundant in +Fh treatments, though not significantly.

Other genes of interest were transcribed but were not significantly different between treatments, including riboflavin biosynthesis (*ribA*, *ribBA*, *ribD*, *yigB*, *ribC*, *ribE_1*, and *ribE_2*; **Figure 4.3; Appendix, Figure 6.20b and Table 6.8**). Additionally, four putative flavin-dependent halogenases, *rebH_1*, *rebH_2*, *rebH_3*, and *prnA*, were identified within transcripts.

Soluble Metabolites

Soluble metabolites were detected via ultra high purity liquid chromatography mass spectrometry (UHPLC-MS) in supernatant collected from two-week incubations No Fh treatments and both two- and eight-week +Fh treatments. Following peak alignment, 18514 features were present amongst all samples, with 725 (3.92%) features passing all quality filters. Of these features, 306 were present in all treatments; No Fh two-week and

+Fh eight-week treatments had 42 unique features each, and 41 features were present in only +Fh treatments (two- and eight-week incubations). No features were unique to two-week +Fh supernatant. Based on hierarchical clustering analysis of normalized abundance of molecular features, extraction and injection replicates grouped together, and No Fh treatments formed a separate statistical clade from +Fh treatments (**Appendix, Figure 6.21**).

Riboflavin was identified within supernatant extracts via comparison to an authentic standard (**Figure 4.4**). A putative riboflavin feature (m/z 377.1461; calculated for $C_{17}H_{21}N_4O_6$ $[M + H]^+$, 377.1462) was detected in all microcosm extracts with a retention time of 3.88-3.89 (**Figure 4.4a**). The MS/MS spectra of the peak were congruent with the fragmentation pattern obtained from the standard (**Figure 4.4b**). This feature was present in greater abundance in +Fh incubations compared to No Fh supernatant (**Figure 4.4c**).

Volatile Metabolites

Over the course of incubation, VOC profiles detected in microcosm headspace differed between treatments (**Figure 4.5a**). In +Fh and No Fh Live treatments, 18 features were significantly higher compared to Dead Cell, No Cell, and N_2 controls (**Appendix, Table 6.9**; $p < 0.001$ for all). After two weeks, three features were more abundant in Live treatments compared to controls, and one feature was significantly more abundant in Live No Fh treatments. After eight weeks, 10 features were significantly higher in both Live treatments compared No Cell and Dead Cell controls ($p < 0.001$ for all). Of these features, five were significantly higher in +Fh compared to No Fh. An additional four features were significantly higher in Live +Fh only. Of all features, 38 were putatively identified (**Figure 4.5a**; **Appendix, Table 6.9**). One feature (m/z 63.71) was putatively identified as DMS, ethanethiol, or 1,2-ethanediol, and was significantly more abundant in +Fh Live headspace following eight weeks (**Figure 4.5b**). Identity of the feature as DMS was supported by comparison to a DMS standard.

Production of DMS was also identified via Membrane Inlet Mass Spectrometry (MIMS). Following both 9 and 13 days of incubation, D_6 -DMS was detected at a higher

abundance in BF02_Schw cultures incubated with both D₆-DMSO and thiosulfate compared to No Cell controls (**Appendix, Figure 6.22**). D₆-DMS was identified via a peak shift from the expected DMS mass (M+6), which supported the utilization of D₆-DMSO by BF02_Schw. When cultures were incubated with thiosulfate and no DMSO, DMS was not detected above background following 13 days of incubation (data not shown).

Discussion

Biogenic Fe reduction

Microbe-mineral interactions through microbial metabolism or the adhesion of microbes to mineral surfaces can alter mineral surface texture, crystal structure, inclusion of organics, or the generation of distinct mineral species (Banfield et al., 2001). For example, aqueous Fe(II) is produced during DIR and adsorbs to unreduced Fe(III), leading to the formation of secondary Fe minerals such as goethite, hematite, and magnetite (Zachara et al., 2002). While secondary Fe minerals may also form abiotically, biogenic Fe mineral phases can be identified by morphological and compositional features that can persist in the rock record. Terrestrially, secondary Fe minerals including hematite and magnetite have been proposed as possible products of DIR in Archaean Banded Iron Formations (e.g., Walker, 1984; Nealson and Myers, 1990; Li et al., 2011; Teixeira et al., 2017). While an abiotic origin cannot be precluded (Rasmussen and Muhling, 2018), Banded Iron Formation mineralogy features from Fe-metabolizing organisms on early Earth may inform features detected on Mars, potentially indicative of Martian life. On Mars, Fe mineralogy and its implications for extant or extinct life have been the subject of multiple investigations. For example, the MSL ChemMin instrument identified magnetite and hematite abundance within Gale Crater, with stratification suggesting the presence of a redoxycline in the possible Hesperian lake (Hurowitz et al., 2017; Rampe et al., 2020). While these minerals are hypothesized to exist due to abiotic processes, redox gradients are key to life as we know it, and thus these features point to a possibly habitable ancient lake within Gale Crater (Grotzinger et al., 2014; Rampe et al., 2020). Large portions of Martian mineralogy remain undetermined due to the abundance

of X-ray amorphous minerals (i.e., non-crystalline features that cannot be easily distinguished via XRD) and have been proposed to contain a number of Fe species, including Fh (Rampe et al., 2020). Fh has been identified within Martian meteorites dated to the Amazonian (Piercy et al., 2022) and possibly the Noachian (Beck et al., 2015), suggesting that Fh has been present on Mars at different points throughout its history. The timing of formation of these meteorites suggest that oxidized Fe minerals were available as potential terminal electron acceptors on early Mars (Nixon et al., 2012).

Shewanella spp. are widely utilized to study microbial DIR, and thus can provide insight into mineral biosignatures generated by modern terrestrial life (Fredrickson et al., 2008). A variety of Fe(II), Fe(III), and Fe(II, III) species have all been observed as products of Fh reduction by *Shewanella* spp. (Zachara et al., 2002; Han et al., 2018; Nims and Johnson, 2022), though the exact composition of mineral products is highly dependent on the environmental context (O'Loughlin et al., 2021). In our BF02_Schw microcosms, goethite and magnetite were the major Fe minerals generated (**Figure 4.1** and **4.2**); formation of these minerals was not observed in Dead or No Cell controls. Additional biological measurements identified an increase in biomass that correlated with an accumulation of total Fe(II), again only in Live treatments. The increase of *mtr* transcripts in +Fh treatments confirms the process is biologically mediated (**Figure 4.3**). Secondary Fe minerals, while detectable by flight-ready methodologies, are ambiguous as biosignatures since they can also be formed from abiotic processes (Banfield et al., 2001). However, detecting additional biosignatures distinguishable from abiotic backgrounds (Neveu et al., 2018) colocalized with Fe mineralogy would strengthen claims of life detection. Thus, we examined concomitant organic biomarkers associated with *Shewanella* DIR targeting soluble and volatile biosignatures that persisted during the production of goethite and magnetite.

Riboflavin

Flavins, including riboflavin, flavin mononucleotide (FMN), and flavin adenine diphosphate (FAD), are electron shuttles ubiquitous across all domains of life (Edwards, 2014; Wallace et al., 2024). During DIR, flavins can be secreted to mediate electron

transfer to external terminal acceptors; electrons are shuttled from membrane-bound cytochromes to flavins, which in turn reduce the external acceptor (Cooper et al., 2016). Flavins increase the extracellular pool of available electron acceptors, as other modes of electron transfer require direct contact for effective transfer. Many species of Fe(III) reducing bacteria, including *Shewanella* spp., utilize riboflavin as an extracellular electron shuttle during Fh reduction (Marsili et al., 2008; Fuller et al., 2014; Okamoto et al., 2014). Thus, detection of riboflavin in conjunction with reduced Fe mineral deposits is a robust concomitant biosignature for DIR. In BF02_Schw microcosms, we detected riboflavin via UHPLC-MS at higher abundance in +Fh treatments (**Figure 4.4**). Additionally, riboflavin appeared to be more abundant in +Fh supernatant following eight weeks of incubation compared to two weeks, which could be due to accumulation within the supernatant over time or higher production.

Linking riboflavin to life detection is not unprecedented. However, several caveats should be considered. Case et al. (Case et al., 2024) and Wallace et al. (Wallace et al., 2024) proposed riboflavin, as well as FMN and FAD, as useful biosignatures due to their intrinsic pigmentation and autofluorescence. In high Fe environments, FMN and FAD are highly sorbent to Fe (hydr)oxides (Swenson et al., 2015; Zhao et al., 2019), though may still be detected via spectral methodologies (e.g., Raman spectroscopy; Jehlička and Edwards, 2008). Riboflavin is highly light sensitive (Sheraz et al., 2014) and would likely be degraded quickly at the Martian surface. Still, certain environmental conditions can increase the stability of riboflavin such as low temperature, inclusion into a mineral matrix, or shielding from UV, which could include a dust layer, presence at depth, or localization in caves. While structurally different and more stable, niacin, a compound with similar redox function, has been detected in meteorites (Oba et al., 2023). It is possible that the extracellular redox shuttles involved in mineral reduction, such as we observed here with riboflavin, can colocalize with transformed mineral products and, under the right environmental conditions, be preserved.

Porphyryns

Heme cofactors are found throughout all domains of life and are composed of a cyclic tetrapyrrole with an Fe center (Ducluzeau and Nitschke, 2016). Heme is essential to the function of bacterial DIR machinery, which utilizes heme cofactors to transfer electrons through the membrane. In addition to their ubiquity, the molecule's porphyrin core is quite stable and has the potential to remain in the rock record for billions of years as geoporphyrins (Czernuszewicz, 2000). During diagenesis, side chains and metal complexes become altered while retaining characteristic porphyrin structures (Falk and Wolkenstein, 2017). Terrestrially, some of the oldest known geoporphyrins were identified in ~1.1 Ga shale deposits, which have been proposed as fossilized remnants of chlorophylls or other Mg-bearing porphyrins (Gueneli et al., 2018). Geoporphyrins uncovered in conjunction with reduced Fe, could have originated as heme and therefore serve as biomarkers for ancient DIR or similar metabolisms.

In *Shewanella*, heme is an integral cofactor in four of the five proteins of the Mtr complex (Beliaev and Saffarini, 1998; Ross et al., 2007). Transcripts associated with heme biosynthesis were significantly higher in +Fh microcosms compared to the No Fh microcosms (**Figure 4.3**). However, we were unable to detect heme in microcosm supernatant via UHPLC-MS. Heme is associated with membrane-bound proteins and unlikely that heme would be present at detectable levels within the supernatant. Confirmation of heme in BF02_Schw microcosms would likely require extraction before analysis. Detection and characterization of terrestrial geoporphyrins similarly require extensive sample preparation (Woltering et al., 2016), and thus identification of Martian geoporphyrins would most likely require a sample return mission. Additionally, challenges may arise in determining the biogenic origin of such compound, as porphyrins have been detected in carbonaceous chondrites (Hodgson and Baker, 1969) and have several plausible mechanisms for abiotic synthesis (as discussed by Rossetto et al., 2024). Regardless, heme, colocalized with reduced Fe bearing signatures, would strengthen the presumption of microbial DIR.

Thiosulfate

Sulfates have been discovered in various locales on Mars (e.g., Bibring et al., 2007), and likely originated during the late Noachian to Hesperian (Gaillard et al., 2013). Both thiosulfate oxidation and reduction have been proposed as a viable metabolism for life in late Noachian Mars (Singh et al., 2019; Macey et al., 2020; Magnuson et al., 2023). *Shewanella* spp. are known to utilize thiosulfate as both an electron donor (Yu et al., 2021) and acceptor (Burns and DiChristina, 2009). Additionally, Zhu et al. (Zhu et al., 2022) showed that soluble S(-II) from thiosulfate reduction reacts with Fe(II) to form FeS surfaces on both cells and insoluble Fh, which greatly accelerates the rate of electron transfer. The high abundance of sulfates on the surface of Mars may therefore have played a key role in early Martian DIR by enhancing the rate of Fe reduction. In our microcosms, we found Fh reduction rates increased when incubated with thiosulfate. This process relies on the expression of the terminal reductase-encoding *psr* pathway, which were significantly more transcribed in +Fh microcosms (**Figure 4.3**). These data support the co-reduction of Fh and thiosulfate and the possible generation of FeS minerals, though we were unable to conclusively identify FeS species through our methodologies. Reduction of thiosulfate by *Shewanella* spp. may also lead to the production of volatile sulfur compounds (e.g., H₂S; Wu et al., 2015) or sulfur-containing VOCs which could serve as useful indicators of Fh and sulfur metabolism on Mars.

DMS

The production of differentially abundant VOCs near mineral deposits may function as indicators of extant microbial activity. Microbial VOCs have a high potential for use as biosignatures and can be detected through a variety of methodologies, including local sampling with mass spectrometry or remote sensing via spectral analysis (Abrahamsson and Kanik, 2022). Of the VOCs identified in BF02_Schw microcosms, DMS has possibly received the most attention from the astrobiological community (Pilcher, 2003; Domagal-Goldman et al., 2011). Terrestrially, DMS is largely produced from the breakdown of the metabolite dimethylsulfoniopropionate (DMSP) and is the largest source of biogenic sulfur in the atmosphere (Watts, 2000; Seager et al., 2012).

While present in relatively high concentrations in the marine environment, DMSP production and metabolism is restricted to specific, typically photosynthetic, taxa (Bullock et al., 2017). DMS is also produced microbially through the methylation of methanethiol (Visscher et al., 1995, 2003; Lomans et al., 1997; Zhang et al., 2024). Although this process has long been understood, its potential importance in the biogeochemical cycling of DMS may be understated, particularly in soil and sediment environments.

Because it is not linked with abiotic processes on Earth, and is potentially detectable via interstellar spectroscopy, DMS has been proposed by multiple studies as a possible indicator of an inhabited planet (Domagal-Goldman et al., 2011; Seager et al., 2016). DMS is readily degraded in Earth's atmosphere and thus does not reach concentrations detectable via interstellar means (Kettle et al., 2001; Domagal-Goldman et al., 2011). However, on planets without O₂-rich atmospheres and lower levels of UV radiation, DMS may accumulate to a greater abundance, and therefore be within spectroscopically detectable ranges. On smaller scales, DMS can be detected in trace amounts using mass spectrometry, such as with the proton-transfer-reaction mass spectrometry (PTR-MS) used in this study. Historically, the sea-to-air transfer of DMS has been considered a relatively small proportion of the oceanic production of DMS and may represent “leakage” from a biological system (Malin et al., 1992; Simó et al., 1995; Simó, 2001). Thus, even if atmospheric DMS concentrations are not sufficiently high enough to be detected on planetary bodies, “hot spots” with higher DMS concentrations associated with aquatic features may be detectable by surface missions.

On Mars, organosulfur compounds were discovered in Gale Crater mudstone via pyrolysis-GC-MS and evolved gas analysis using the SAM instrument (Eigenbrode et al., 2018) with DMS or its isomeric equivalent ethanethiol detectable in the pM range, though laboratory experiments suggested that ethanethiol was the primary isomer (Millan et al., 2022). In BF02_Schw microcosms, DMS was detected in greater abundance in +Fh microcosms following an eight-week incubation (**Figure 4.5**). While not detected at the two-week time point, increased transcript abundance for *dms* gene clusters in +Fh

microcosms suggests that DMS was produced during two-week incubation, though not at detectable levels (**Figure 4.3**). The *dms* pathway encodes terminal reductases that are utilized by *Shewanella* spp. during respiration of DMSO, leading to production of DMS (Xiong et al., 2016). DMSO reduction by BF02_Schw was observed in incubations with D₆-DMSO as an isotopically labeled sulfur source. However, DMS production was not detected during 14-day incubations with thiosulfate as the sole sulfur source via MIMS, and DMSO was not provided in the medium during PTR-MS experiments. These data suggest that DMS production by BF02_Schw under our +Fh conditions likely requires time (e.g., our eight-week incubation) to accumulate to within detection limits of our instrumentation; as a DIR biosignature, detection of DMS would likely require collection and long-term monitoring of gas evolution from soils. DMS may also form by yet-unknown abiotic processes; recent evidence for DMS was identified on comet 67P/Churyumov-Gerasimenko (Hänni et al., 2024). So, while detection of DMS would not provide unequivocal evidence for life, its detection in association with other mineral or chemical biosignatures on Mars may guide more targeted sampling for life detection missions and sample return.

Halogenated compounds

On Earth, chloromethane and dichloromethane are produced as a result of both biotic and abiotic processes, such as the nonspecific degradation of organic matter, often by haloperoxidases (Chen and van Pée, 2008; Field, 2016). Halogenated compounds including chlorate and perchlorate may be reduced by microorganisms, including *Shewanella* spp., leading to the generation of O₂ and Cl⁻ (Clark et al., 2014; Youngblut et al., 2016). Halogens can also be incorporated into microbial natural products, such as antibiotics (Chen and van Pée, 2008) which, due to their complexity, are strong indicators of biological synthesis. During preliminary PTR-MS experiments performed in complex media, we detected the production of chloromethane by BF02_Schw when grown under both aerobic and anaerobic conditions, as well as bromomethane during aerobic incubation (see supplemental material in **Appendix; Appendix, Figure 6.23**). While the Viking GC-MS identified chloro- and dichloromethane, experiments performed using

terrestrial analog soils suggest that these compounds likely formed from reactions of organic matter with soil perchlorates, which are abundant on Mars (Navarro-González et al., 2010). Thus, volatile organohalides alone would be challenging to interpret in a Martian setting.

Interestingly, we were also able to detect transcription of a putative tryptophan halogenase (*prnA*) in minimal media treatments. Flavin-dependent halogenases have been shown to add halogen atoms to a variety of substrates (Chen and van Pée, 2008). Of transcripts annotated as flavin-dependent halogenases, only *prnA* contained conserved binding sites for tryptophan and FAD (as reviewed in Chen and van Pée, 2008; **Appendix, Figure 6.24**), and had high structural homology to characterized tryptophan halogenases (**Appendix, Figure 6.25** and **Table 6.10**). While *prnA* is likely not associated with the observed chloro- or bromomethane volatiles, halogenation of organic compounds by BF02_Schw appears to occur under all examined conditions. Considering the hypothesized global distribution of halogenated compounds across the surface of Mars (Georgiou et al., 2017), these results suggest that further investigation targeting the production of halogenated compounds in Martian contexts are certainly warranted.

Conclusion

Microorganisms isolated from extreme environments can be utilized to validate signals from astrobiologically relevant instrumentation, test how biosignatures vary under a range of environmental conditions, and potentially discover novel biosignatures. Life on Mars may have occurred early in its history under warmer, wetter conditions. In these early ecosystems, Fe reduction may have been a dominant primitive metabolism, facilitated in part by the Martian sulfur cycle, leaving biologically altered Fe minerals in the rock record. When grown under iron reducing conditions, BF02_Schw produced reduced Fe species including goethite and magnetite as well as VOCs including DMS and chloro- and bromomethane and the electron shuttle riboflavin. Simultaneous detection of multiple biosignatures associated with mineral transformations on another planetary body would provide far more compelling evidence of life. However, detection of biosignatures such as volatiles or flavins with flight-ready instrumentation likely would require sample

incubation for long-term headspace monitoring if utilizing methods such as PTR-MS. While not within the capabilities of current surface missions, future missions may endeavor to develop analyses that target biosignatures that utilize incubation or extraction methodologies in high priority sampling locations or instrumentation to detect ambient volatiles to target sampling efforts for *in situ* experiments or even sample return. These methodologies are reminiscent of the experiments on the *Viking 1* and *2* landers and highlight the promise of deploying similar payloads, albeit with a modern approach.

Data availability

The genomic sequence of BF02_Schw was published previously in Boles et al. (2021) under NCBI accession number JABKAW000000000, and transcripts from No Fh and +Fh treatments will be deposited as raw sequences on NCBI. Analysis scripts and raw data files are available upon request.

