Geomicrobiology of Terrestrial Subsurface Fluids

and Potential Applications in Biotechnology

BY

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THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Earth and Environmental Sciences
in the Graduate College of the
University of Illinois at Chicago, 2017

Chicago, Illinois

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ACKNOWLEDGEMENTS

I would like to thank my thesis committee, consisting of Dr. D'Arcy Meyer-Dombard, Dr. Fabien Kenig, Dr. Fred A. Rainey, Dr. Kathryn L. Nagy, and Dr. Max Berkelhammer, for guiding me through my academic career and providing positive encouragement through the many setbacks experienced in research. Special thanks to Professor Meyer-Dombard for her patient guidance and for largely funding my research expenses. Completion of this degree would not have been possible without the unwavering love and support of my friends and family.

I am also grateful to many friends and colleagues that assisted in sample collection and data analysis. Caitlin Boblitt, M.S. for indispensable aid in constructing figures for this manuscript; Dr. Caloy Arcilla at The University of the Philippines, Diliman for coordinating sample collection in the Philippines; Lionel Mojekwu, M.S. at Argonne National Laboratory for ICP analyses; Dr. Dawn Cardace at University of Rhode Island for field assistance and geochemical analyses of environmental samples; Dr. Everett Shock for geochemical data collected over the years from field sites in Yellowstone National Park; Dr. Kristin Woycheese and Caitlin Casar, M.S. for laboratory aid, field support, productive academic discussions, and moral support.

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SUMMARY

The purpose of this study was to culture bacteria from two distinct extreme ecosystems, and to assess their physiological and biochemical characteristics as they pertain to biotechnology. Specifically, the focus was on applications in bioremediation/biometallurgy and degradation of cellulose. Two dissimilar field sites chosen for this study included alkaline serpentinizing environments in the Philippines and acidic hydrothermal environments in Yellowstone National Park. The unifying theme of both sites is that fluids undergo extensive interactions with the subsurface, resulting in unique respective geochemistry and microbial communities.

The first investigation involves microbe-metal interactions, with a focus on isolating and identifying highly metal-tolerant bacteria, and determining the maximum extent of their metal tolerance. Implications of studying these bacteria and their associated mechanisms range from applications in bioremediation to extracting metal from ores, a process known as biomining. Tolerance to five metals, Cr, Cu, Ni, Co, and Zn, was determined for 20 isolated strains. Findings indicate that most metal-tolerant isolates from both environments are *Bacillus* species. Furthermore, many organisms from both types of environment can tolerate extremely high concentrations (>200 mg/L) of toxic heavy metals. In addition, several were observed to transform or otherwise remove metals from solution. Most of the isolates appear to use efflux pumps to regulate internal metal concentrations, while some are capable of intracellular accumulation of at least one metal.

The second investigation focuses on the ability of microorganisms from these same environments to degrade cellulose, the most abundant organic compound on Earth. Cellulose degradation at extreme pH and temperatures has implications in the conversion of this abundant renewable resource to biofuels and other useful chemicals like acetic acid and glucose. Results demonstrate that in both alkaline and acidic conditions, most cellulolytic organisms belong to the phylum *Firmicutes*, specifically the families *Clostridiaceae* and *Bacillaceae.* The results may support targeted culturing efforts for cellulolytic bacteria that are active at a specific pH range. Additionally, a cellulolytic consortium of *Thermoanaerobacterium* and *Caldicellulosiruptor* was observed to efficiently degrade paper substrate at low pH (~4.0).