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Appendix: Tables and Figures

Table 0.1 Summary of utilized treatments

Treatments	Components
+Fh	Minimal medium + Fh + lactate + thiosulfate
No Fh	Minimal medium + lactate + thiosulfate
Additional mineralogy controls	Minimal medium + Fh + lactate

Fh – ferrihydrite

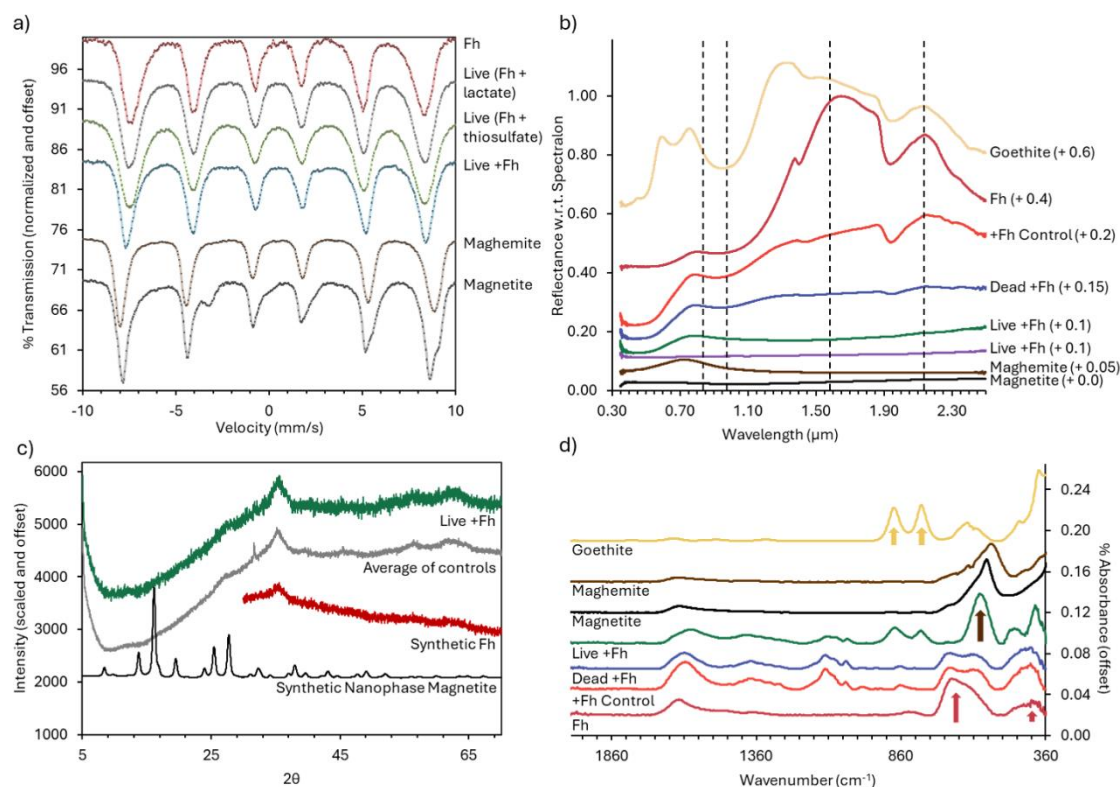


Figure 0.1. Summary of mineralogical changes during incubation. (a) Mössbauer spectra for all Live Cell treatments. Vertical lines highlight the shift in peaks consistent with the presence of Fe^{2+} . (b) Visual and near infrared (VNIR) spectra for +Fh treatments. Two Live Cell +Fh treatments are shown due to slight differences in absorption. (c) X-ray diffraction (XRD) spectra for Live cells in +Fh medium compared to averaged Dead and No Cell controls. Observed features are largely consistent with Fh. (d) Fourier transform infrared (FTIR) spectra of +Fh treatments. Standard peaks consistent with observed features in treatments are highlighted with arrows. +Fh medium contains *Fh* + lactate + thiosulfate.

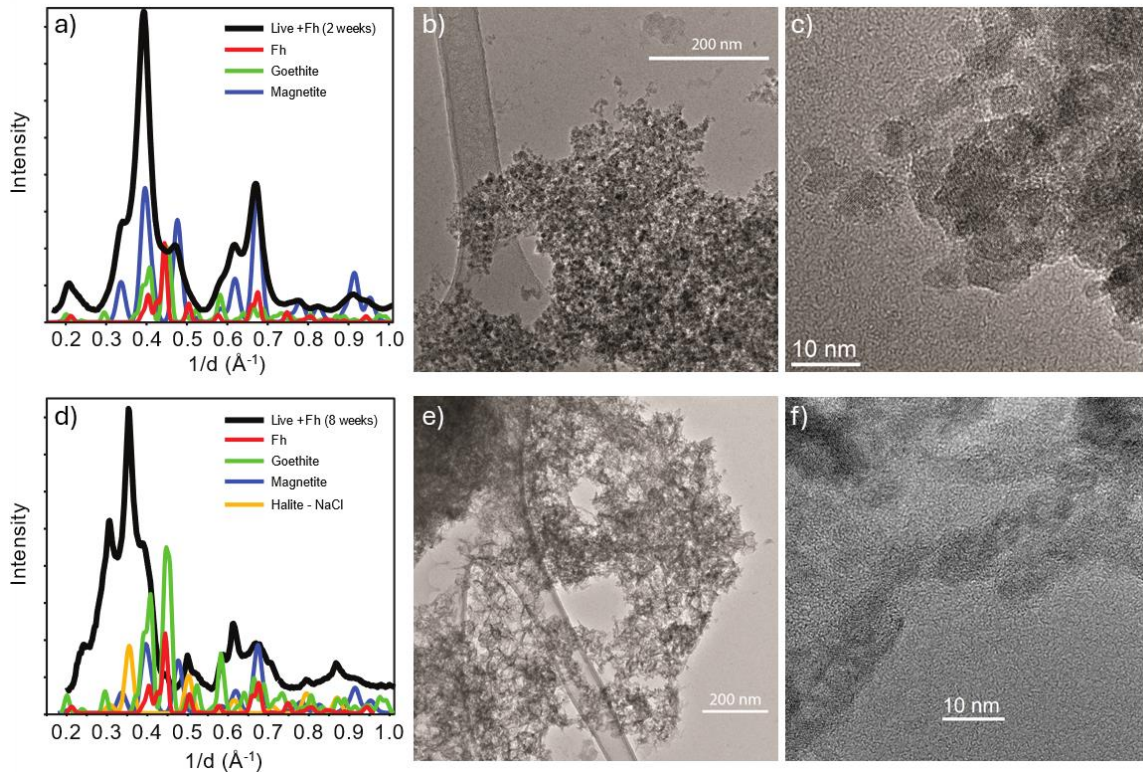


Figure 0.2. Identification of mineralogy of +Fh Live Cells following two weeks (a, b, and c) and eight weeks of incubation (d, e, and f) by transmission electron microscopy (TEM) and selected area electron diffraction (SAED). (a) Two-week incubations feature peaks consistent with both magnetite and Fh, with minor peaks associated with goethite, as seen in both (b) TEM images and (c) SAED diffraction patterns. (d) Eight-week incubations show an increase in features consistent with goethite, in addition to the presence of magnetite and the appearance of peaks consistent with halite formation, which are consistent with both (e) TEM images and (f) SAED patterns.

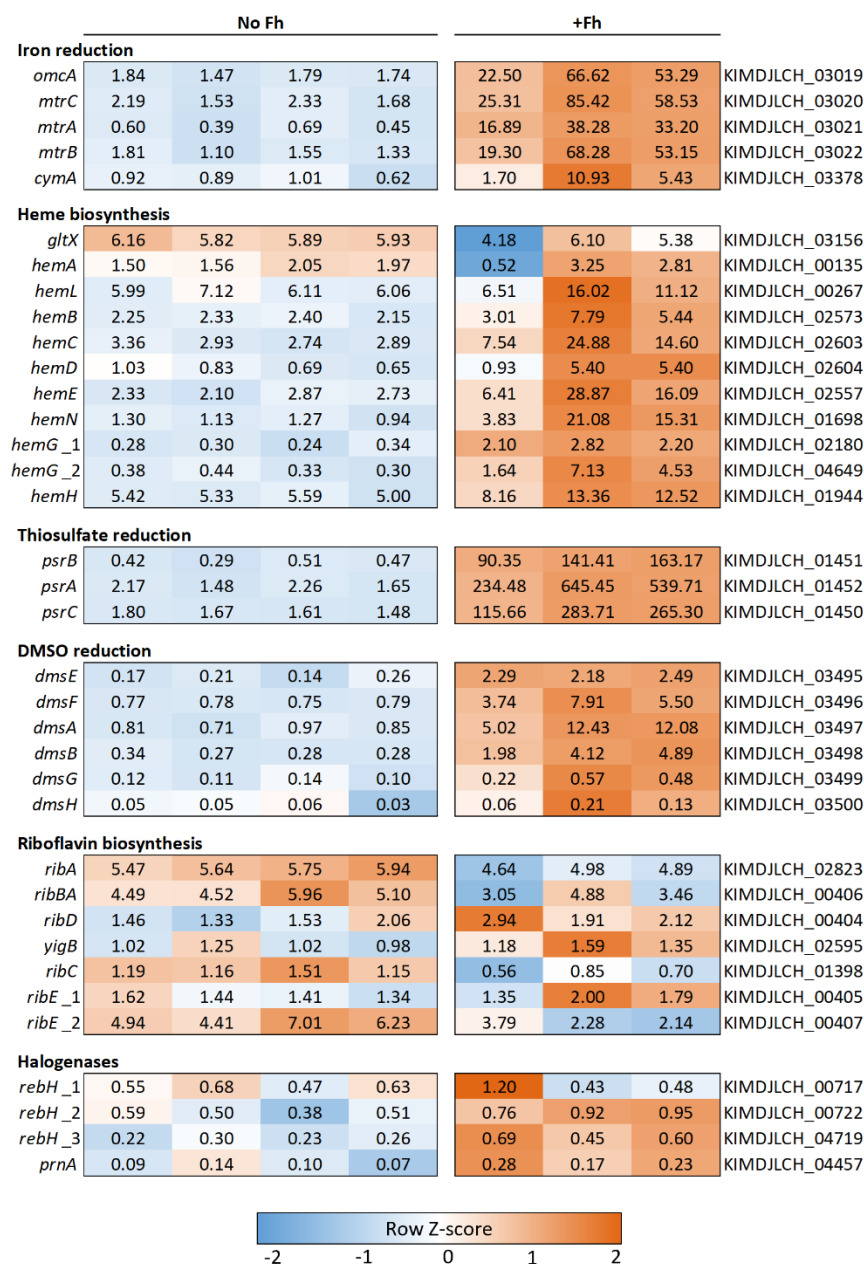


Figure 0.3 Abundance of transcripts of interest between +Fh and No Fh treatments. Transcript identities were determined based on both annotation by Prokka (120) and sequence alignment to characterized genes and are grouped by putative function. Numeric values for each sample represent normalized transcript abundance (median of ratios) per thousand transcripts. Shading represents the Z-score for each gene (standard deviations from mean).

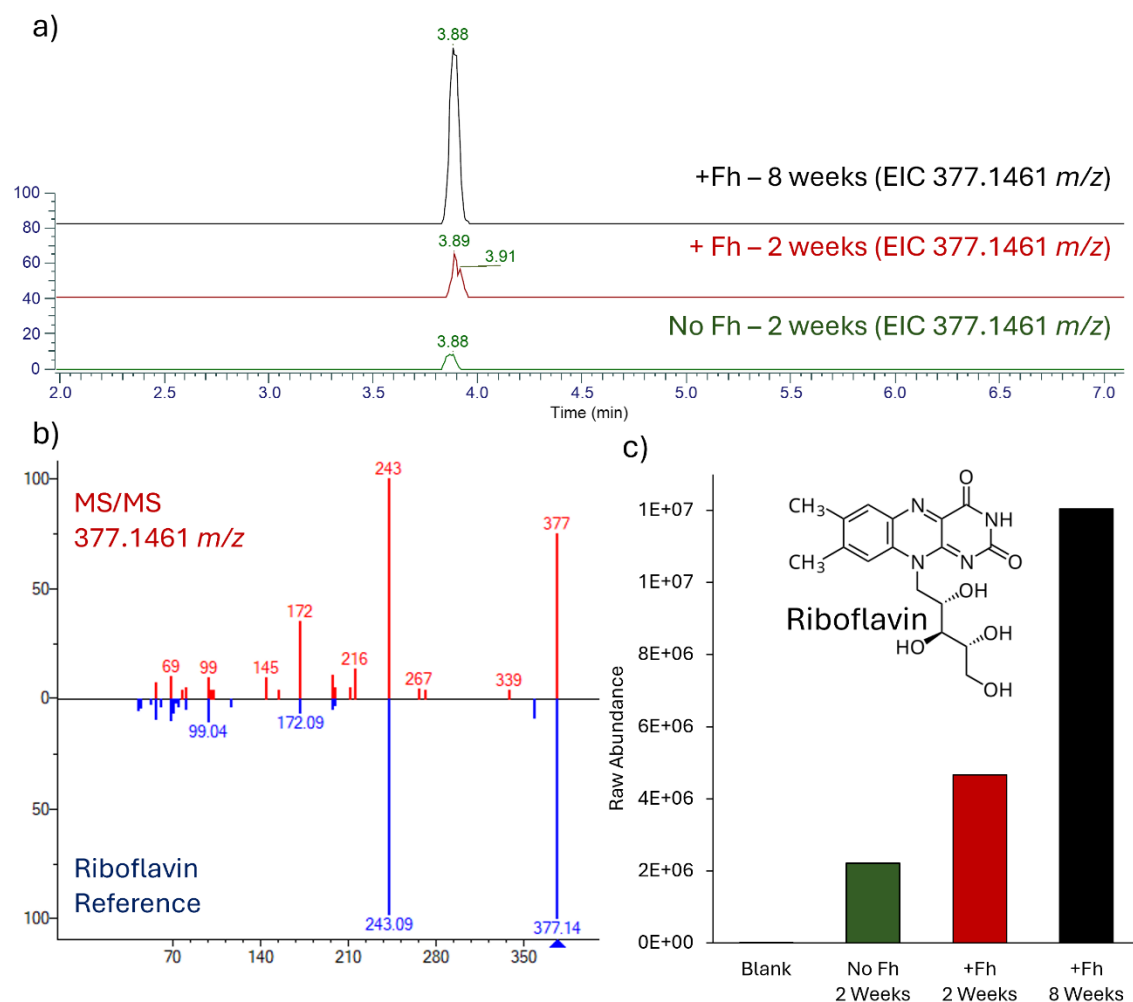


Figure 0.4 Differential abundance of riboflavin detected via UHPLC-MS. (a) Extracted Ion Chromatograms (EIC) for a feature with m/z 377.1461. (b) Mirrored MS/MS fragmentation patterns for predicted riboflavin feature mirrored with a known standard for riboflavin. (c) Raw abundance of putative riboflavin feature across the examined supernatants. (inset) Molecular structure of riboflavin.

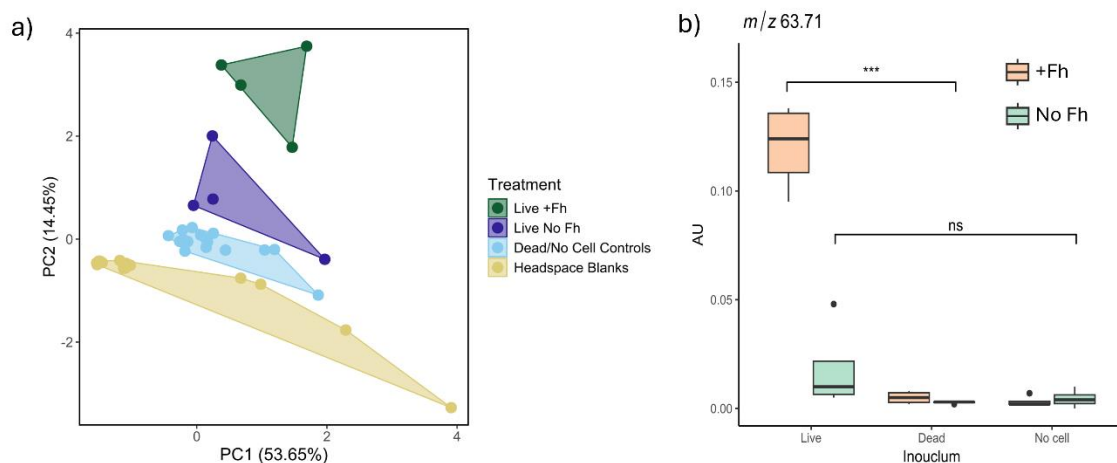


Figure 0.5 Volatile organic compounds (VOCs) detected in microcosm headspace following eight weeks of incubation. (a) PCA plot showing differences between treatments. Polygons represent range of observed scores within a treatment group. (b) Differential abundance of feature with m/z 63.71, putatively identified as dimethyl sulfide (DMS). *ns* – not significant.

CONCLUSION

This dissertation addressed how three representative and cultivated bacteria from the Antarctic polar desert interact with their environment. Earth's cryosphere plays a significant role in global climate and biogeochemical cycling (Meredith, 2022), and efforts to describe the responses of polar communities to change are a high priority for the international cryosphere research community (Kennicutt et al., 2015; NAESM, 2024). Microbial life in the McMurdo Dry Valleys (MDV) are exposed to a wide range of extremes (e.g., temperature, salinity, UV radiation) that may serve as useful analogs for studying life on other planets (Salvatore and Levy, 2021). Environmental changes actively occurring within the MDV are already altering community structure and function (Cary et al., 2010; Fountain et al., 2014; Gooseff et al., 2017; Barrett et al., 2024). Given the rapid pace of change in the cryosphere, studies into the role of microbial life in these environments, and how biosignatures are imprinted under analogous conditions, are more urgent than ever.

Due the thin ozone layer and high solar radiation at extreme latitudes, microbial communities in the MDV are exposed to large amounts of UV radiation during the austral summer (Frederick and Snell, 1988). Long term climate monitoring has identified that solar radiation in the MDV is increasing in variability (Obryk et al., 2018), thus efficient mechanisms of UV protection will become more important for both individuals and communities. The genome of *Massilia frigida* DJPM01, described in **Chapter Two**, encoded numerous putative genomic adaptations to life in cold environments, including fatty acid desaturases and chaperones for protein folding. A host of secondary metabolite biosynthetic gene clusters (BGCs) were coded for in the genome and we conclusively identified the synthesis of a red pigment as prodigiosin, which degraded following constant incubation with UV-A radiation. We concluded that in Antarctic *Massilia*, this pigment may function as an UV protectant for the larger microbial mat community in which it was isolated from (Gareia-Pichel and Castenholz, 1994; Borić et al., 2011), as a bactericidal or nematocidal agent (Danevčič et al., 2016; Habash et al., 2020), or as a yet-undefined player in the modulation of low-temperature energetics (Amato and Christner, 2009; Haddix and Shanks, 2018).

M. frigida DJPM01 has potential as a model organism for studying the role of secondary metabolism in Antarctic bacteria. In general, extreme environments are of strong interest by the natural products communities due to their potential to shape the evolution of novel bioactive compounds (Giddings and Newman, 2015), and investigation of Antarctic microorganisms have

already revealed a wide variety of novel compounds and enzymes with pharmaceutical and industrial applications (Silva et al., 2018; Correa and Abreu, 2020). However, the ecological function of secondary metabolites in these systems remains poorly constrained (Medeiros et al., 2025). As a culturable bacterial isolate with significant potential for secondary metabolite production, *M. frigida* DJPM01 provides a unique opportunity for elucidating the ecological function of these compounds.

Several unknowns remain regarding the function of prodigiosin in the Antarctic. While we were able to identify compound photolysis, alteration in pigment production remains uncharacterized. Additionally, the impact of other wavelengths of UV radiation should be investigated, particularly UV-B, which has increased in recent decades as a result of Antarctic ozone thinning (Robinson et al., 2024). While the utility of genetic tools for generating mutant strains of *M. frigida* DJPM01 remains unknown, long-term incubations (>1 yr at 4 °C) has resulted in the isolation of a visually unpigmented strain, as well as a purple pigmented strain (Shaffer, unpublished data). Through a targeted metabolomics approach, we were able to confirm that the purple-pigmented strain produces the pigment violacein, which was hypothesized based on the presence of the violacein BGC in the *M. frigida* DJPM01 genome, as well as the isolation of purple-pigmented *Massilia* spp. from other Antarctic and glaciated regions (Agematu et al., 2011; Wang et al., 2018; Sedláček et al., 2022). While initial genomic characterization of these strains is required to identify any mutations within pigment BGCs or in other coding sequences, the existence of a prodigiosin-producing, a violacein-producing, and non-pigmented strain of *M. frigida* DJPM01 provides nearly limitless opportunities for laboratory studies investigating the ecological functions of these compounds and the relative fitness of these strains.

The melting of buried permafrost within the MDV leads to the formation of meltwater seeps (Ball et al., 2011; Ball and Virginia, 2012). Wicking processes mobilize salts upwards to the surface, where they are left behind as the water evaporates. Warmer conditions within the MDV will lead to greater melt of near surface permafrost, and thus an increased abundance of saline soil patches (Ball et al., 2011; Fountain et al., 2014). In **Chapter Three**, we identified that community structure within a Fryxell basin seep did not have large, phylum-level differences as seen across other MDV soil salinity gradients (Geyer et al., 2014; Van Horn et al., 2014; George et al., 2021; Jiang et al., 2022; Monteiro et al., 2022), though the phyla Bacillota and

Bacteroidota were positively correlated with soil conductivity. Metagenomic sequencing identified several putative salt-response genes that were in greater abundance in the moderately conductive soil metagenome. Growing *Gillisia* sp. FRX26, an isolate positively correlated with salinity in the seep and present in several MDV basins, in 0.2 M and 0.5 M NaCl elicited several transcriptional and metabolic changes, many of which were hypothesized to be related to the production of extracellular polymeric substances (EPS).

As soils throughout the MDV are extremely heterogeneous, additional efforts in describing seep communities are required to build a more comprehensive picture of the impacts of seep formation. In addition to other archived materials collected from within this same seep, further sequencing and bacterial culturing should be directed at soils collected from other seeps for comparisons at basin, valley, and inter-valley scale. As biofilm formation has been identified as a possible response to increases in salinity within the seep, efforts to identify the distribution of cells within the soil matrix, as well as the quantification of any extant biofilms, should also be performed. Similarly, further experimentation with *Gillisia* sp. FRX26 should focus on quantification and identification of synthesized EPS components. Microbial life adapted to cold conditions can produce EPS and capsule components with unique characteristics, such as interactions with ice for the sea ice-associated *Colwellia psychrerythraea* 34H (Marx et al., 2009; Carillo et al., 2015), thus description of the EPS components of strain FRX26 may reveal additional insight into responses to salinity. Finally, as experiments performed *in vitro* are an abstraction from the natural conditions of the soil matrix, investigation of both *Gillisia* and whole community responses to seep formation can be explored in soil microcosms, similar to Shaw and Wall (2019) and Monteiro et al. (2023). Using a sterile soil matrix approximating the physicochemistry of Fryxell basin soils, seep formation can be simulated via addition of water with a range of salinities. These microcosms can be utilized to explore production of EPS in a soil matrix through microscopy and multi-omics approaches to provide a more environmentally-relevant understanding of how community members respond to the physicochemistry of seeps within the MDV.

Shewanella sp. BF02_Schw, isolated from Blood Falls, Antarctica (Mikucki and Priscu, 2007; Boles et al., 2021) was utilized as a model analog extremophile in **Chapter Four** to explore biosignature detection via Mars-relevant instrumentation. A suite of spectral and microscopic analyses identified that ferrihydrite was reduced to form biogenic goethite and

magnetite, and possibly small amounts of akaganéite. Riboflavin concentrations were elevated in microcosm supernatants as detected via liquid chromatography-mass spectrometry. Signals consistent with the mass of volatile compounds dimethyl sulfide, chloromethane, and bromomethane were detected in the headspace of incubations. Detected biosignatures were validated with abiotic controls and transcriptomic analyses.

Incubations with *Shewanella* sp. BF02_Schw produced identifiable biogenic mineral species and organic compounds. However, no detected biosignature would be considered conclusive, and thus we would be unable to definitively report a positive result for life detection (Neveu et al., 2018). In part, this is due to the inherent challenge of the search for life, which is built on the premise that the presence of life must always be a hypothesis of last resort. As outlined by Neveu et al. (2018), a positive detection for life requires spectral evidence for pigments, detection of complex nucleic acids, or evidence for at least structural preference (e.g., chirality) or patterns of complexity in organics (e.g., specific C chain lengths). Interestingly, patterns of complexity may have been detected in our microcosms via Raman spectroscopy (Sapers et al., 2019), though the high concentrations of Fe required for the suite of mineral analyses overwhelmed any organic signatures. Thus, future attempts at *Shewanella* sp. BF02_Schw microcosms should include treatments with lower concentrations of Fe.

As execution of these microcosms occurred over several years of observation and refinement, many tangential findings excluded from the final narrative are worth future investigation. Rates of ferrihydrite reduction significantly increased in treatments containing thiosulfate compared to those without. In *Shewanella oneidensis* MR-1, this acceleration was demonstrated to be mediated by the production of biogenic mackinawite following thiosulfate reduction (Zhu et al., 2022). However, we were unable to conclusively identify any FeS minerals in *Shewanella* sp. BF02_Schw microcosms, though we did observe significantly increased transcription of the *psr* pathway in treatments with both ferrihydrite and thiosulfate, as was observed in *S. oneidensis*. In addition to the description of any biogenic FeS minerals, future studies should be conducted to characterize the distribution of this synergistic metabolism, especially in light of the high concentrations of Fe and S observed at Blood Falls (Mikucki et al., 2009; Lyons et al., 2019) and the relevance of Fe and S to both early Earth (Wächtershäuser, 1990) and Mars (Magnuson et al., 2023).

Together, these chapters highlight how culture-based laboratory experiments provide insight into the ecology of the MDV and how microorganisms interact with their environment. The use of untargeted metabolomic approaches can identify compounds of interest in ecological (**Chapter Three**) or astrobiological contexts (**Chapter Four**), which can be further analyzed via targeted approaches (**Chapter Two**). Target molecules serving as biomarkers for response to environmental change (e.g., compatible solutes, EPS, or pigments) can be quantitatively identified via field-portable methodologies, such as the detection of antibiotics in MDV soils via colorimetric immunoassays (Shaffer, unpublished data). These techniques can be utilized to both monitor the impacts of climate change and to guide sampling efforts for more in depth, laboratory-based experimentation. Additionally, the use of cultivatable bacteria from extreme environments can be utilized to identify changes to subtle or gradual perturbations that might not be detectable in the environment. In a rapidly changing cryosphere, understanding how individual organisms respond to subtle environmental challenges is critical for understanding the impact of climate change on entire ecosystems.

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APPENDIX: SUPPLEMENTARY MATERIALS

Chapter Two Supplementary Materials

Table 0.1 RefSeq *Massilia* genomes used in comparative analysis. Average nucleotide identity (ANI) was calculated using the enviomics collection (Rodriguez-R and Konstantinidis, 2016) and digital DNA-DNA hybridization (dDDH) was performed using Genome-to-Genome Distance Calculator v3.0 (Meier-Kolthoff et al., 2022). *C.I.* – confidence interval.

RefSeq Genome (Accession Number)	Isolation Site	Comparison to strain DJPM01	
		ANI (%)	dDDH [CI] (%)
<i>Massilia agilis</i> JCM 31605 (GCF 024756255)	Forest soil; Suwon, South Korea	79.41	21.1 [18.9 - 23.6]
<i>Massilia agri</i> JCM 31661 (GCF 024753255)	Grassland Soil; Morang, Nepal	79.02	20.6 [18.4 - 23]
<i>Massilia alkalitolerans</i> DSM 17462 (GCF 000427785)	Soil; Yunnan Province China	79.25	20.8 [18.6 - 23.2]
<i>Massilia antarctica</i> P9640 (GCF 015689335)	Brook water; James Ross Island, Antarctica	91.92	46.5 [44 - 49.1]
<i>Massilia aquatica</i> CCM 8693 (GCF 011682045)	Small stones; James Ross Island, Antarctica	87.94	34.4 [31.9 - 36.9]
<i>Massilia arenae</i> GEM5 (GCF 008014745)	Sand; Qinghai Province, China	79.06	20.5 [18.3 - 23]
<i>Massilia arenosa</i> MC02 (GCF 004614185.1)	Sandy-loam maize soil; United States	79.07	20.6 [18.4 - 23]
<i>Massilia aromaticivorans</i> ML15P13 (GCF 013003915)	Arctic Soil; Svalbard, Norway	79.19	21.1 [18.8 - 23.5]
<i>Massilia atriviolacea</i> SOD (GCF 003953935)	Soil; Hefei, China	88.04	34.5 [32.1 - 37]
<i>Massilia aurea</i> JCM 13879 (GCF 024753205)	Drinking water distribution system; Seville, Spain	78.92	20.3 [18.1 - 22.7]
<i>Massilia cavernae</i> K1S02-61 (GCF 003590855)	Soil from karst cave; Guizhou, China	81.25	23.2 [20.9 - 25.7]
<i>Massilia eburnea</i> JCM 31587 (GCF 009720745)	Lagoon sediments; Japan	78.53	20 [17.8 - 22.4]
<i>Massilia eurypsychrophila</i> JCM 30074 (GCF 002760655)	Ice core; Muztagh Glacier, Xinjiang, China	81.41	23.1 [20.8 - 25.6]
<i>Massilia forsythiae</i> GN2-R2 (GCF 012849555)	Flower of <i>Forsythia</i> <i>koreana</i> ; Gongju-si, South Korea	79.47	21.5 [19.3 - 24]
<i>Massilia frigida</i> CCM 8695 (GCF 011682175)	Water; James Ross Island, Antarctica	97.36	75.6 [72.6 - 78.4]

Table 6.1 (continued)

RefSeq Genome (Accession Number)	Isolation Site	Comparison to strain DJPM01	
		ANI (%)	dDDH [CI] (%)
<i>Massilia glaciei</i> B448-2 (GCF 003011895)	Thawed water; Muztagh Glacier, Xinjiang, China	81.21	23.2 [21 - 25.7]
<i>Massilia horti</i> ONC3 (GCF 004614195)	Garden soil subsurface; United States	79.39	21.2 [19 - 23.6]
<i>Massilia mucilaginoso</i> CCM 8733 (GCF 011682145)	Water; James Ross Island, Antarctica	88.41	35.2 [32.8 - 37.7]
<i>Massilia niastensis</i> DSM 21313 (GCF 000382345)	Air Sample; Suwon, South Korea	79.88	21.4 [19.2 - 23.9]
<i>Massilia norwichensis</i> LMG 28164 (GCF 024753245)	Air sample; Norwich, United Kingdom	79.20	20.8 [18.6 - 23.3]
<i>Massilia oculi</i> CCUG 43427 (GCF 003143515)	Human eye; Linköping, Sweden	79.10	20.8 [18.6 - 23.3]
<i>Massilia pinisoli</i> JCM 31316 (GCF 024753285)	Forest soil; Suwon, South Korea	78.55	20.4 [18.1 - 22.8]
<i>Massilia polaris</i> RP-1-19 (GCF 012927275)	Arctic Soil; Svalbard, Norway	81.30	22.9 [20.6 - 25.4]
<i>Massilia psychrophila</i> JCM 30813 (GCF 002760665)	Ice core; Muztagh Glacier, Xinjiang, China	80.81	22.4 [20.2 - 24.9]
<i>Massilia puerhi</i> SJY3 (GCF 009861485)	Soil; China	79.41	20.7 [18.5 - 23.2]
<i>Massilia putida</i> 6NM-7 (GCF 001941825)	Wolfram mining tailing; Jiangxi, China	78.81	20.9 [18.7 - 23.4]
<i>Massilia rhizosphaerae</i> NEAU- GH312 (GCF 016093545)	Soil; Heilongjiang, China	78.65	20.4 [18.2 - 22.8]
<i>Massilia rubra</i> CCM 8692 (GCF 011682065)	Stone; James Ross Island, Antarctica	88.31	35.1 [32.6 - 37.6]
<i>Massilia soli</i> R798 (GCF 016809835)	Soil; South Korea	81.09	22.8 [20.5 - 25.2]
<i>Massilia solisilvae</i> JCM 31607 (GCF 024753215)	Forest soil; Suwon, South Korea	79.47	21.2 [18.9 - 23.6]
<i>Massilia terrae</i> JCM 31606 (GCF 024753145)	Forest soil; Suwon, South Korea	79.42	21.3 [19.1 - 23.8]
<i>Massilia timonae</i> NEU (GCF 001866515)	Forest soil; Neuchatel, Switzerland	79.26	21 [18.7 - 23.4]

Table 6.1 (continued)

RefSeq Genome (Accession Number)	Isolation Site	Comparison to strain DJPM01	
		ANI (%)	dDDH [CI] (%)
<i>Massilia violaceinigra</i> B2 (GCF 002752675)	Glacial permafrost; Tianshan Glacier, Xinjian, China	91.38	44.5 [42 - 47.1]
<i>Massilia yuzhufengensis</i> CGMCC 1.12041 (GCF 900112225)	Ice core; Yuzhufeng Glacier, China	79.29	21 [18.8 - 23.4]

Table 0.2 Summary of genomic adaptations present in DJPM01. Coding sequences were identified using Prokka, and KEGG identities were provided using GhostKOALA.

Gene Product name	Sequence ID	KO	GhostKOALA Annotation	Score
Cold stress				
Cold Shock Protein CspA	HPNLJDEO_02266	K03704	<i>cspA</i> ; cold shock protein	135
Cold Shock Protein CspA	HPNLJDEO_03891	K03704	<i>cspA</i> ; cold shock protein	138
ATP-dependent Clp protease	HPNLJDEO_00228	K03695	<i>clpB</i> ; ATP-dependent Clp protease ATP-binding subunit ClpB	1410
ATP-dependent Clp protease	HPNLJDEO_05808	K03695	<i>clpB</i> ; ATP-dependent Clp protease ATP-binding subunit ClpB	310
molecular chaperone DnaJ	HPNLJDEO_04280	K03686	<i>dnaJ</i> ; molecular chaperone DnaJ	62
molecular chaperone DnaJ	HPNLJDEO_01662	K03686	<i>dnaJ</i> ; molecular chaperone DnaJ	608
molecular chaperone DnaK	HPNLJDEO_01663	K04043	<i>dnaK</i> , HSPA9; molecular chaperone DnaK	1087
molecular chaperone DnaK	HPNLJDEO_01779	K04043	<i>dnaK</i> , HSPA9; molecular chaperone DnaK	876
molecular chaperone DnaK	HPNLJDEO_05795	K04043	<i>dnaK</i> , HSPA9; molecular chaperone DnaK	1014
chaperonin GroEL	HPNLJDEO_05678	K04077	<i>groEL</i> , HSPD1; chaperonin GroEL [EC:5.6.1.7]	903
chaperonin GroES	HPNLJDEO_05679	K04078	<i>groES</i> , HSPE1; chaperonin GroES	169
molecular chaperone GrpE	HPNLJDEO_01664	K03687	GRPE; molecular chaperone GrpE	249
stearoyl-CoA desaturase	HPNLJDEO_04730	K00507	SCD, <i>desC</i> ; stearoyl-CoA desaturase (Delta-9 desaturase) [EC:1.14.19.1]	662

Table 6.2 (continued)			GhostKOALA	
Gene Product name	Sequence ID	KO	Annotation	Score
acyl-CoA thioesterase TesA	HPNLJDEO_03409	K10804	<i>tesA</i> ; acyl-CoA thioesterase I [EC:3.1.2.- 3.1.2.2 3.1.1.2 3.1.1.5]	261
acyl-CoA thioesterase YciA	HPNLJDEO_02203	K10806	<i>yciA</i> ; acyl-CoA thioesterase YciA [EC:3.1.2.-]	224
ATP-dependent RNA helicase DbpA	HPNLJDEO_00246	K05591	<i>dbpA</i> ; ATP-dependent RNA helicase DbpA [EC:3.6.4.13]	707
ATP-dependent RNA helicase DeaD	HPNLJDEO_05195	K05592	<i>deaD</i> , <i>cshA</i> ; ATP- dependent RNA helicase DeaD [EC:3.6.4.13]	845
ATP-dependent RNA helicase RhIE	HPNLJDEO_01449	K11927	<i>rhIE</i> ; ATP-dependent RNA helicase RhIE [EC:3.6.4.13]	573
ATP-dependent RNA helicase RhIE	HPNLJDEO_02635	K11927	<i>rhIE</i> ; ATP-dependent RNA helicase RhIE [EC:3.6.4.13]	657
ATP-dependent RNA helicase RhIE	HPNLJDEO_02720	K11927	<i>rhIE</i> ; ATP-dependent RNA helicase RhIE [EC:3.6.4.13]	634
DNA Photorepair				
deoxyribodipyrimidine photolyase <i>phr</i>	HPNLJDEO_05006	K01669	<i>phr</i> , PHR1; deoxyribodipyrimidine photo-lyase [EC:4.1.99.3]	655
(6-4)DNA photolyase <i>phrB</i>	HPNLJDEO_04024	K06876	<i>phrB</i> ; (6-4)DNA photolyase [EC:4.1.99.13]	434
Oxidative Stress				
catalase	HPNLJDEO_00224	K03781	<i>katE</i> , CAT, <i>catB</i> , <i>srpA</i> ; catalase [EC:1.11.1.6]	737
catalase	HPNLJDEO_02565	K03781	<i>katE</i> , CAT, <i>catB</i> , <i>srpA</i> ; catalase [EC:1.11.1.6]	1056
catalase	HPNLJDEO_04366	K03781	<i>katE</i> , CAT, <i>catB</i> , <i>srpA</i> ; catalase [EC:1.11.1.6]	677

Table 6.2 (continued)		GhostKOALA		
Gene Product name	Sequence ID	KO	Annotation	Score
catalase	HPNLJDEO_04511	K03781	<i>katE</i> , CAT, <i>catB</i> , <i>srpA</i> ; catalase [EC:1.11.1.6]	66
catalase	HPNLJDEO_04578	K03781	<i>katE</i> , CAT, <i>catB</i> , <i>srpA</i> ; catalase [EC:1.11.1.6]	783
catalase	HPNLJDEO_04622	K03781	<i>katE</i> , CAT, <i>catB</i> , <i>srpA</i> ; catalase [EC:1.11.1.6]	717
lipoyl-dependent peroxiredoxin	HPNLJDEO_01672	K04063	<i>osmC</i> , <i>ohr</i> ; lipoyl-dependent peroxiredoxin [EC:1.11.1.28]	189
lipoyl-dependent peroxiredoxin	HPNLJDEO_04258	K04063	<i>osmC</i> , <i>ohr</i> ; lipoyl-dependent peroxiredoxin [EC:1.11.1.28]	191
superoxide dismutase	HPNLJDEO_03214	K04564	SOD2; superoxide dismutase, Fe-Mn family [EC:1.15.1.1]	358
Osmotic Stress				
sodium/hydrogen antiporter (NHA1)	HPNLJDEO_02400	K24160	NHA1, SOD2; sodium/hydrogen antiporter	509
Na ⁺ :H ⁺ antiporter (NhaA)	HPNLJDEO_04494	K03313	<i>nhaA</i> ; Na ⁺ :H ⁺ antiporter, NhaA family	520
monovalent cation:H ⁺ antiporter	HPNLJDEO_05830	K03316	TC.CPA1; monovalent cation:H ⁺ antiporter, CPA1 family	493
citrate synthase	HPNLJDEO_05492	K01647	CS, <i>gltA</i> ; citrate synthase [EC:2.3.3.1]	656
glutamate synthase – large chain	HPNLJDEO_05872	K00265	<i>gltB</i> ; glutamate synthase (NADPH) large chain [EC:1.4.1.13]	289
glutamate synthase – small chain	HPNLJDEO_05871	K00266	<i>gltD</i> ; glutamate synthase (NADPH) small chain [EC:1.4.1.13]	637
glutamate/aspartate transport system	HPNLJDEO_05974	K10001	<i>gltI</i> , <i>aatJ</i> ; glutamate/aspartate	791

Table 6.2 (continued)		GhostKOALA		
Gene Product name	Sequence ID	KO	Annotation	Score
substrate-binding protein			transport system substrate-binding protein	
glutamate/aspartate transport system permease protein	HPNLJDEO_05973	K10003	<i>gltJ, aatQ</i> ; glutamate/aspartate transport system permease protein	2712
glutamate/aspartate transport system permease protein	HPNLJDEO_05972	K10002	<i>gltK, aatM</i> ; glutamate/aspartate transport system permease protein	854
glutamate/aspartate transport system ATP-binding protein	HPNLJDEO_02196	K10004	<i>gltL, aatP</i> ; glutamate/aspartate transport system ATP-binding protein [EC:7.4.2.1]	373
glutamate/aspartate transport system ATP-binding protein	HPNLJDEO_05971	K10004	<i>gltL, aatP</i> ; glutamate/aspartate transport system ATP-binding protein [EC:7.4.2.1]	338
formimidoylglutamate deiminase	HPNLJDEO_01030	K05603	<i>hutF</i> ; formimidoylglutamate deiminase [EC:3.5.3.13]	320
N-formylglutamate deformylase	HPNLJDEO_01029	K01458	<i>hutG</i> ; N-formylglutamate deformylase [EC:3.5.1.68]	401
histidine ammonia-lyase	HPNLJDEO_06057	K01745	<i>hutH, HAL</i> ; histidine ammonia-lyase [EC:4.3.1.3]	422
imidazolonepropionase	HPNLJDEO_01032	K01468	<i>hutI, AMDHD1</i> ; imidazolonepropionase [EC:3.5.2.7]	554
urocanate hydratase	HPNLJDEO_01027	K01712	<i>hutU, UROC1</i> ; urocanate hydratase [EC:4.2.1.49]	350

Table 0.3 antiSMASH output table for strain DJPM01.

antiSMASH Category	Contig	Position	Closest known match (% Similarity)
Acyl Amino Acids	2	208027 – 272514	
Acyl Amino Acids, RiPP-like	38	1 – 34955	
Arylpolyene	77	1 – 27333	APE Ec (36%)
Homoserine Lactone	16	98016 – 118603	
Indole, TransAT-PKS, NRP	12	2347 – 102882	Violacein (100%)
Lanthipeptide Class iv	4	13765 – 36443	
Lanthipeptide Class-iv	6	1 – 14584	
NRPS	28	50388 – 91004	Cephameycin C (36%)
NRPS	36	1 – 37992	Myxochelin (41%)
NRPS-like	96	1 – 11867	
Prodigiosin	10	53690 – 90993	Prodigiosin (42%)
RiPP-like	123	1 – 4714	
RiPP-like	29	67380 – 78210	
RiPP-like	53	31685 – 43283	
Terpene	62	26592 – 43142	
TransAT-PKS	3	1 – 50887	Migrastatin / Dorrigocin (45%)
TransAT-PKS-like, Butyrolactone	89	1 – 19037	

AT - acetyltransferase. *NRPS* - nonribosomal peptide synthetase. *PKS* - polyketide synthase. *RiPP* - ribosomally synthesized and post-translationally modified peptide.