I. Introduction

1.1 Microbiology of the subsurface and implications in biotechnology

The study of life in the deep subsurface has far-reaching implications ranging from elucidating the origin of life on Earth to assessing the mechanisms by which life may exist elsewhere in the universe. Despite the profundity of these implications, the concept of life existing in the deep subsurface has only gained traction in recent decades. For much of scientific history, the subsurface was believed to be a hostile environment exhibiting geochemical and physical extremes rendering it completely inhospitable to life. In the 1930s, subsurface microbiology emerged in the field of petroleum geology when sulfate-reducing bacteria were isolated from water flowing from an oil well (Bastin 1926); however, sampling and analytical techniques at the time, as well as misinformed assumptions about distribution of life on Earth, cast doubt on the validity of the findings. It was not until the end of the 20^{th} century that technological advancements allowed for convincing, accurate analysis and interpretation of subsurface ecosystems. Since then, numerous studies have demonstrated that microbial life can exist in the deep underground (Fredrickson et al. 1991; Fredrickson and Onstott 1996; Whitman et al. 1998; Colwell and D'Hondt 2013), and that it exists in great quantities despite the harsh, inhospitable conditions. Studies of microbial biomass in subseafloor sediments estimate that there are 2.9×10^{29} total cells, corresponding to 0.6% of Earth's total biomass (Kallmeyer et al. 2012). Organisms adapted to living in subsurface habitats are capable of coping with extremes in temperature, pressure, nutrient availability, pH, metal toxicity, salinity, and radiation (Pikuta et al. 2007). The mechanisms by which bacteria overcome such extremes are not only complex and varied, but often unique when compared with eukaryotes. Studying these microorganisms and their unique functions presents enormous potential for applications in biotechnology. Bacteria and archaea possess billions of years of adaptations to virtually every type of environment on the planet, making them some of Earth's most specialized inhabitants. The enzymes and

associated mechanisms that subsurface microbes have developed to tolerate and alter their surroundings may be exploited for human interests. Subsurface ecosystems therefore present largely untapped potential for discovering novel solutions to some of the most critical environmental and economic issues such as energy production, waste management, pollution remediation, and manufacturing of valuable resources. To find sustainable, low-cost solutions to these issues, it is necessary to learn from Earth's most ancient creatures that have been undertaking such endeavors for far longer than humans.

Subsurface habitats can be broadly divided into endolithic and aqueous varieties, though the two may coincide in the case of groundwater ecosystems (Amy et al. 1992; Hahn 2009). The present study will focus on aqueous subsurface habitats, specifically terrestrial springs. Terrestrial springs allow for easy sampling of subsurface microorganisms, similar to the way in which mines and drill cores serve as simple, low-cost sampling methods of endolithic subsurface habitats. Fluids in terrestrial springs may often be altered by geochemical interactions with the local bedrock, and in some cases, may be heated by a geothermal source. These changes in geochemistry affect the microbial communities inhabiting these fluids, resulting in diverse consortia that are highly adapted to the unique conditions of the subsurface.

1.2 Terrestrial hydrothermal springs

Hydrothermal environments can broadly be divided into terrestrial and marine systems. While both types may share a unifying theme in a mantle-driven heat source, other geochemical parameters exhibit significant variation. Among these, the fluid source is likely the critical differentiator, as terrestrial hydrothermal environments receive primarily meteoric input, while marine systems involve recirculating seawater. For the purposes of this study, the focus will be on terrestrial hydrothermal springs as a proxy for subsurface fluids. Yellowstone National Park, WY, USA exhibits a unique diversity of terrestrial hydrothermal systems that have been extensively studied since the late 1800s. Thermal springs in Yellowstone can generally be categorized into two main classes: vapor-dominated systems characterized

by acidity and high concentrations of sulfur, and hot water dominated systems characterized by alkalinity, carbonates, and chlorides (Fournier 1989).