Table 0.4 Summary of post hoc tests on BGC abundance in Massilia genomes.

	Comparison	Estimated Difference	Std. Error	t value	Pr(> t)
Raw abundance	Arctic/alpine to Antarctic	-6.643	1.541	-4.31	< 0.001
	Mesophile to Antarctic	-6.318	1.276	-4.95	< 0.001
	Mesophile to Arctic/alpine	0.325	1.202	0.27	0.960
Normalized to genome size*10 ⁶	Arctic/alpine to Antarctic	-0.495	0.201	-2.467	0.048
	Mesophile to Antarctic	-0.464	0.166	-2.793	0.023
	Mesophile to Arctic/alpine	0.031	0.156	0.198	0.978
Normalized to number of coding sequences*10 ³	Arctic/alpine to Antarctic	-0.598	0.236	-2.536	0.041
	Mesophile to Antarctic	-0.548	0.195	-2.808	0.021
	Mesophile to Arctic/alpine	0.050	0.184	0.272	0.955

Table 0.5 Table of prodiginine features detected by MPACT. *FC* – fold change. *RT* – retention time.

Compound	m/z	RT (min)	FC	max	-logp	-logq	log FC
6.883_266.12851	266.12851	6.883	0.338	2.62E+07	4.97	4.62	-1.57
6.894_282.15964	282.15964	6.894	0.625	4.34E+07	3.68	3.52	-0.68
5.963_294.1597	294.1597	5.963	2.167	2.67E+07	2.12	2.04	1.12
7.328_296.17532	296.17532	7.328	0.010	1.39E+07	6.35	5.76	-6.64
7.328_298.19122	298.19122	7.328	0.010	1.84E+07	5.80	5.32	-6.64
6.88_308.17499	308.17499	6.880	0.335	1.58E+07	3.80	3.63	-1.58
7.328_308.17569	308.17569	7.328	0.014	2.74E+07	5.64	5.18	-6.20
6.739_310.19122	310.19122	6.739	0.265	2.48E+07	0.98	0.94	-1.91
7.325_310.1915	310.1915	7.325	0.010	2.37E+09	4.02	3.79	-6.64
6.886_322.19125	322.19125	6.886	0.328	2.06E+07	4.60	4.30	-1.61
6.635_322.19153	322.19153	6.635	0.097	1.06E+07	2.15	2.06	-3.37
6.881_324.20724	324.20724	6.881	0.083	8.89E+09	3.59	3.44	-3.58
7.326_326.18634	326.18634	7.326	0.017	2.36E+09	6.04	5.51	-5.87
6.992_326.1864	326.1864	6.992	0.010	2.74E+08	5.39	5.00	-6.64
6.746_326.18655	326.18655	6.746	0.010	5.62E+08	5.99	5.47	-6.64
5.977_326.18665	326.18665	5.977	0.016	2.26E+08	2.86	2.74	-6.01
6.893_340.20172	340.20172	6.893	0.601	1.33E+09	3.78	3.61	-0.73
6.461_340.20197	340.20197	6.461	5.475	4.77E+07	5.44	5.03	2.45
6.521_340.20209	340.20209	6.521	0.407	3.61E+07	1.56	1.51	-1.30
6.146_340.20221	340.20221	6.146	1.837	2.18E+07	1.84	1.77	0.88
7.317_352.23792	352.23792	7.317	0.013	1.10E+08	7.44	6.38	-6.22
7.265_352.23822	352.23822	7.265	0.039	7.42E+07	7.57	6.41	-4.70
7.854_354.21735	354.21735	7.854	0.010	6.42E+07	3.61	3.46	-6.64
7.088_354.21753	354.21753	7.088	0.245	5.82E+07	3.00	2.87	-2.03
7.275_368.23285	368.23285	7.275	0.116	3.53E+08	6.06	5.51	-3.11

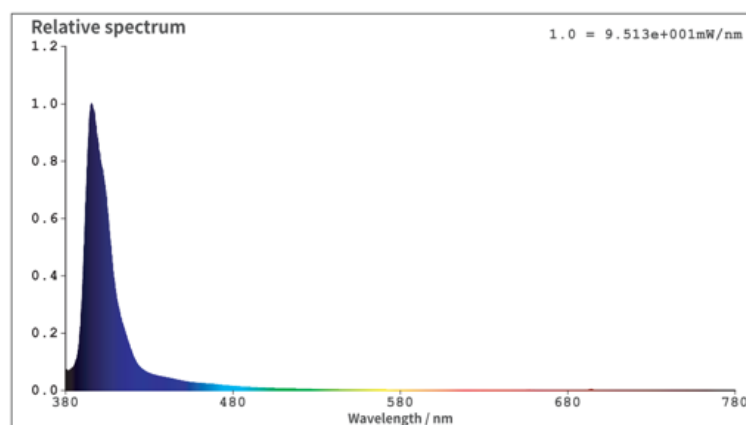


Figure 0.1 Output spectrum of UV-A light, as sourced from the Item User Guide (SANSEI Lighting 5W LED Black Light Bulb; Item Model Number C21BB-TE26-5W-UVA).

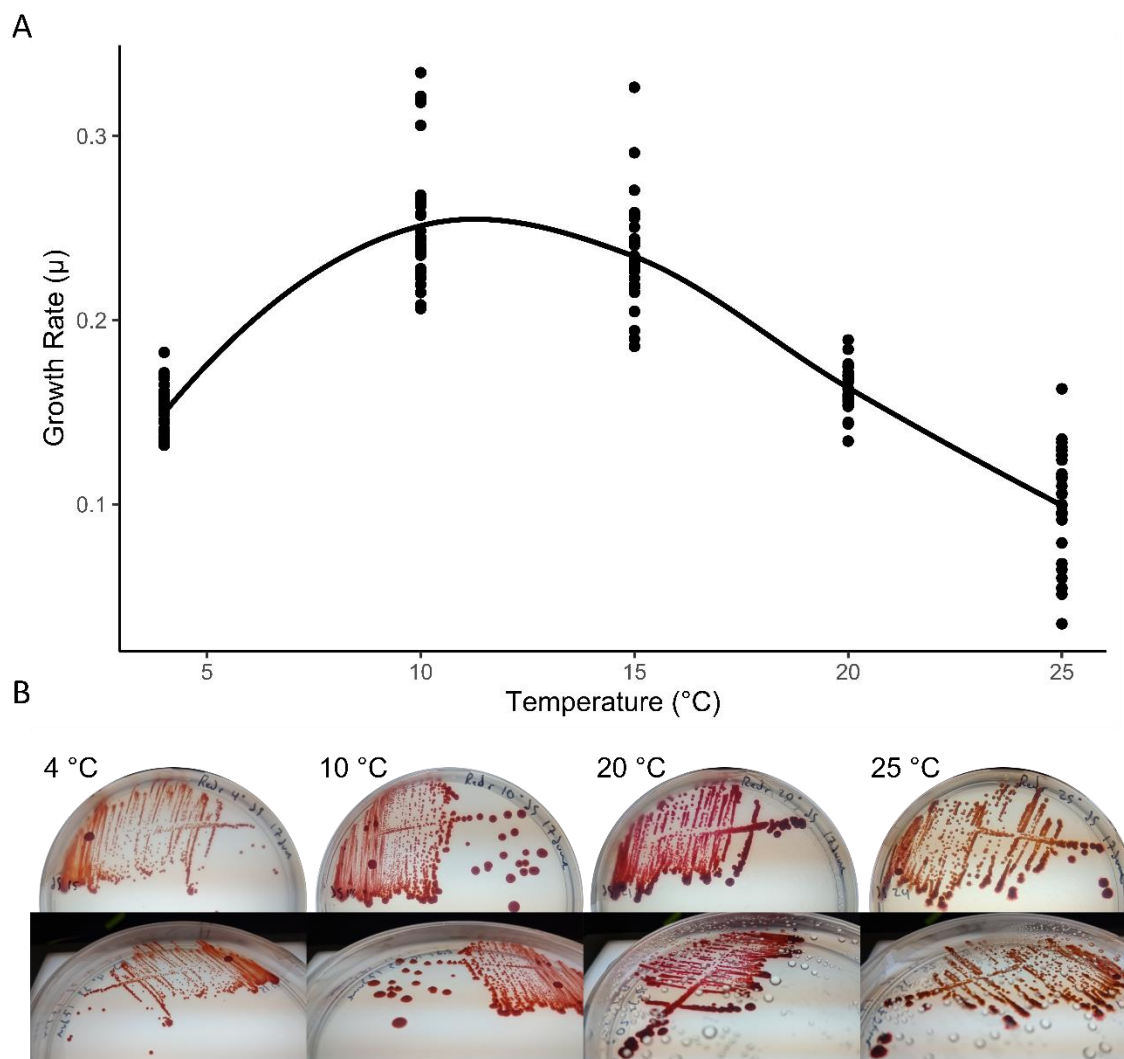


Figure 0.2 Effect of temperature on DJPM01. (A) Calculated growth rates for strain DJPM01 grown at temperatures 4, 10, 15, 20, and 25 °C. Growth curves were performed in a 96-well plate and measurements were taken once per day. Wells on the outside of the plate were excluded from final calculations (n=30). (B) Images of streak plates of strain DJPM01 incubated at 4, 10, 20, and 25 °C, respectively.

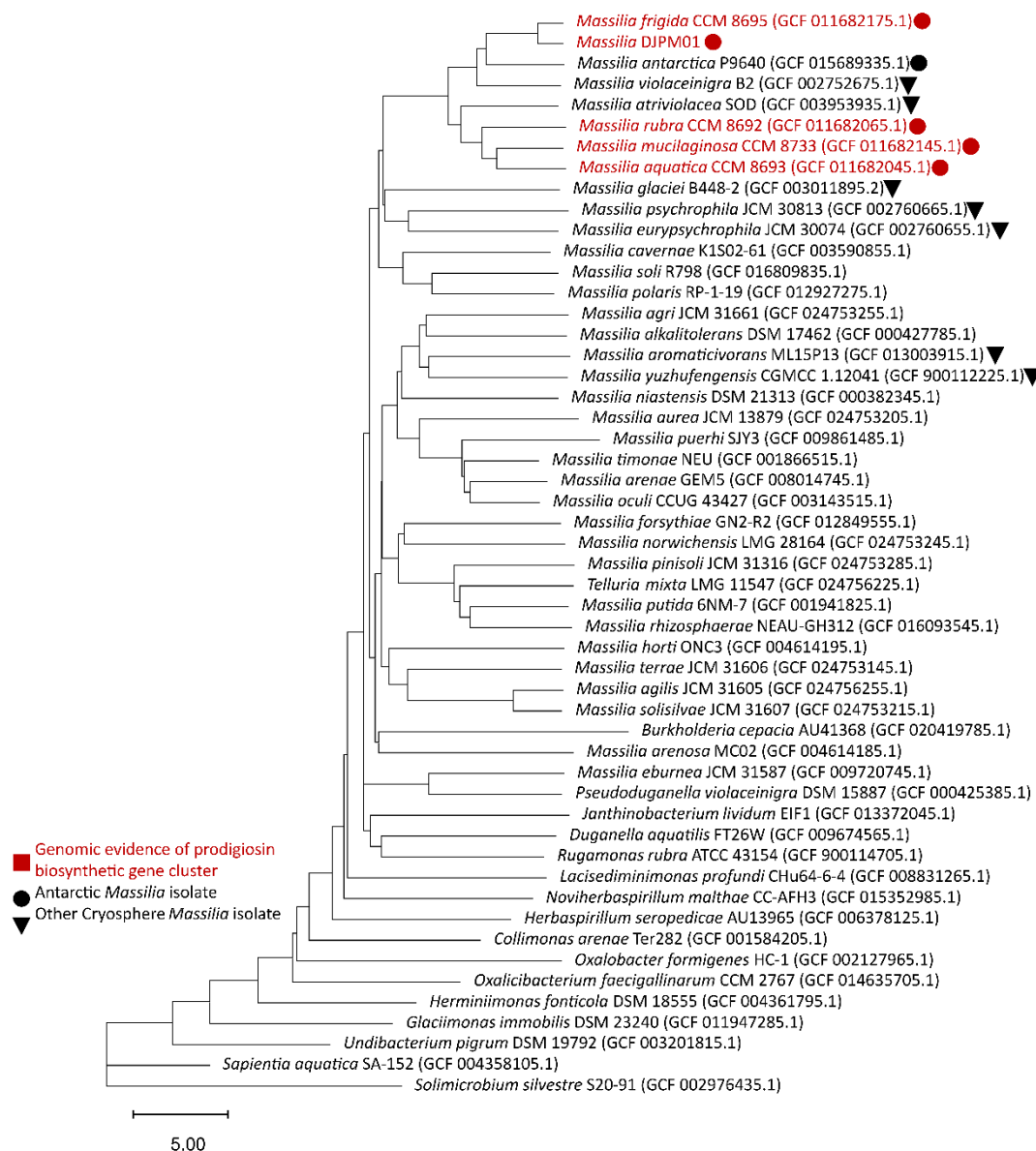


Figure 0.3 Maximum likelihood phylogenetic tree constructed using 16S rRNA genes from members of the Oxalobacteraceae family and strain DJPM01. Sequences from strains with phenotypic and genotypic evidence for prodigiosin production are indicated in red. Strain DJPM01 was highly similar to other Antarctic and cryosphere *Massilia* isolates. Bootstrap values >50 (9999 replicates) are indicated at nodes. An Antarctic *Shewanella* species (strain BF02_Schw) was used as an outgroup (DQ677870.1).

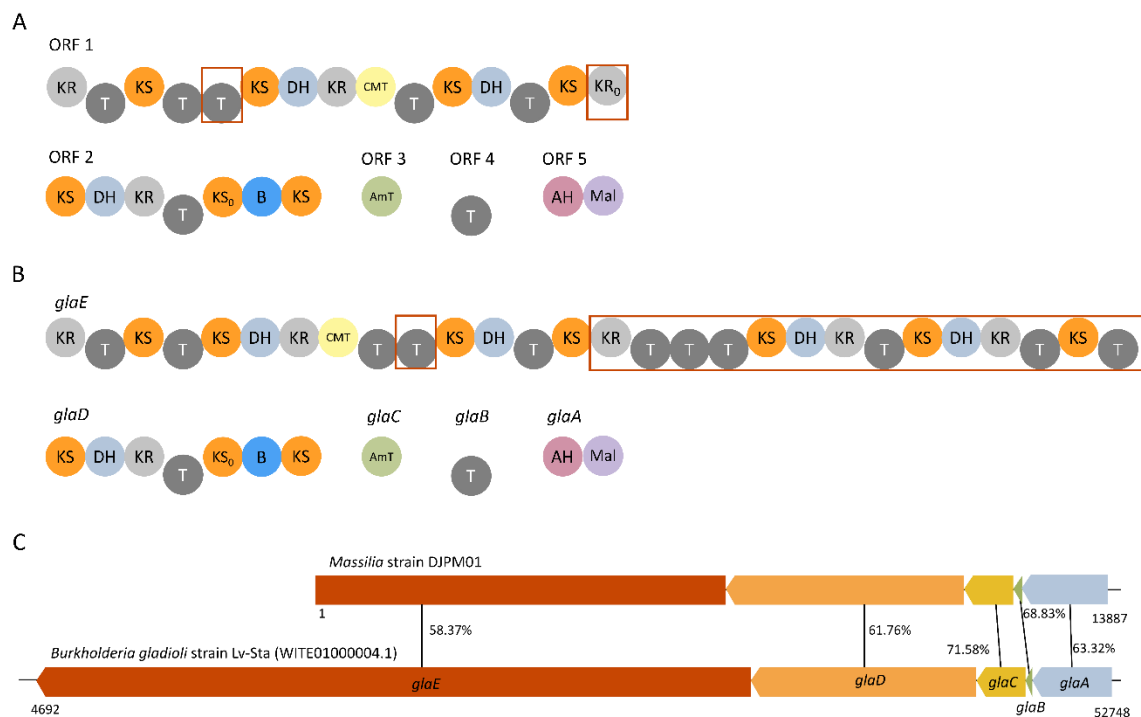
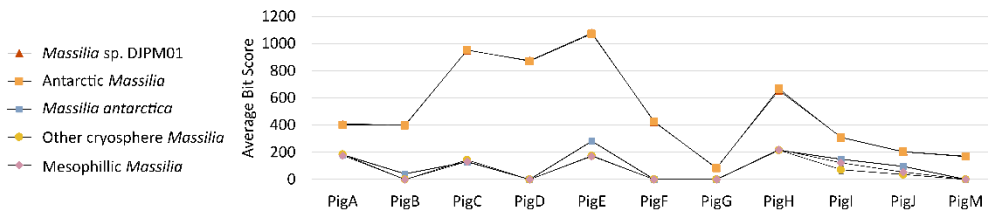


Figure 0.4 Proposed DJPM01 gladiofungin BGC. PRISM v4.4.5 (Skinnider et al., 2020) output for (A) putative iso-migrastatin BGC in strain DJPM01 genome and (B) gladiofungin BGC in *Burkholderia gladioli* strain Lv-Sta (WITE01000004.1), with missing PKS modules boxed in red. (C) Alignment of BGCs, highlighting similarity between individual genes. *AH* - acylhydrolase. *AmT* - aminotransferase. *B* - branching domain. *CMT* - C-methyltransferase. *DH* - dehydrotase. *KR* - ketoreductase. *KR0* - inactive ketoreductase. *KS* - ketosynthase. *Mal* - acyltransferase (malonyl-CoA). *T* - Thiolation.

A



B

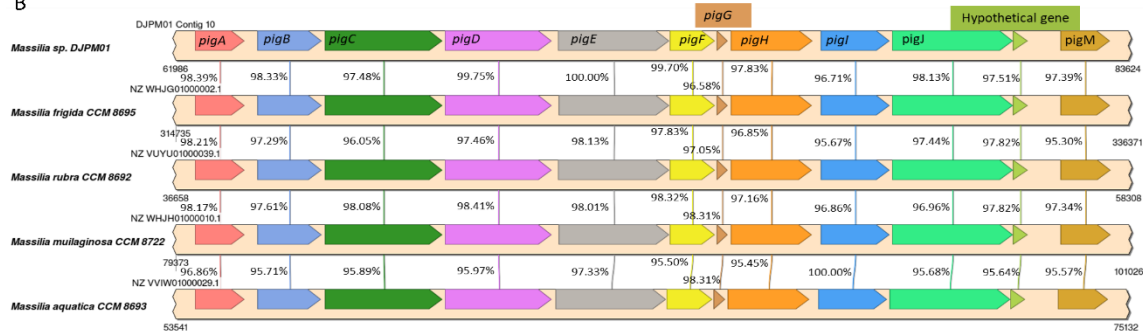


Figure 0.5 Prodigiosin gene comparisons between *Massilia* strains. (A) Summary of sequence comparisons between *Serratia* sp. strain ATCC 39006 and RefSeq *Massilia* genomes. Similarities are expressed in Average bit score for each gene per group of genomes. (B) Comparison of putative prodigiosin BGCs for Antarctic *Massilia* species. Lines between individual genes highlight percent identity between those two genes.

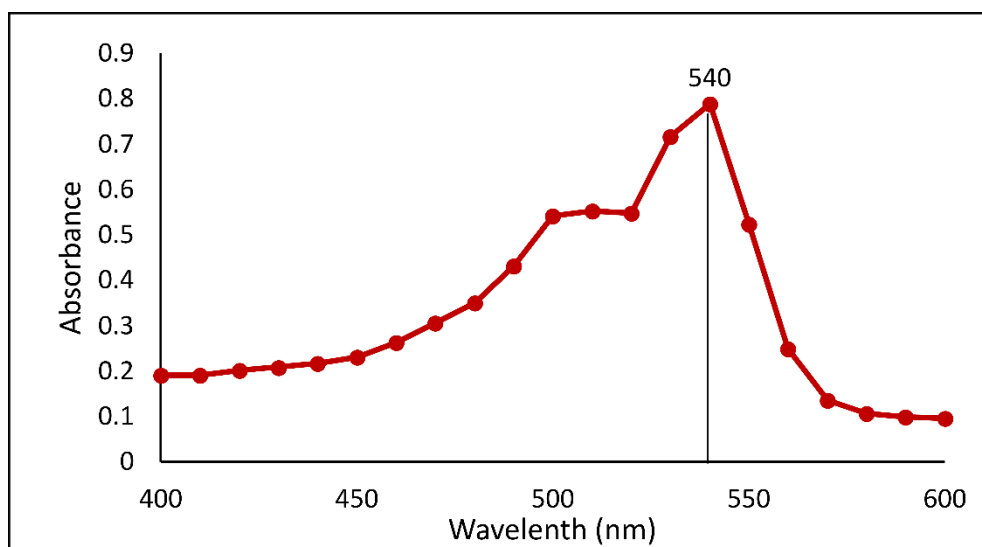


Figure 0.6 Absorbance spectrum of extracted pigment from colonies of strain DJPM01 in butanol. The absorbance maximum is at 540 nm, which corresponds to literature values for prodigiosin at acidic pH.

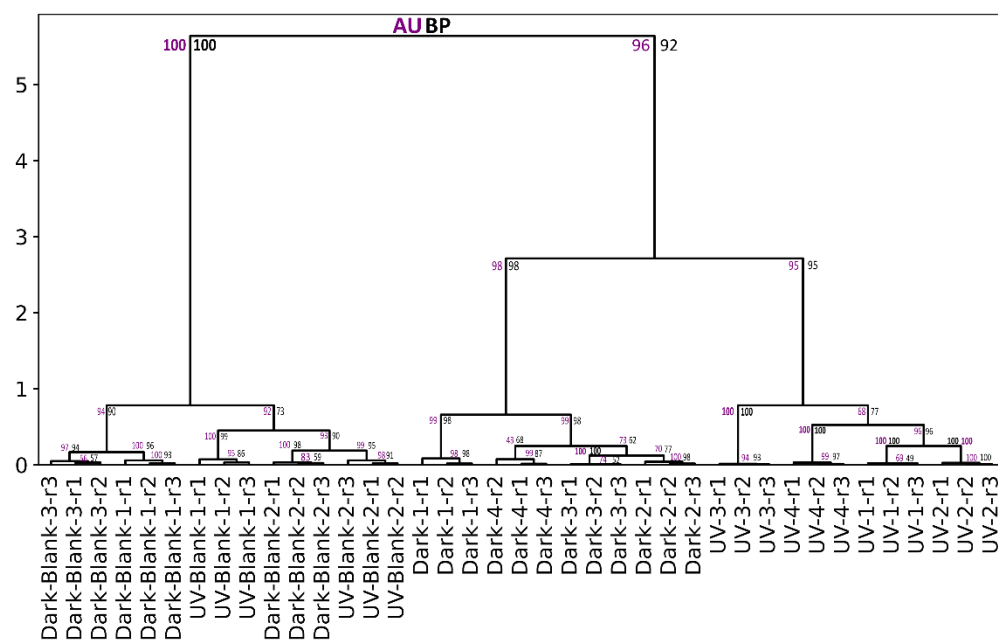


Figure 0.7. Statistically significant differences between blanks, controls, and UV-treated samples. Hierarchical clustering analysis of filtered LC/MS data demonstrating the statistically significant differences between samples (4 biological replicates injected in triplicate). Media blanks, UV-treated DJPM01 on R2A, and DJPM01 on R2A controls are indicated by black, red, and blue, respectively.

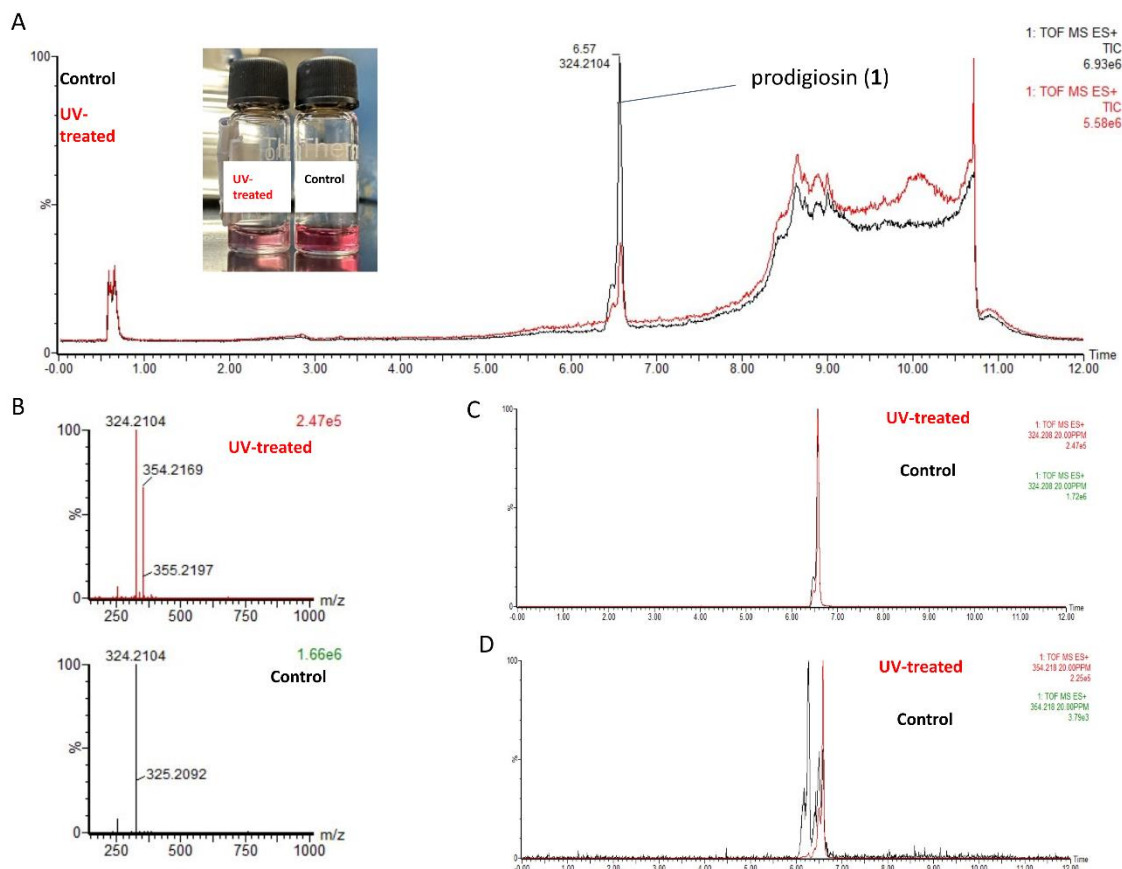


Figure 0.8 UV Photolysis of prodigiosin standard. (A) TIC chromatograms of prodigiosin standard (black) and UV-exposed prodigiosin standard (red) Prodigiosin is present at 6.57 minutes. Inset: Image of degraded standard (left) compared to control standard (right). (B) MS1 spectra at 6.57 minutes for UV-exposed (black) and unexposed (red) prodigiosin standards showing the appearance of a coeluting photolysis product with m/z 354.2169. (C) EIC for prodigiosin (324.208 m/z) in UV-exposed (black) and unexposed (red) prodigiosin standards showing decreased prodigiosin content. (D) EIC for a prodigiosin photolysis product featuring addition of CH₂O (324.208 m/z) in UV-exposed (black) and unexposed (red) prodigiosin standards showing an approximate 100-fold increase in abundance of the photolysis product with tR of 6.59 minutes.

Chapter Four Supplementary Materials

Methods and results for preliminary VOC experiments.

Methods. Preliminary headspace analyses were performed via PTR-MS as described in the main text. Two sets of incubations were analyzed: the first included Live, Dead, or No Cell treatments inoculated in Difco Marine Broth 2216 (BD) under an aerobic headspace flushed with ultra-pure air. The second utilized Live, Dead, and No Cell treatments and were performed in Difco Marine Broth 2216 (BD) supplemented with 100 mM Fh and incubated with an ultra-pure N₂ headspace.

Results. In preliminary Marine Broth incubations, 14 features were identified as significantly higher in aerobic Live treatments, and 5 features were significantly higher in anaerobic Live treatments. Anaerobic features included m/z 44.3, 46.25, 48.23, 49.21, 51.17, 62.95, 78.54, 80.5, 92.21, 94.18, 96.13, 109.72, 125.33, and 171.93. The feature with m/z 96.13 was putatively identified as bromomethane, 62.95 as DMS, 51.17 as chloromethane, and 49.21 as methanethiol (Fig. S15). Features significantly higher in anaerobic incubations include m/z 48.97, 50.97, 62.99, 78.93, and 94.95. Putative identities include DMS (m/z 62.99), chloromethane (m/z 50.97), and methanethiol (m/z 48.97).

Table 0.6. Mössbauer Parameters: 295K

295K	CS	QS	W	Area	CS	QS	W	Area	CS	QS	W	Area
	Fe³⁺				Fe³⁺				Fe²⁺			
Fh + lactate – No Cells	0.35	0.57	0.47	67	0.35	1.02	0.46	33				
Fh + lactate – Dead Cells	0.35	0.56	0.41	60	0.35	0.98	0.45	40				
Fh + lactate – Live Cells	0.35	0.55	0.55	67	0.35	1.04	0.5	33				
Fh + lactate + thiosulfate – No Cells	0.35	0.55	0.45	64	0.35	1	0.44	36				
Fh + lactate + thiosulfate – Dead Cells	0.35	0.58	0.45	69	0.35	1.01	0.44	32				
Fh + lactate + thiosulfate – Live Cells	0.35	0.52	0.41	47	0.35	0.92	0.53	48	1.31	2.02	0.58	5

Table 0.7. Mössbauer Parameters: 4K

4K	CS	QS	W	Field	Area	CS	QS	W	Field	Area	CS	QS	W	Field	Area
	Fe³⁺					Fe³⁺					Fe³⁺				
Fh + lactate – No Cells	0.44	-0.06	0.58	450	26	0.47	-0.07	0.75	480	46	0.47	-0.01	0.68	508	28
Fh + lactate – Dead Cells	0.44	-0.1	0.75	450	25	0.46	-0.06	0.93	482	48	0.47	-0.03	0.83	511	27
Fh + lactate – Live Cells	0.45	-0.08	0.55	463	51	0.48	-0.06	0.52	485	25	0.47	-0.02	0.55	509	24
Fh + lactate + thiosulfate – No Cells	0.44	-0.09	0.52	465	42	0.48	-0.09	0.67	491	40	0.46	-0.02	0.53	514	18
Fh + lactate + thiosulfate – Dead Cells	0.44	-0.1	0.71	456	27	0.47	-0.07	0.87	485	44	0.47	-0.04	0.78	512	29
Fh + lactate + thiosulfate – Live Cells	0.42	-0.02	0.44	476	45	0.5	-0.23	0.44	494	22	0.45	-0.05	0.52	512	24
Fh + lactate + thiosulfate – Live Cells <i>continued</i>	Fe^{2.x+}					0.80*	0.80*	1.00*	276*	6	1.30*	3.00*	0.90*		3

Table 0.8. Differential expression of transcripts of interest.

Pathway	Gene Name	Gene ID	log2 Fold Change	Padj
Iron reduction	<i>omcA</i>	KIMDJLCH_03019	4.79	> 0.001
	<i>mtrC</i>	KIMDJLCH_03020	4.87	> 0.001
	<i>mtrA</i>	KIMDJLCH_03021	5.79	> 0.001
	<i>mtrB</i>	KIMDJLCH_03022	5.02	> 0.001
	<i>cymA</i>	KIMDJLCH_03378	2.81	0.005
Heme biosynthesis	<i>gltX</i>	KIMDJLCH_03156	-0.19	1
	<i>hemA</i>	KIMDJLCH_00135	0.31	1
	<i>hemL</i>	KIMDJLCH_00267	0.83	1
	<i>hemB</i>	KIMDJLCH_02573	1.25	1
	<i>hemC</i>	KIMDJLCH_02603	2.40	0.003
	<i>hemD</i>	KIMDJLCH_02604	2.29	0.109
	<i>hemE</i>	KIMDJLCH_02557	2.77	0.001
	<i>hemN</i>	KIMDJLCH_01698	3.53	> 0.001
	<i>hemG_1</i>	KIMDJLCH_02180	3.04	> 0.001
	<i>hemG_2</i>	KIMDJLCH_04649	3.62	> 0.001
	<i>hemH</i>	KIMDJLCH_01944	1.09	1
Thiosulfate reduction	<i>psrB</i>	KIMDJLCH_01451	8.29	> 0.001
	<i>psrA</i>	KIMDJLCH_01452	7.97	> 0.001
	<i>psrC</i>	KIMDJLCH_01450	7.08	> 0.001
DMSO reduction	<i>dmsE</i>	KIMDJLCH_03495	3.58	> 0.001
	<i>dmsF</i>	KIMDJLCH_03496	2.89	> 0.001
	<i>dmsA</i>	KIMDJLCH_03497	3.56	> 0.001
	<i>dmsB</i>	KIMDJLCH_03498	3.64	> 0.001
	<i>dmsG</i>	KIMDJLCH_03499	1.85	0.133
	<i>dmsH</i>	KIMDJLCH_03500	1.55	1
Riboflavin biosynthesis	<i>ribA</i>	KIMDJLCH_02823	-0.24	1
	<i>ribBA</i>	KIMDJLCH_00406	-0.40	1

Table 6.8 (continued)

Pathway	Gene Name	Gene ID	log2 Fold Change	p_{adj}
	<i>ribD</i>	KIMDJLCH_00404	0.54	1
	<i>yigB</i>	KIMDJLCH_02595	0.36	1
	<i>ribC</i>	KIMDJLCH_01398	-0.83	1
	<i>ribE_1</i>	KIMDJLCH_00405	0.24	1
	<i>ribE_2</i>	KIMDJLCH_00407	-1.05	1
Halogenases	<i>rebH_1</i>	KIMDJLCH_00717	0.27	1
	<i>rebH_2</i>	KIMDJLCH_00722	0.82	1
	<i>rebH_3</i>	KIMDJLCH_04719	1.19	1
	<i>prnA</i>	KIMDJLCH_04457	1.17	1

**Table 0.9. Abundance and putative identities of VOC features differentially abundant between treatments.
Day 14**

<i>m/z</i>	Log(2) Fold Change	ANOVA (<i>p</i>)	Putative ID's (assigned by Progenesis MALDI)
41.03	128.78	1.47E-06	Cyclopropene (C ₃ H ₄) Propyne (C ₃ H ₄) Propa-1,2-diene (C ₃ H ₄)
42.43	64853.36	0.000321	Acetonitrile (CH ₃ CN)
45.26	169.45	<1.1E-16	Ethanal (CH ₃ CHO) Ethylene oxide (C ₂ H ₄ O) Propane (CH ₃ CH ₂ CH ₃)
53.72	19.23	3.17E-12	1-Oxoprop-2-ynyl (C ₃ HO) 2-Propenenitrile (C ₃ H ₃ N)
55.09	156.58	5.01E-09	1,3-Butadiene (C ₄ H ₆) 1,2-Butadiene (C ₄ H ₆) Cyclobutene (C ₄ H ₆) 1-Butyne (C ₄ H ₆) 2-Butyne (C ₄ H ₆) 1-Methylcyclopropene (C ₄ H ₆)
56.58	372.91	5.08E-12	1-Oxoprop-2-enyl (C ₃ H ₃ O) Propanenitrile (C ₃ H ₅ N) Vinylimine (C ₃ H ₅ N) Propargylamine (C ₃ H ₅ N) Cyclopropanimine (C ₃ H ₅ N)
59.4	89.86	1.63E-12	Acetone (CH ₃ COCH ₃) Ethanedial (C ₂ H ₂ O ₂) Propanal (CH ₃ CH ₂ CHO) Propen-2-ol (CH ₃ COHCH ₂) 2-Propen-1-ol (CH ₂ CHCH ₂ OH) Dimethyldiazene (C ₂ H ₆ N ₂)

Table 6.9 (continued)

<i>m/z</i>	Log(2) Fold Change	ANOVA (<i>p</i>)	Putative ID's (assigned by Progenesis MALDI)
62.25	2290.81	0.000421	Butane (C ₄ H ₁₀)
			Methylpropane (C ₄ H ₁₀)
			N-Methoxy-methanamine (C ₂ H ₇ NO)
			Monoethanolamine (C ₂ H ₇ NO)
			N-Hydroxy-N-methylmethanamine (C ₂ H ₇ NO)
76.69	47.08	6.99E-14	Thioformamide (CH ₃ NS)
			Acetohydroxamic acid (C ₂ H ₅ NO ₂)
			Glycine (C ₂ H ₅ NO ₂)
			Glycoamide (C ₂ H ₅ NO ₂)
			Methylcarbamate (C ₂ H ₅ NO ₂)
			Hydrazinecarboxamide (CH ₅ N ₃ O)
			2-Methoxyethanamine (C ₃ H ₉ NO)
			Trimethylamine N-oxide (C ₃ H ₉ NO)
			Dimethylaminomethanol (C ₃ H ₉ NO)
			3-Amino-1-propanol (C ₃ H ₉ NO)
			2-Amino-1-propanol (C ₃ H ₉ NO)
			1-Amino-2-propanol (C ₃ H ₉ NO)
			Ethanethioamide (C ₂ H ₅ NS)
79.55	199.51	1.09E-06	Benzene (C ₆ H ₆)
			Dimethylsulfoxide (CH ₃ SOCH ₃)
			2-Mercaptoethanol (CH ₂ OHCH ₂ SH)
82.44	77.05	3.10E-05	1-Methyl-(1H)-pyrrole (C ₅ H ₇ N)
			2-Methyl-(1H)-pyrrole (C ₅ H ₇ N)
			3-Methyl-(1H)-pyrrole (C ₅ H ₇ N)
			Methallyl cyanide (C ₅ H ₇ N)
94.12	17.85	0.000226	3-Methyl-pyridine (C ₅ H ₆ N ₂)
			3-Methyl-pyridine (C ₅ H ₆ N ₂)
			Phenylamine (C ₆ H ₇ N)
99.96	127.71	0.000911	Piperidinone (C ₅ H ₉ NO)
			1-Butylisocyanate (C ₅ H ₉ NO)