Gases emanating from vapor-dominated systems are sourced from underlying magmatic material as shallow as 5 – 10 km from the surface, and typically consist of H_2S, CO_2, and/or NH_3 (Fournier 1989). Upon reaching the surface, these gases may be expressed as fumaroles, boiling pools of sulfuric acid, and mud pots, all of which harbor specialized communities of microorganisms. Bacteria and archaea in such environments not only tolerate the harsh conditions, but actively participate in altering the geochemistry through metabolic processes, especially via oxidation of available reduced gases like H_2S. Oxidation of sulfides results in the production of sulfuric acid, attributing to the extreme acidity in these features. The Greater Obsidian Pool Area in Yellowstone is an example of a primarily vapor-dominated, acid-sulfate system. Previous studies based in this area have described a diverse group of microorganisms including *Aquificales*, *Thermodesulfobacteriales*, *Thermocrinis*, and *Crenarchaea* (Meyer-Dombard et al. 2005; Spear et al. 2005).

Hot water-dominated systems involve a greater degree of water-rock interactions and are typically neutral to slightly alkaline in pH, but can also exhibit acid-sulfate pools like those seen in vapor-dominated systems. The more well-known features of Yellowstone such as its many geysers and multi-colored springs are characterized as hot water-dominated systems. These waters may be elevated in trace metals derived from water-rock interactions, and are rich in chlorides and SiO_2 that may be deposited as siliceous sinter. In Mammoth Hot Springs and Calcite Springs in the northern area of the park, fluid interactions with underlying limestone and dolostone leach calcium which is then deposited as large travertine terraces at the surface. In many instances, water-dominated systems may interact with gas-dominated systems and result in springs with geochemical characteristics of both systems.

1.3 Serpentinization and Serpentinizing Environments

Serpentinizing systems are currently under scientific scrutiny for their potential in providing insight into origin of life on Earth, exobiology, and global carbon cycling. Serpentinization is a low-temperature exothermic reaction involving the hydrologic alteration of ultramafic mantle rocks containing olivine and pyroxene to produce serpentine (Eq. 1). Resulting fluids from this reaction are reduced, extremely depleted in dissolved inorganic carbon, and are highly alkaline with typical pH values above 10 (Schrenk et al. 2013; Cardace et al. 2015); major byproducts of the serpentinization reaction include iron oxides, hydrogen, methane (Eq. 2), and small concentrations of organic molecules, all of which are excellent sources of chemosynthetic energy for microorganisms.

Eq. 1 $(Fe,Mg)_2SiO_4 + H_2O_{(l)} \rightarrow (Fe,Mg)_3Si_2O_5(OH)_4 + (Mg,Fe)(OH)_2 + Fe_3O_4 + H_{2(g)}$

Eq. 2 $4H_{2(g)} + CO_{2(g)} \rightarrow CH_{4(g)} + 2H_2O$

Studies indicate that serpentinizing ecosystems are not only able to support life, but may have facilitated the genesis of life billions of years ago in Earth's primordial oceans (Sleep et al. 2004; Schulte et al. 2006; Martin et al. 2008; Russell et al. 2010; Schrenk et al. 2013). This process may theoretically occur anywhere that ultramafic material contacts liquid water, even on extraterrestrial bodies. The detection of methane in the Martian atmosphere, as well as serpentine minerals in numerous locations, indicates that serpentinization was actively occurring at some point in Mars' history (Ehlmann et al. 2010), and is likely still active in the subsurface considering the presence of transient liquid water and ultramafic material in the Martian crust. As such, serpentinizing systems are the newest and most promising of the many potential habitats that have been identified in the Martian subsurface (Sleep et al. 2004; Schulte et al. 2006; Martin et al. 2008; Russell et al. 2010; Schrenk et al. 2013). Organisms inhabiting serpentinizing environments are capable of withstanding extreme pH, surviving on very little nutrients, and display a

variety of metabolisms including methanotrophy, methanogenesis, hydrogenotrophy, and iron cycling (Schrenk et al. 2013; Cardace et al. 2015).