Table 6.9 (continued)

<i>m/z</i>	Log(2) Fold Change	ANOVA (<i>p</i>)	Putative ID's (assigned by Progenesis MALDI)
111.56	431.53	2.33E-08	Isothiocyantocyclopropane (C ₄ H ₅ NS)
			Allylthiocyantate (C ₄ H ₅ NS)
			5-Methyl-isothiozole (C ₄ H ₅ NS)
			1-(2-Furany)-ethanone (C ₆ H ₆ O ₂)
			2-Methyl-2-furancarboxaldehyde (C ₆ H ₆ O ₂)
			1-(1H-Pyrazol-4-yl)-ethanone (C ₅ H ₆ N ₂ O)
			2-Ethyl-5-methylfuran (C ₇ H ₁₀ O)
			2,3,5-Trimethylfuran (C ₇ H ₁₀ O)
114.49	1205.38	3.20E-08	3-Methyl-2-cyclohexen-1-one (C ₇ H ₁₀ O)
			1-Methyl-2-piperidinone (C ₆ H ₁₁ NO)
117.44	238.07	1.28E-07	4-Isothiocyantato-1-butene (C ₅ H ₇ NS)
			Nitrosomorpholine (C ₄ H ₈ H ₂ O ₂)
			Hexanoic acid (C ₆ H ₁₂ O ₂)
120.58	89.46	0.00015	5-Methyl-3-hexanol (C ₇ H ₁₆ O)
			Trichoromethane (CHC ₃)
123.35	34.44	2.22E-16	Benzoic acid (C ₇ H ₆ O ₂)
			2-Ethyl-phenol (C ₈ H ₈ O)
			4-Ethyl-phenol (C ₈ H ₈ O)
141.33	52.51	3.60E-05	2,2,6-Trimethylcyclohexanone (C ₉ H ₁₆ O)
			N-Acetyl-L-glutamic acid (C ₇ H ₁₁ NO ₅)
190.55	10.51	3.23E-08	a-Cyano-3-hydroxycinnamic acid (C ₁₀ H ₇ NO ₃)
			1H-Indole-3-acetic acid methyl ester (C ₁₁ H ₁₁ NO ₂)
			1H-Indole-3-propanoic acid (C ₁₁ H ₁₁ NO ₂)
			Tricyclazole (C ₉ H ₇ N ₃ S)
			Lesquerellin (C ₈ H ₁₅ NS ₂)

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Table 6.9 (Continued)

<i>m/z</i>	Log(2) Fold Change	ANOVA (<i>p</i>)	Putative ID's (assigned by Progenesis MALDI)
36.91	23.75	8.88E-16	
46.68	3.1	2.20E-09	Nitrosomethane (CH ₃ NO) Dimethylamine (CH ₃ NHCH ₃) Ethylamine (CH ₃ CH ₂ NH ₂)
48.09	5.91	<1.1E-16	N-Methyl-hydroxylamine (CH ₅ NO) O-Methyl-hydroxylamine (CH ₅ NO)
55.11	148.97	<1.1E-16	1,3-Butadiene (C ₄ H ₆) 1,2-Butadiene (C ₄ H ₆) Cyclobutene (C ₄ H ₆) 1-Butyne (C ₄ H ₆) 2-Butyne (C ₄ H ₆) 1-Methylcyclopropene (C ₄ H ₆) 1-Oxoprop-2-enyl (C ₃ H ₃ O) Propanenitrile (C ₃ H ₅ N) Vinylimine (C ₃ H ₅ N) Propargylamine (C ₃ H ₅ N) Cyclopropanimine (C ₃ H ₅ N)
56.55	433.61	<1.1E-16	Acetone (CH ₃ COCH ₃) Ethanedial (C ₂ H ₂ O ₂) Propanal (CH ₃ CH ₂ CHO) Propen-2-ol (CH ₃ COHCH ₂) 2-Propen-1-ol (CH ₂ CHCH ₂ OH) Dimethyldiazene (C ₂ H ₆ N ₂) Butane (C ₄ H ₁₀) Methylpropane (C ₄ H ₁₀)
59.39	183.57	<1.1E-16	
60.82	78.05	1.29E-09	
62.27	1345.47	<1.1E-16	N-Methoxy-methanamine (C ₂ H ₇ NO) Monoethanolamine (C ₂ H ₇ NO)

Table 6.9 (Continued)

<i>m/z</i>	Log(2) Fold Change	ANOVA (<i>p</i>)	Putative ID's (assigned by Progenesis MALDI)
			N-Hydroxy-N-methylmethanamine (C ₂ H ₇ NO)
			Thioformamide (CH ₃ NS)
63.71	9.08	4.44E-16	1,2-Ethanediol (C ₂ H ₆ O ₂)
			Dimethylsulfide (C ₂ H ₆ S)
			Ethanethiol (C ₂ H ₆ S)
65.13	31.58	1.11E-16	
			2-Hydroxy-propanenitrile (C ₃ H ₅ NO)
			3-Hydroxy-propanenitrile (C ₃ H ₅ NO)
			Isocyanatoethane (C ₃ H ₅ NO)
			Methoxyacetonitrile (C ₃ H ₅ NO)
72.22	1.31	1.19E-06	2-Propanamide (C ₃ H ₅ NO)
			Pyrrolidine (C ₄ H ₉ N)
			2-Methyl-2-propen-1-amine (C ₄ H ₉ N)
			N-Methyl-2-propen-1-amine (C ₄ H ₉ N)
			N-Methylallylamine (C ₄ H ₉ N)
			Acetohydroxamic acid (C ₂ H ₅ NO ₂)
			Glycine (C ₂ H ₅ NO ₂)
			Glycoamide (C ₂ H ₅ NO ₂)
			Methylcarbamate (C ₂ H ₅ NO ₂)
			Hydrazinecarboxamide (CH ₅ N ₃ O)
76.72	95.9	<1.1E-16	2-Methoxyethanamine (C ₃ H ₉ NO)
			Trimethylamine N-oxide (C ₃ H ₉ NO)
			Dimethylaminomethanol (C ₃ H ₉ NO)
			3-Amino-1-propanol (C ₃ H ₉ NO)
			2-Amino-1-propanol (C ₃ H ₉ NO)
			1-Amino-2-propanol (C ₃ H ₉ NO)
			Ethanethioamide (C ₂ H ₅ NS)
78.17	12.3	2.01E-11	
83.82	30.84	<1.1E-16	

Table 6.9 (Continued)

<i>m/z</i>	Log(2) Fold Change	ANOVA (<i>p</i>)	Putative ID's (assigned by Progenesis MALDI)
105.76	18.54	<1.1E-16	Diethanolamine (C ₄ H ₁₁ NS)
			2-Amino-1,3-butanediol (C ₄ H ₁₁ NS)
			N-(Methyl)mercaptoacetamide (C ₃ H ₇ NOS)
			DL- α -aminothiopropionic acid (C ₃ H ₇ NOS)
			b-Mercaptopropionamide (C ₃ H ₇ NOS)
			2-(Dimethylamino)-ethanethiol (C ₄ H ₁₁ NS)
111.61	210.58	<1.1E-16	1-(2-Furany)-ethanone (C ₆ H ₆ O ₂)
			2-Methyl-2-furancarboxaldehyde (C ₆ H ₆ O ₂)
			1-(1H-Pyrazol-4-yl)-ethanone (C ₅ H ₆ N ₂ O)
			2-Ethyl-5-methylfuran (C ₇ H ₁₀ O)
			2,3,5-Trimethylfuran (C ₇ H ₁₀ O)
			3-Methyl-2-cyclohexen-1-one (C ₇ H ₁₀ O)
114.55	338.54	<1.1E-16	1-Methyl-2-piperidinone (C ₆ H ₁₁ NO)
			4-Isothiocyanato-1-butene (C ₅ H ₇ NS)
117.49	75.17	1.11E-16	Nitrosomorpholine (C ₄ H ₈ H ₂ O ₂)
			Hexanoic acid (C ₆ H ₁₂ O ₂)
			5-Methyl-3-hexanol (C ₇ H ₁₆ O)

Table 0.10. Protein Data Bank (PDB) matches with structural similarity to predicted BF02_Schw PrnA (KIMDJLCH_04457) structure.

Protein description	Chain	Lali (# of equivalent residues)	# of residues in target structure	% Identity to PDB match
Putative Tryptophan Halogenase	6y1w-b	479	493	48
Putative Tryptophan Halogenase	6y1w-a	483	505	48
Tryptophan Halogenase Superfamily	6frl-a	483	491	46
Tryptophan Halogenase Superfamily	6frl-b	481	492	46
Tryptophan 5-Halogenase	2wet-c	476	494	33
Tryptophan 5-Halogenase	2wet-d	476	494	33
Tryptophan 5-Halogenase	2wet-a	476	496	33
Tryptophan 5-Halogenase	8fov-a	480	496	33
Tryptophan 5-Halogenase	8fov-b	482	500	33
Tryptophan 6-Halogenase	5hy5-b	473	490	33
Tryptophan 5-Halogenase	2wes-c	476	496	33
Tryptophan 5-Halogenase	2wes-b	476	496	33
Tryptophan 5-Halogenase	2wes-d	476	496	33
Tryptophan 5-Halogenase	2wes-a	476	496	33
Tryptophan 5-Halogenase	2wet-b	474	502	33
Tryptophan Halogenase	6nsd-b	474	498	33
Tryptophan Halogenase	6nsd-a	475	503	33
Flavin-Dependent Tryptophan Halogenase PrnA	4z43-a	481	505	34
Tryptophan 5-Halogenase	8fox-d	477	491	33
Tryptophan Halogenase PrnA	2ard-a	481	503	34
Tryptophan 5-Halogenase	8fox-a	470	488	33
Tryptophan 5-Halogenase	8fox-c	476	489	34
Tryptophan 5-Halogenase	8fox-b	474	495	33
Tryptophan 6-Halogenase	5hy5-a	476	510	33
Tryptophan 6-Halogenase	6sls-b	477	517	31
Tryptophan Halogenase PrnA	2jkc-a	482	517	34
Tryptophan 5-Halogenase	2weu-c	474	500	32
Tryptophan 5-Halogenase	2weu-a	467	495	33
Tryptophan Halogenase PrnA	2apg-a	479	517	34
Tryptophan 6-Halogenase	6sls-a	479	524	30
Tryptophan Halogenase PrnA	2aqj-a	481	518	34
Tryptophan 5-Halogenase	2weu-d	469	499	32
Tryptophan Halogenase PrnA	2ar8-a	479	518	34
Tryptophan 6-Halogenase	7cu2-b	480	520	31

Table 6.10 (continued)		Lali (# of equivalent residues)	# of residues in target structure	% Identity to PDB match
Protein description	Chain			
Flavin-Dependent Tryptophan Halogenase PrnA	4z44-a	481	516	34
Tryptophan 6-Halogenase	6slt-b	477	525	31
Tryptophan 5-Halogenase	2weu-b	471	500	32
Thermophilic Tryptophan Halogenase	5lv9-b	474	513	34
Thermophilic Tryptophan Halogenase	5lv9-a	467	509	34
Tryptophan 6-Halogenase	7cu1-a	476	535	30
Tryptophan 6-Halogenase ThaL	6h43-b	477	521	31
Flavin-Dependent Tryptophan Halogenase RebH	6p00-b	476	519	32
Tryptophan 6-Halogenase	7aqv-a	473	520	31
Flavin-Dependent Tryptophan Halogenase RebH	6p00-a	474	518	31
Tryptophan 6-Halogenase	8ad8-b	473	515	30
Tryptophan 6-Halogenase	7cu1-b	477	524	30
Tryptophan 6-Halogenase	7cu2-a	478	522	31
Flavin-Dependent Tryptophan Halogenase RebH	6p2v-b	474	521	32
Tryptophan 6-Halogenase	8ttk-d	474	517	32
Tryptophan 6-Halogenase	8ttk-b	472	519	32
Tryptophan Halogenase	2oal-b	477	528	32
Tryptophan 6-Halogenase	8tti-d	470	519	32
Tryptophan 6-Halogenase	8ttk-c	474	519	32
Tryptophan 6-Halogenase	6ib5-b	476	524	31
Flavin-Dependent Tryptophan Halogenase RebH	6p2v-a	476	523	31
Tryptophan Halogenase	2oam-b	476	524	32
Tryptophan 6-Halogenase	8ad8-a	478	524	29
Tryptophan 6-Halogenase	7aqu-b	477	527	31
Tryptophan 6-Halogenase	8ttk-a	472	521	32
Tryptophan 6-Halogenase	6h44-b	478	529	31
Tryptophan Halogenase	2o9z-a	474	524	31
Flavin-Dependent Tryptophan Halogenase RebH	7ju0-b	476	528	31
Tryptophan 6-Halogenase	8ad7-b	475	525	31
Tryptophan 6-Halogenase	8tti-c	471	520	32
Tryptophan Halogenase	2oa1-a	476	529	32
Tryptophan Halogenase	2oam-a	475	523	31
Tryptophan 6-Halogenase	6ib5-a	477	526	30

Table 6.10 (continued)		Lali (# of equivalent residues)	# of residues in target structure	% Identity to PDB match
Protein description	Chain			
Tryptophan 6-Halogenase	7aqv-b	477	530	30
Flavin-Dependent Tryptophan Halogenase RebH	7ju0-a	476	527	31
Tryptophan 6-Halogenase	8tti-a	472	522	32
Tryptophan Halogenase	2oa1-b	476	527	32
Tryptophan Halogenase	2e4g-b	477	529	32
Tryptophan Halogenase	2o9z-b	475	526	31
Tryptophan 6-Halogenase	8ttj-d	472	521	32
Tryptophan 6-Halogenase	6h44-a	476	529	30
Tryptophan 6-Halogenase ThaL	6h43-a	476	528	31
Tryptophan 6-Halogenase	6ul2-a	477	527	32
Tryptophan Halogenase	2oal-a	476	527	31
Flavin-Dependent Tryptophan Halogenase RebH	4lu6-b	476	527	32
Tryptophan 6-Halogenase	8ttj-c	473	522	32
Tryptophan Halogenase	2pyx-b	476	525	32
Tryptophan Halogenase	2e4g-a	477	529	31
Tryptophan 6-Halogenase	8ttj-a	472	523	32
Tryptophan 6-Halogenase	6ul2-d	468	517	32
Tryptophan 6-Halogenase	8ttj-b	472	523	32
Tryptophan Halogenase	2pyx-a	476	526	32
Flavin-Dependent Tryptophan Halogenase RebH	4lu6-a	476	527	32
Tryptophan 6-Halogenase	6ul2-b	471	521	32
Tryptophan 6-Halogenase	7aqu-a	476	530	31
Tryptophan 6-Halogenase	8tti-b	472	522	32
Tryptophan 6-Halogenase	7cu0-b	475	531	30
Tryptophan 6-Halogenase	8ad7-a	477	529	30
Tryptophan 6-Halogenase	7cu0-a	475	532	30
Tryptophan 6-Halogenase	6slt-a	477	529	30
Tryptophan 6-Halogenase	6ul2-c	471	520	32
Tryptophane-5-Halogenase	5uao-c	459	514	25
Tryptophane-5-Halogenase	5uao-d	461	514	25
Tryptophane-5-Halogenase	5uao-a	460	519	25
VirX1	6qgm-c	474	522	28
Tryptophane-5-Halogenase	5uao-b	459	516	25

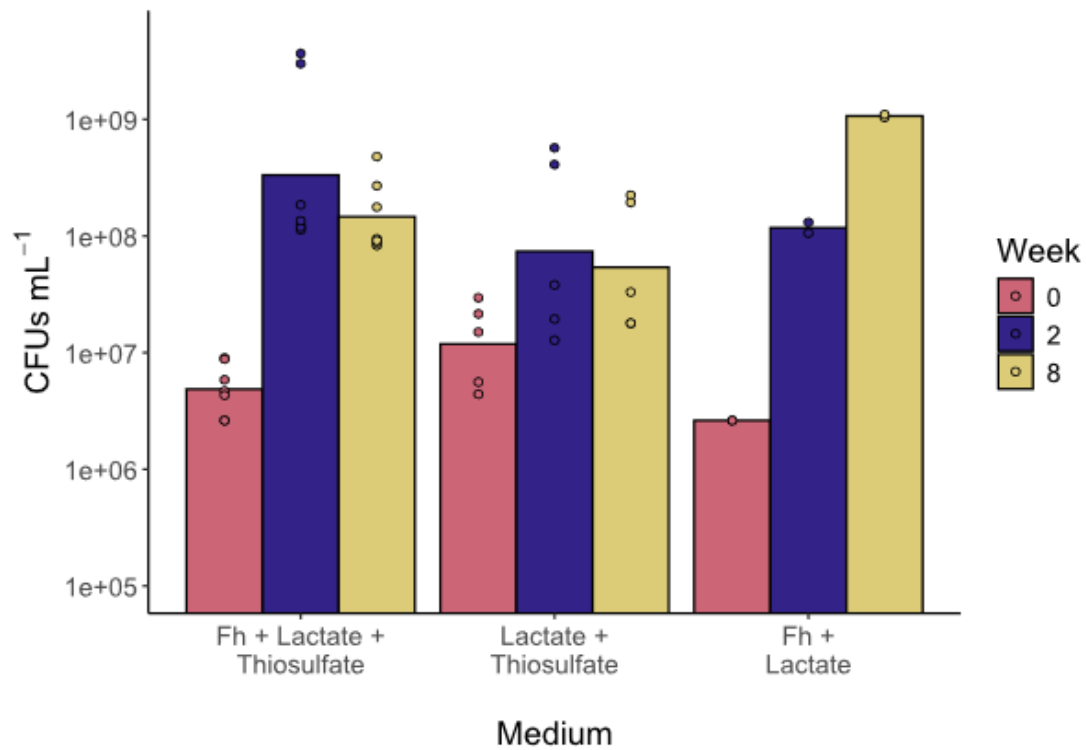


Figure 0.9. Increase in CFUs following two and eight weeks of incubation for all experiments.

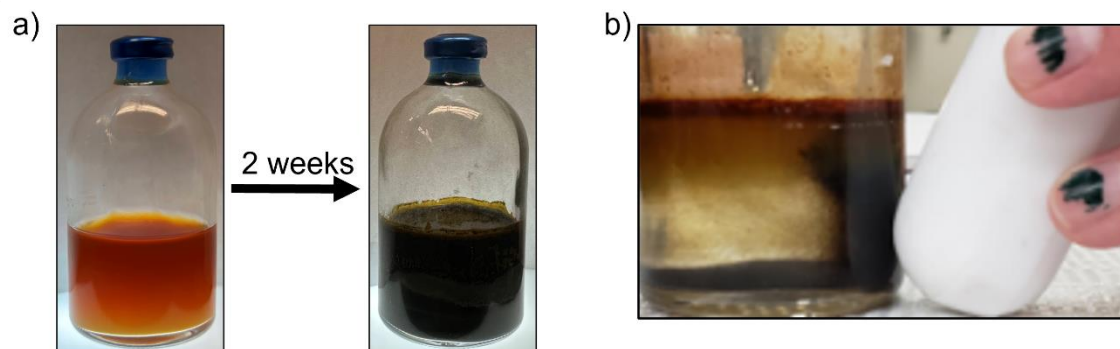


Figure 0.10. Visual transformation of insoluble in Fe following 2 weeks of incubation in Live +Fh incubations.

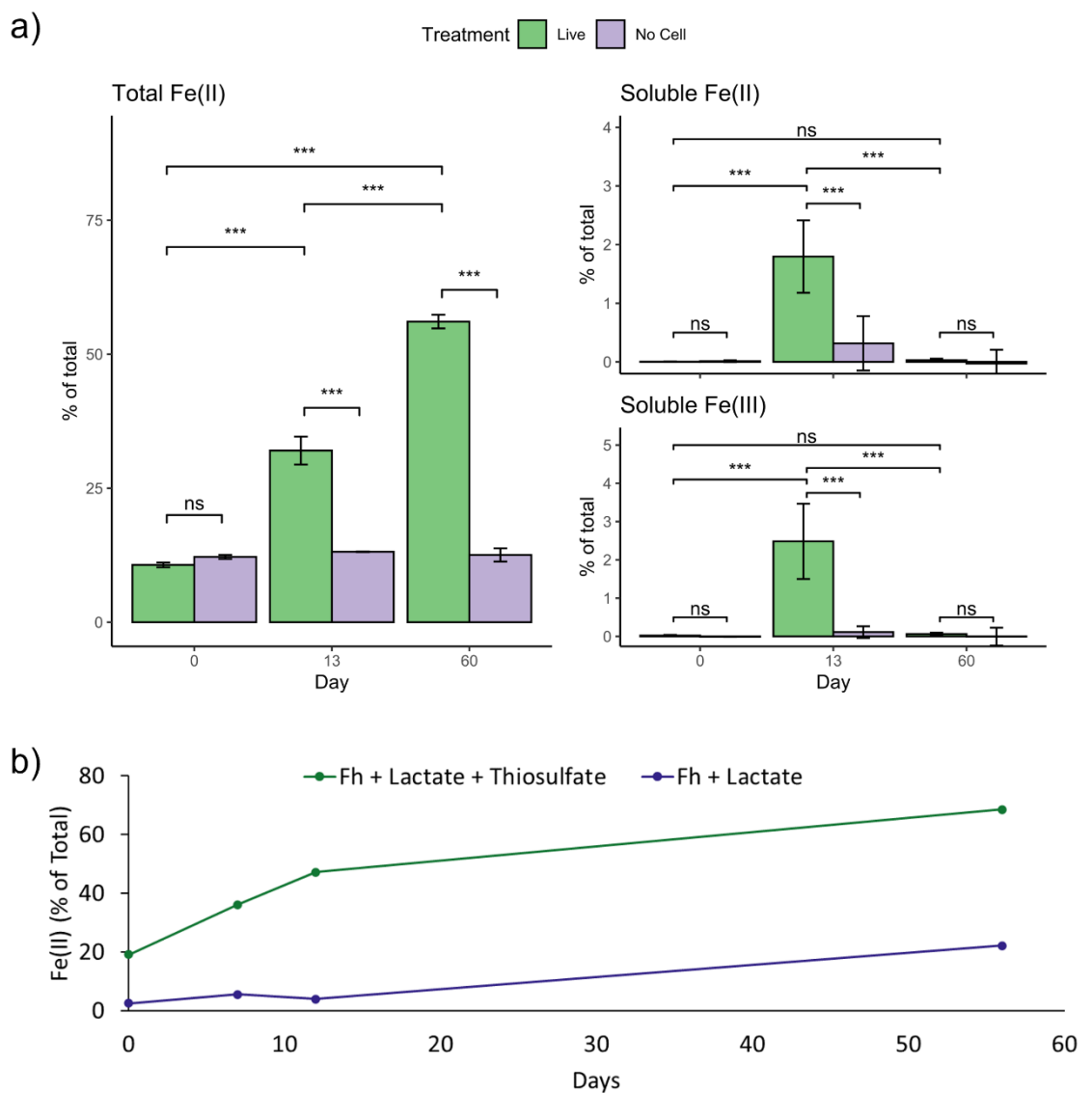


Figure 0.11. Reduction of Fe(III) to Fe(II) as evidenced by FerroZine™ colorimetric assay. All values are normalized to total Fe. (a) Fe(III) reduction in microcosms utilized for RNA-Seq. Total Fe(II) increased in Live microcosms over the course of incubation (t-test, *** = $p < 0.001$, ns = not significant). Soluble Fe(II) and Fe(III) increased only on Day 13. (b) Fe(III) reduction in Live mineral microcosms. Treatments containing Fh + lactate + thiosulfate had greater Fe(III) reduction than those without thiosulfate. ns – not significant

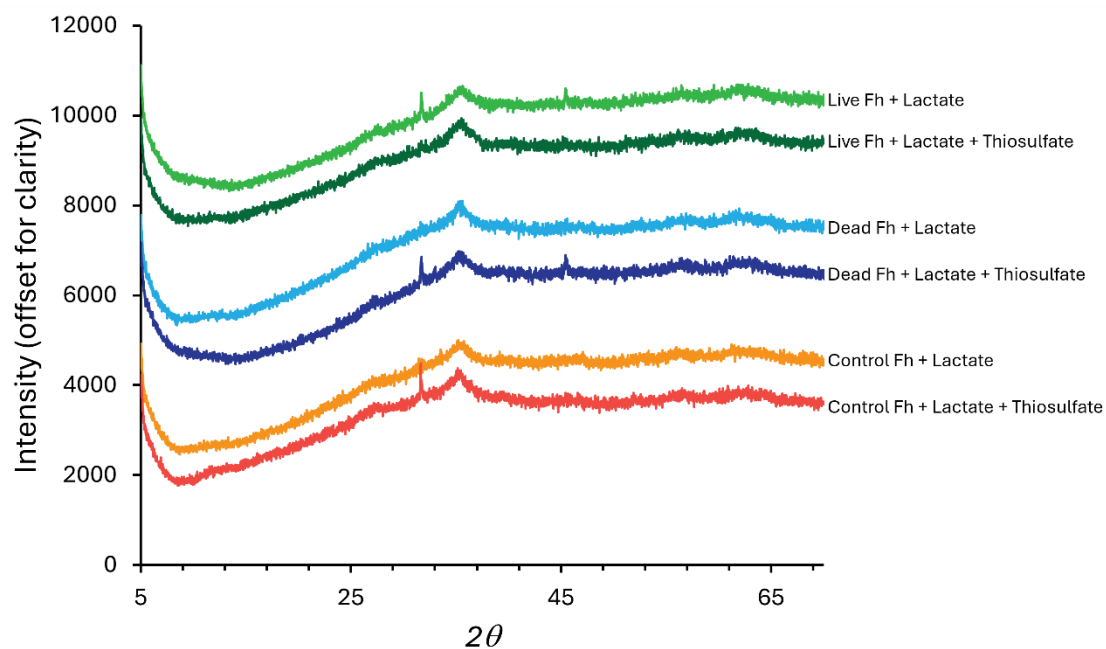


Figure 0.12 XRD spectra collected for all treatments.

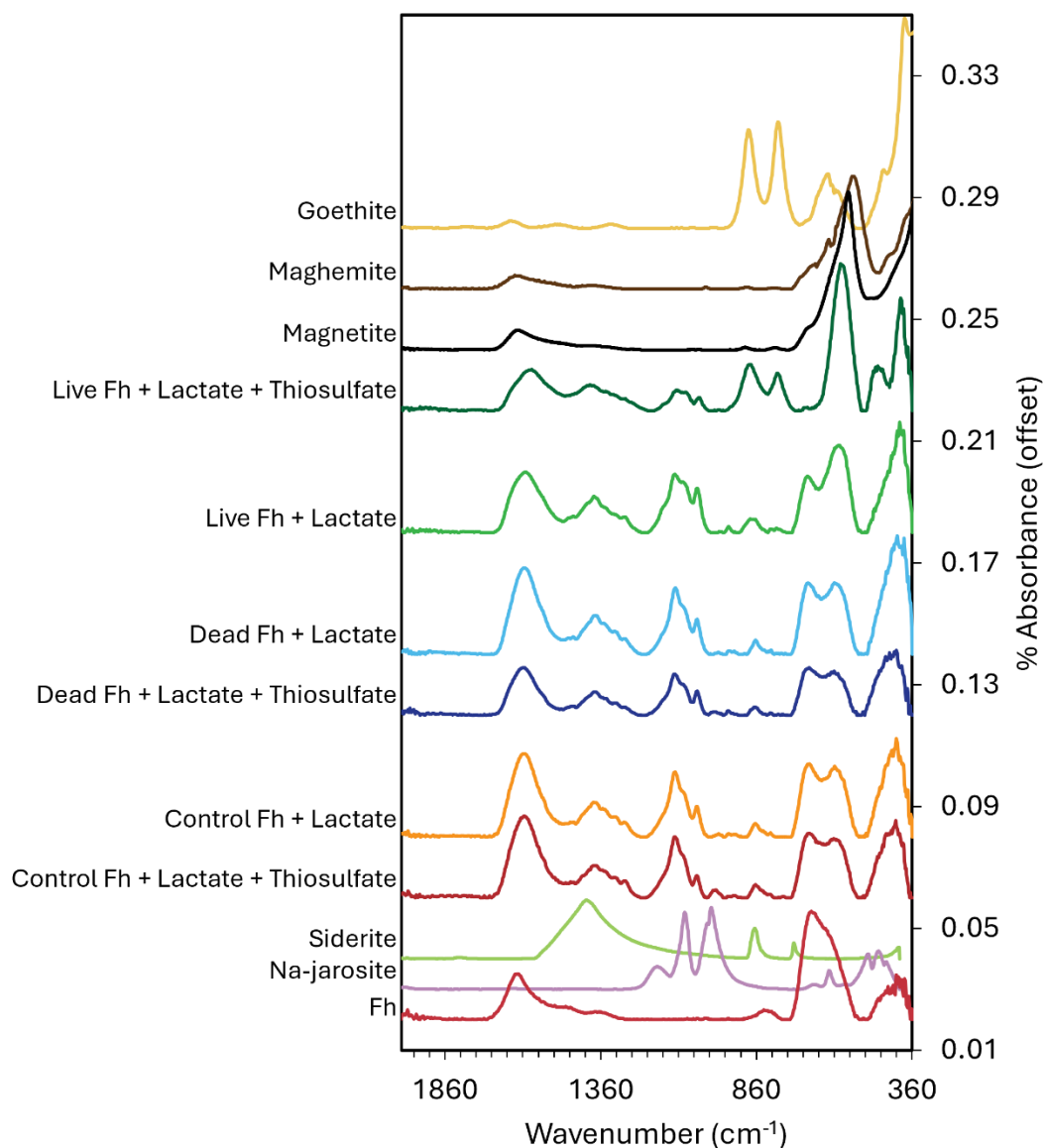


Figure 0.13. FTIR spectra of eight-week reaction products and controls for experiments with Fh + Lactate and Fh + Lactate + Thiosulfate. One replicate of each condition is shown except for the Live cell FLT experiment, where the products differed spectrally. Spectra of siderite from the USGS specli 7 (Kokaly et al., 2017) and for several nanophase Fe oxides spectra from Sklute et al. (Sklute et al., 2018) are shown for reference.

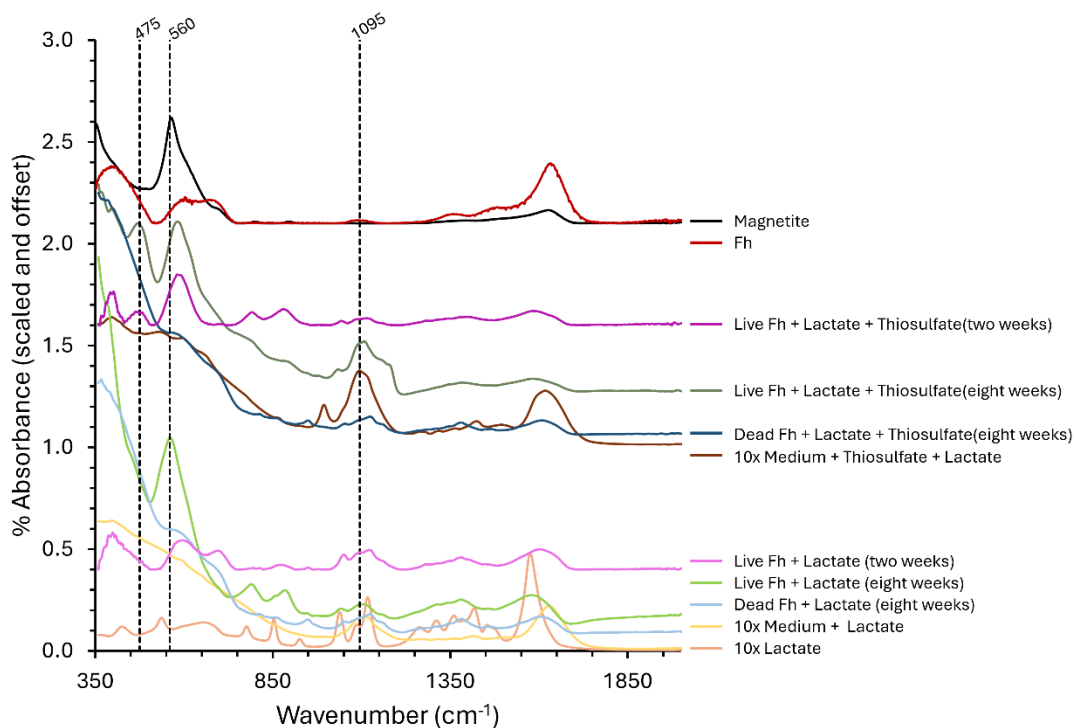


Figure 0.14. FTIR spectra of two- and eight-week Live treatments, eight-week Dead treatments, and 10x concentrated marine minimal medium with lactate, thiosulfate, or lactate + thiosulfate (no Fh), in addition to 10x concentrated lactate or thiosulfate. Plot is grouped by experimental condition and scaled and offset for clarity. Magnetite and Fh are shown in red for reference. Note only reference oxides and two-week incubations have been baseline removed.

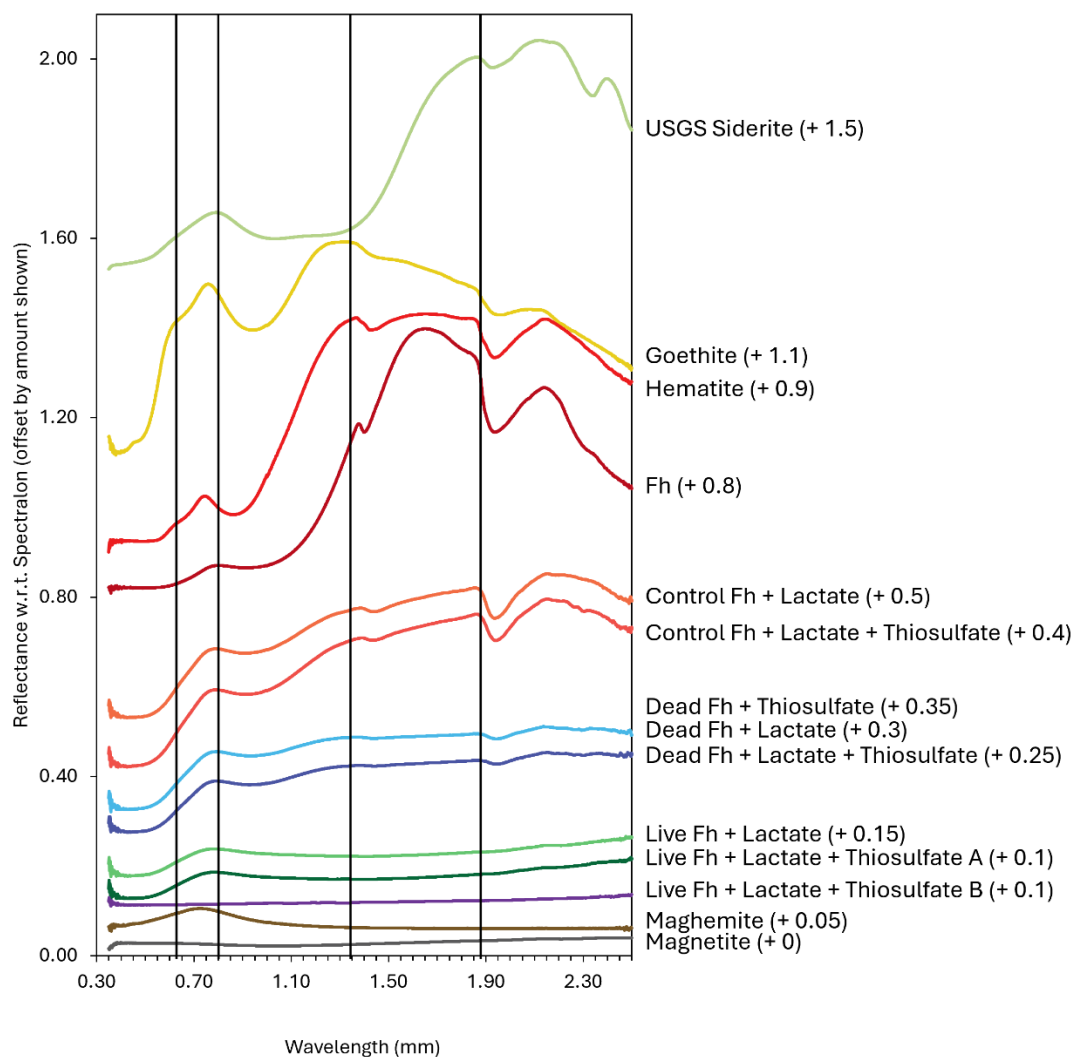


Figure 0.15. VNIR spectra of two-week incubations and controls for experiments with Fh + lactate and Fh + lactate + thiosulfate. One replicate of each condition is shown except for the Live Cell Fh + lactate + thiosulfate experiment, where the products differed spectrally. Spectra of siderite from the USGS specli 7 (Kokaly et al., 2017) and for several nanophase Fe oxides spectra from Sklute et al. (Sklute et al., 2018) are shown for reference.

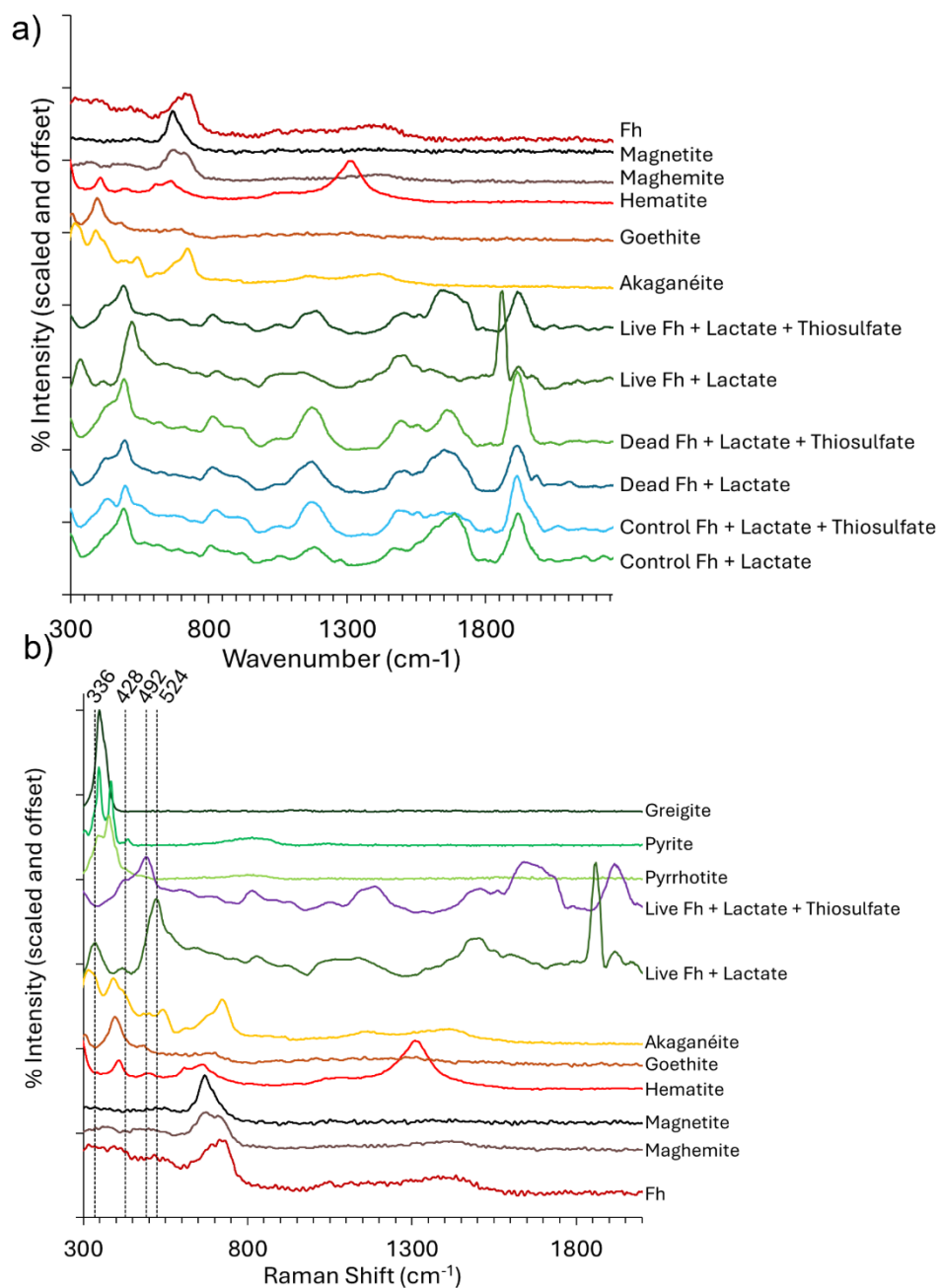


Figure 0.16. Raman spectra of Fh treatments. (a) Raman spectra acquired from mineral treatments compared to oxides from Sklute et al. (Sklute et al., 2018). (b) The Live Cell Fh + lactate treatment showed some spectral changes on one analysis. That spectrum, along with Live Cells Fh + lactate + thiosulfate are shown compared to (hydr)oxides from Sklute et al. (Sklute et al., 2018) and RRUFF sulfide samples pyrite R050190, pyrrhotite R606440, and greigite R120103 from the RRUFF database (Lafuente et al., 2015).