Examples of common environments where serpentinization occurs include mid-ocean ridges, subduction zones, and terrestrial ophiolites (Schrenk et al. 2013). While the study of deep sea serpentinization in off-axis environments is useful and necessary, accessing locations and sample collection is extremely difficult and expensive. Terrestrial ophiolites provide a cost-effective way to study these deep subsurface processes at the surface. Ophiolites are sections of oceanic crust and upper mantle that have been emplaced onto land, typically at continental margins, as a result of tectonic processes like subduction, extension, or plume activity (Schrenk et al. 2013). Oftentimes, these terrestrial ophiolites are actively serpentinizing, and can be sampled via fluid seeps at the surface. Thus, ophiolites serve as convenient analogs to study subsurface processes and microbiology. Ophiolite complexes can be found in several locations around the world including the Western US, the Philippines, Oman, Turkey, and Canada.

Serpentinizing seeps on terrestrial ophiolites present a unique transitional habitat for microorganisms in which reduced, nutrient-depleted fluids from the deep subsurface come into contact with the surface environment. Although it is believed that the fluids exiting these springs were initially meteoric in origin (Schrenk et al. 2013), they emerge with an entirely different chemistry reflective of the process of serpentinization. It is a reasonable assumption that microorganisms percolating down through sediments with meteoric fluid have been isolated from the surface for an extended period, and thus have evolved to adapt to subsurface conditions. It is currently unknown how long it takes for meteoric water to cycle through these systems and re-emerge at the surface, but it may be assumed that it varies depending on hydrological characteristics of specific locales. At the surface, microbes in these fluids must cope with a number of geochemical and physical changes that occur along rapid gradients. For example, temperatures associated with serpentinization in the subsurface may reach upwards of 60 °C, while surface temperatures are significantly cooler. Nutrient-poor fluid from the subsurface system is suddenly

overwhelmed with a large surficial input of plants, insects, and other organic matter; furthermore, a major change that could affect metabolism and growth of many organisms is exposure to a comparatively oxygen-rich atmosphere. Recent work by Woycheese et al. (Woycheese et al. 2015) indicates that transposon activity in bacteria increases with distance down the outflow channel of a serpentinizing seep, suggesting that microorganisms undergo rapid genetic changes in response to this transitional environment over the course of a few meters. These genetic changes may potentially be encoding for new metabolisms or other coping mechanisms to survive conditions of the exposed surface. Thus, this transitional environment hosts a plethora of yet undiscovered and poorly understood microbial processes.

1.4 Geomicrobiology and potential applications in biotechnology

Earth's most extreme environments harbor some of the most functionally unique microorganisms. They have adapted over time to not only survive in those environments, but alter and utilize the harsh conditions to their advantage. Many of these microbial adaptations can be exploited for use in various industries and applications. In fact, people have benefited from targeted use of microorganisms for thousands of years, especially in medicine and the food industry. Ancient people discovered the utility of microorganisms and microbial enzymes in fermentation of dairy products, bread, and alcohol. Biologically sourced antibiotics found in various natural products and soils have been used medicinally throughout human history without any knowledge of the underlying mechanism. Eventually, the accidental discovery of Penicillin by Alexander Fleming in 1928 sparked increased interest in the study of environmental microbiology in relation to the production of natural antibiotics; today, the majority of antibiotics used in medicine are produced by a single genus of bacteria known as *Streptomyces* (Watve et al. 2001; de Lima Procópio et al. 2012), a common soil-dwelling organism. Through significant advances in science and technology in the last hundred years, the potential applications of microorganisms have now grown far beyond food and medicine.

A primary advantage to using microorganisms in industrial and environmental applications is that biological waste products are typically far less destructive to the environment than those produced from anthropogenic manufacturing practices. This is not always the case, as evidenced by phenomena like biocorrosion and acid mine drainage in which microbial processes may escalate anthropogenically initiated processes. Generally, however, natural microbial processes may be used to deal with environmental contaminants or production of valuable resources in a manner that is both environmentally friendly and less expensive than alternatives. As such, biotechnology is becoming increasingly necessary to address many current environmental concerns such as pollution, hazardous waste management, and discovering alternatives to fossil fuels.

A currently popular area of research involves the microbially-mediated conversion of lignocellulosic biomass to biofuels and other chemicals. Net primary production of biomass in terrestrial and marine ecosystems is estimated to be approximately 113 Gt/year, and about half of all terrestrial biomass is in the form of cellulose (Schwarz 2001). This means that cellulose is the most abundant organic compound and source of renewable natural energy available on Earth. While cellulose is not easily degraded, numerous microorganisms have developed the ability to metabolize this substrate, producing ethanol and other sugars as byproducts. Biofuel research focuses on discovering new enzymes with better efficiency and new strategies for conversion of cellulose into biofuel.

Another popular field of study is microbial tolerance to and transformation of metals. Studying interactions between microorganisms and metals has a range of applications from bioremediation of contaminated sites to bioleaching of valuable metals from ores. Metals such as Cr, Cu, Ni, Pb, and Hg derived from both natural and anthropogenic sources pose a severe threat to both human health and the environment. Anthropogenic activities such as burning of fossil fuels, mining, smelting, steel manufacturing, and production of municipal wastes are significant sources of environmental metal pollution; alternatively, natural sources of metals in the environment from the weathering of ultramafic

rocks at the surface can cause similar concerns. For example, weathering of serpentine rocks is known to produce laterite soils containing high concentrations of Cr, Ni, and Co (Oze et al. 2004; Pal et al. 2005; Rajkumar et al. 2009). Furthermore, many metals are difficult to remove from soil due to their mobility and general recalcitrance to be transformed to inert forms. Natural mechanisms by which metals are immobilized and transformed to less toxic species are primarily mediated by microorganisms (Haferburg and Kothe 2007). For this reason, remediation efforts have turned to naturally metal-resistant bacterial communities.

A lesser known application of metal-tolerant microorganisms is microbially-mediated metal extraction from ores and mine tailings. While underrated, biomining potentially dates back to before the first century BCE, and compared with conventional mining processes is both economically superior and more environmentally friendly (Rawlings et al. 2003; Johnson 2015). For these reasons, the minerals biotechnology industry is currently exhibiting a strong increase in feasibility and popularity. Many companies have already successfully implemented this technology, using microbial processes to recover large quantities of copper from low-grade deposits that are otherwise too costly to process. The biological processes in question involve "mineral-eating" microbes, or chemolithotrophs, capable of oxidizing iron and sulfur in the ore. Similar to processes active in many hydrothermal springs in Yellowstone, the oxidation of sulfur produces sulfuric acid, acidifying the environment and solubilizing iron which can act as an electron donor. These processes promote dissolution of valuable metals locked in mineral phases, such as Cu, Ni, Zn, Ag, and Au that can be recovered from leachate. The most relevant bacteria in these processes belong to the *Acidithiobacillales* and *Nitrospirales*, but at higher temperatures archaeal species typically dominate (Norris et al. 2000). Biomining has proven potential, and with growing environmental concern and stricter regulations, its viability and implementation may continue to increase in the coming years.

1.5 Justification and objectives

The purpose of this study is to cultivate extremophiles from two geochemically distinct subsurface ecosystems and investigate their phylogenetic, physiological, and biochemical characteristics as it pertains to applications in biotechnology. It is anticipated that a comparison of similar investigations of hydrothermal sites in Yellowstone National Park and serpentinizing seeps in the Philippines will result in a unique and comprehensive dataset.

The field sites chosen for this study include two natural, dissimilar environments well-suited to investigate processes associated with microbial cellulose degradation and interactions between microorganisms and metals. The Zambales ophiolite region in the Philippines is a serpentinizing system with associated fluid seeps that exhibit extremely alkaline and low-nutrient conditions; however, there is a transition to high nutrient availability at the surface where there is a large input of organic carbon, including cellulose from the surrounding rainforest. It is expected that investigating microbial cellulose-degradation in this unexplored extreme habitat may result in identification of novel taxa relevant to biofuel research. In addition, weathering of serpentinite rocks associated with this ophiolitic complex may result in leaching of various metals into soils and fluids. Therefore, studying microbial metal tolerance and potential for metal removal is justified in this setting.