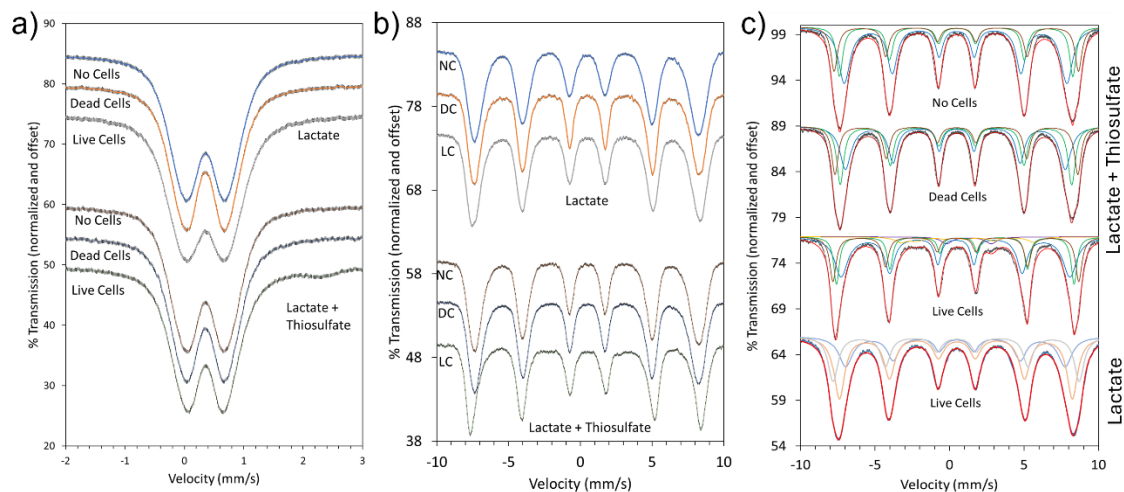


Figure 0.17. Mössbauer spectra acquired at (a) 295K and (b) 4K. (c) Fits of Mössbauer spectra at 4K. All treatments contain Fh.

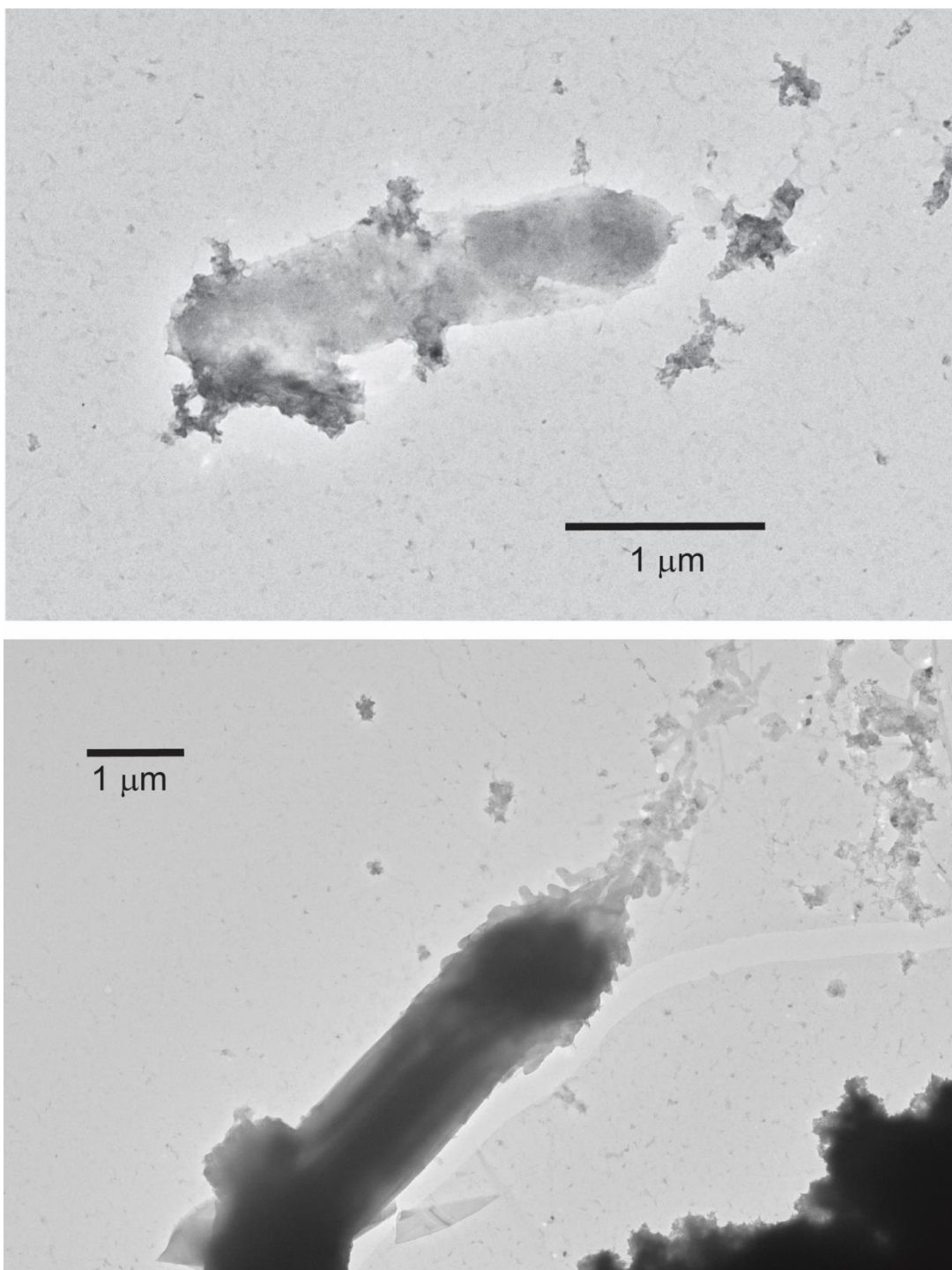


Figure 0.18. TEM images of a BF02_Schw cell associated with Fe oxides.

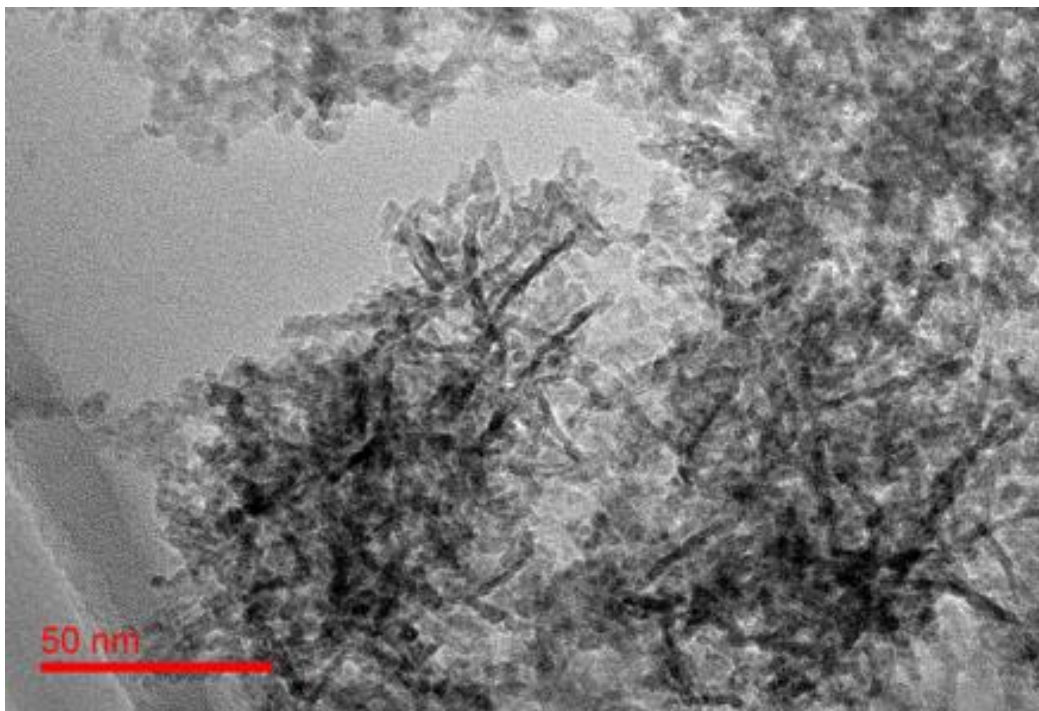


Figure 0.19. TEM image of akaganéite laths visible with Fh in Fh + lactate treatments following two-week incubations.

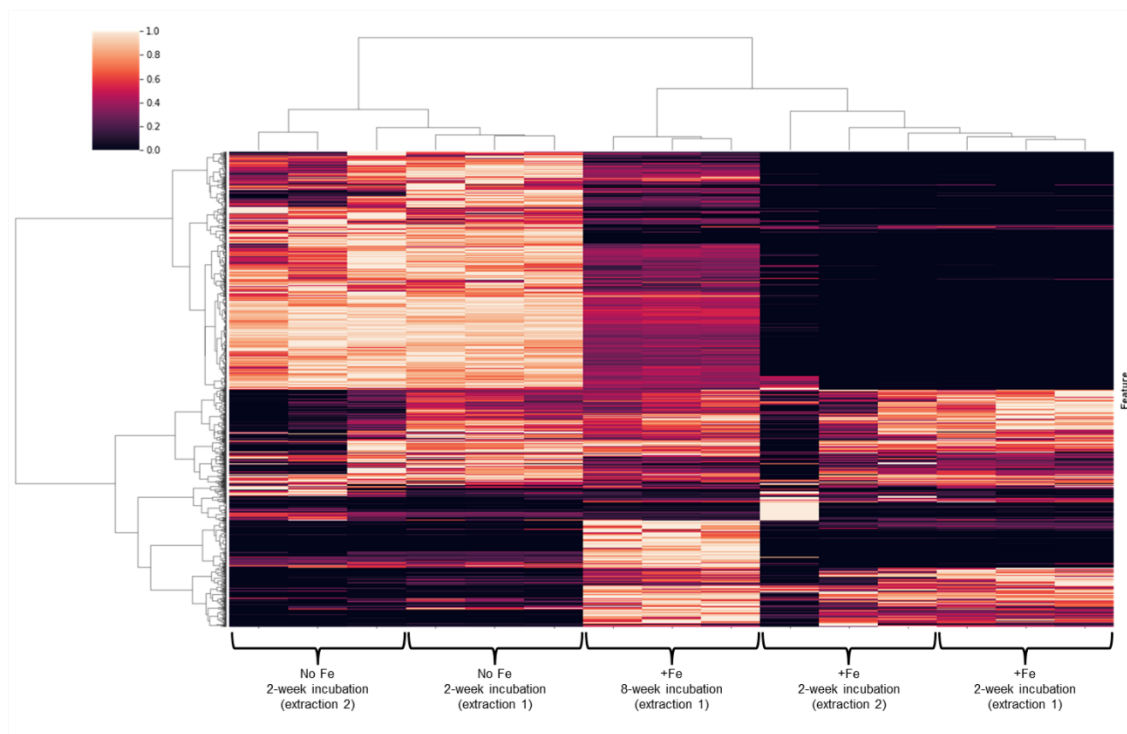


Figure 0.21. Hierarchical clustering analysis of normalized molecular feature abundance. Supernatant from No Fh treatments cluster separately from both two- and eight-week +Fh supernatants.

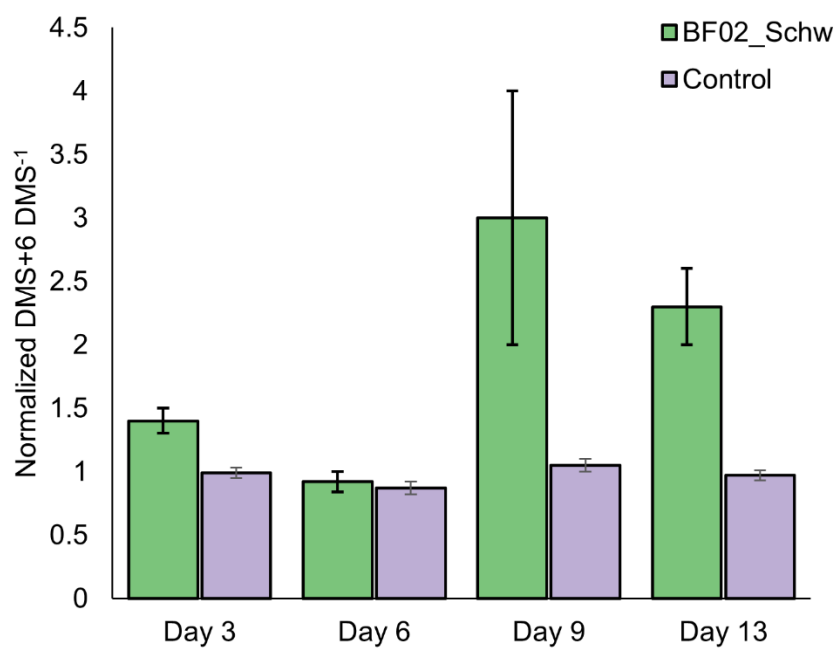


Figure 0.22. Reduction of D₆-DMSO by BF02_Schw and production of D₆-DMS (M + 6) measured by MIMS.

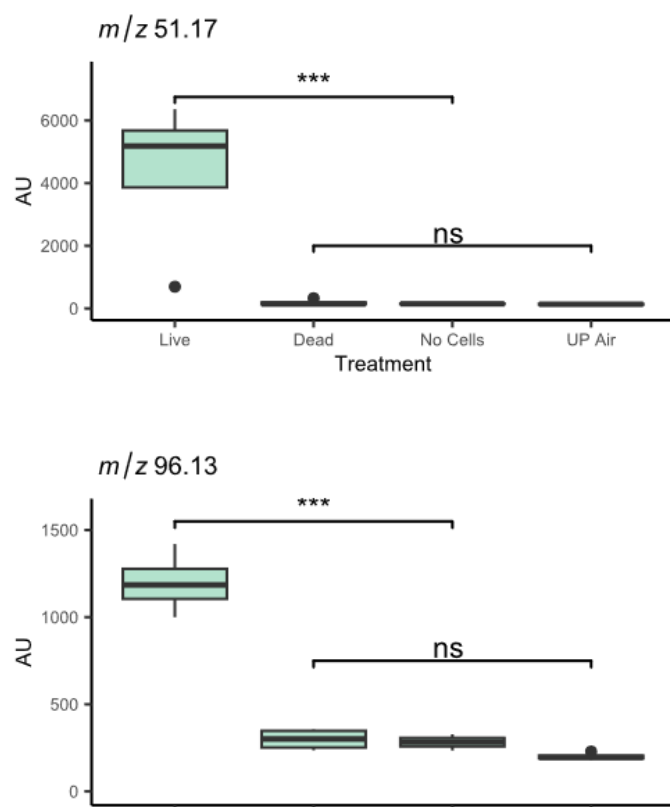


Figure 0.23. Putative halogenated compounds. Raw abundance of putative chloromethane (m/z 15.17) and bromomethane (m/z 96.13) features in headspace of aerobic incubations. *ns* – not significant.



Figure 0.24. Sequence alignment of putative BF02_Schw halogenases against characterized tryptophan halogenases. Conserved FAD-binding site and tryptophan residues are annotated in red. *Xanthomonas campestris* strain B100 putative tryptophan halogenase 6Y1W (Xcc4156) sourced from RCSB PDB. Sequences for *Streptomyces rugosporus* NRRL 21084 tryptophan 5-halogenase TRP5H_STRRG (A4D0H5), *S. toxytricini* NRRL 15443 tryptophan 6-halogenase TRP6H_STRT5 (E9P162), and *Pseudomonas fluorescens* B1915 tryptophan 7-halogenase TRP7H_PSEFL (P95480) were sourced from UniProt.

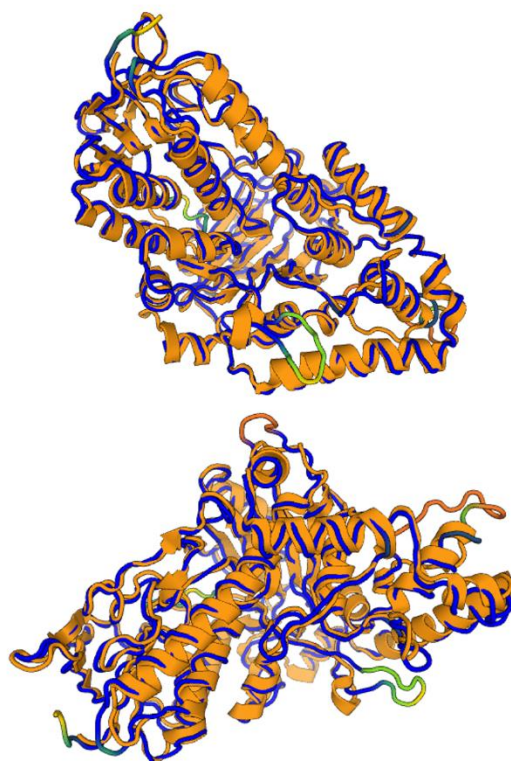


Figure 0.25. Predicted structure of BF02_Schw PrnA aligned with flavin-dependent halogenase from *Xanthomonas campestris* (PDB Chain 6Y1W-B; orange). Coloration of PrnA is based on structural conservation, ranging from blue (high) to red (low).

VITA

Jacob Shaffer spent most of his early life running around the woods and scabland of eastern Washington, constantly asking questions like “why?” and “how come?” when looking at the world around him. Thanks to his father, an amateur astronomer, these questions soon began to be directed towards scientists, engineers, and educators across a variety of fields (but mostly space). While the topic of interest changed over the years (astronomy to geology to entomology to pathology), the curiosity and questioning never really stopped. Thanks to Spokane Public Schools, Jacob took two introductory biology classes in high school that inspired enough of an interest to pursue a bachelor’s degree in it over the Cascades at the University of Puget Sound. Thanks to a tardigrade-patterned phone case and a connection through the UPS School of Music, he learned that the Biology Department had research labs, and thus the “whys?” and “how comes?” were directed towards microorganisms. After some adventures with tardigrades, nematodes, and hydrogen sulfide, Jacob decided to ask such questions professionally, and discovered that they really, *really* like inspiring students to ask “why?” and “how come?” in both the classroom and the laboratory. So again, Jacob moved back over the Cascades, over the Rockies, and down to the Smokies to earn a PhD in the University of Tennessee Department of Microbiology, where, despite (or maybe because of?) the heat, they fell in love with the cold.