In Yellowstone, spring fluids typically range from acidic to neutral, and contain low concentrations of organic carbon. Addition of exogenous carbon is limited to infrequent rainfall events in which surrounding debris is washed into hydrothermal pools. Some of this debris is likely to be nutrients in the form of cellulose derived from vegetation and bison excrement. Studying the ability of hot spring microbial communities to degrade cellulose may elucidate biogeochemical processes in these springs, and may result in discovery of novel cellulolytic consortia capable of functioning in extreme conditions that may prove to be more efficient than current strategies for biofuel production from cellulosic biomass. Furthermore, subsurface water-rock interactions in these springs leach metals in a similar fashion to

weathering in serpentine environments, resulting in toxicity, but also a source of chemosynthetic energy. Therefore, hydrothermal ecosystems are appropriate settings to find metal-tolerant organisms that may be capable of sequestering or transforming metals in solution.

1.6 Geologic Setting, Field Site Characterization, and Sample Collection - Philippines

Samples for this study were collected from an alkaline serpentinizing spring in the Zambales Ophiolite complex located in western Luzon, the Philippines. The Philippine archipelago is located amidst a tectonically complex region consisting of a convergent boundary between the Eurasian plate in the west and the Philippine Sea plate in the east, as well as several smaller microplates. The Zambales Ophiolite consists of a typical sequence of mantle peridotite, gabbro, oceanic pillow basalts, and marine sedimentary units (Hawkins and Evans 1983; Evans and Hawkins 1989). The ophiolite itself consists of Eocene Age material believed to have been emplaced between the Oligocene and early Miocene. In previous studies it was concluded that the ophiolite is actively serpentinizing based on gas chemistry and isotopic ratios (Cardace et al. 2015).

The field site is located in Manleluag Spring National Park in Mangatarem, Pangasinan Province (N15°42’16” E120°16’52”). The region exhibits a tropical climate with an average annual temperature of 27.4 °C and average rainfall of 222.3 cm. A field campaign was conducted in September 2013 during the wet season in which samples were collected from an actively serpentinizing fluid seep, designated Manleluag 2 (ML2), for geochemical and microbiological analysis. ML2 is a small, half-meter wide natural spring located in a densely-vegetated area. Fluid from the seep forms an outflow channel that flows tens of meters into the rainforest, depositing travertine terraces downstream. The fluid temperature is about 34°C, and pH at the seep and outflow is recorded as 10.8 (see Table I for additional geochemical data). Compared with other serpentinizing springs in the region, ML2 exhibits slightly lower pH and Ca^{2+} concentration, suggesting that the underlying material might represent a contact between mafic and

ultramafic rocks; gas analysis reveals a nearly equal ratio of H_2:CH_4 (Fig. 1), indicating a more typical serpentinization signal (Cardace et al. 2015). Serpentinizing fluids in this site are still within the range of Type II waters that are characterized by higher Ca^{2+} and OH^-. It has also been suggested that heavy precipitation significantly influences the input of DOC into this system, causing considerable fluctuations in carbon availability, and distinguishing this site from other terrestrial serpentinizing seeps.

Table I. Geochemical data for ML2 and CC1 for 2012 and 2013. Sampling in 2012 was performed during the dry season, and 2013 sampling was during the wet season. 'nd' refers to no data available.

Site Name	Year	T (°C)	pH	Cond. (mS/cm)	DO (%)	DIC (ppm)	DOC (ppm)	ORP	Cl^- (ppm)	Na^+ (ppm)	Ca^{2+} (ppm)
					26.6						
ML2	2012	34.3	10.8	0.337	0	0.4	0.42	-355	18.7	24.4	3.9
CC1	2012	33.8	10.8	nd	0.81	1.53	0.54	-245	nd	nd	nd
ML2	2013	34.4	10.8	0.388	0.90	0.4	0.10	-425	16.9	18.7	6.0

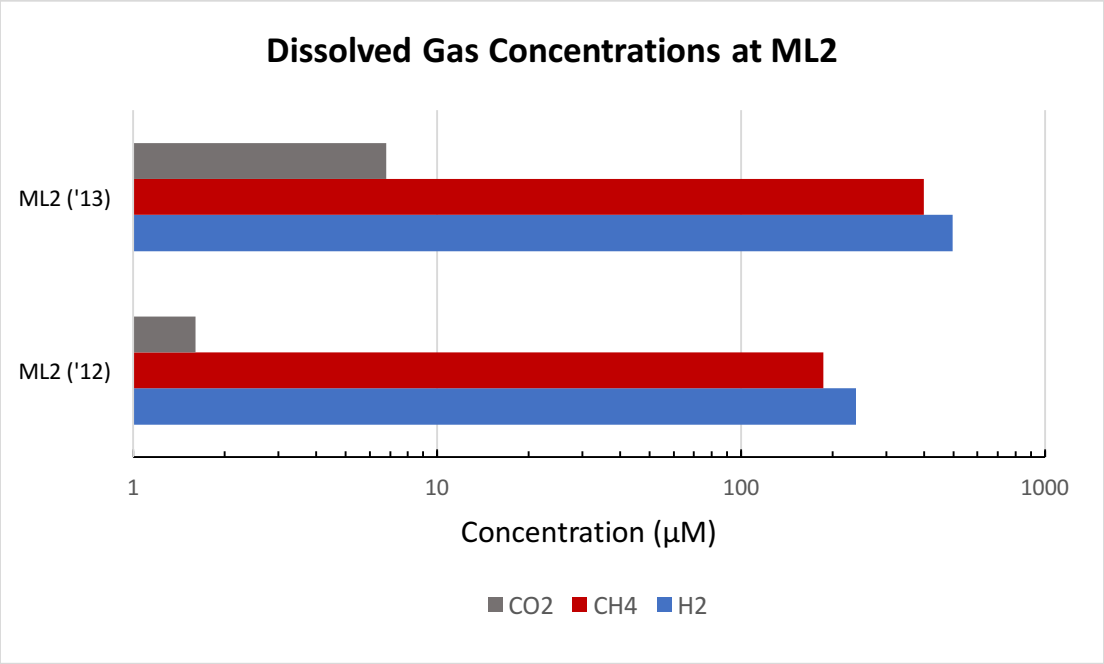

Figure 1. Dissolved gas concentrations (µM) at ML2 for 2012 and 2013. Measurements were made via gas-stripping of aqueous samples.

Fluid and sediment were collected at the source of ML2 (Fig. 2), as well as points downstream in the outflow channel. Sediment for culturing was collected from the bottom of the source pool with a sterile scoop and transferred to a Whirl-Pak® bag that was immediately stored at 4°C. Fluid emanating from the seep was continuously flowing through the sampled sediment, and the top ~5-10 cm was collected. Sediment consisted of brown silt to sand sized particles. The second sampling location, designated Caustic Cascade 1 (CC1), is located approximately 10 m down the outflow channel from ML2 near the first travertine deposit (Fig. 3). At CC1, the top 3 cm of sediment was collected with a sterile scoop and transferred to a Whirl-Pak® bag for storage at 4°C. Environmental parameters including temperature, pH, conductivity, ORP and DO were measured in the field using a YSI 556 multiprobe meter. All other sampling for geochemical analyses including DIC, DOC, and major cations and anions was performed as described by Cardace et al. (2015).

Figure 2. Manleluag 2 (ML2) source pool with fluid outflow towards the top of the photo (4x6 inch field book for scale).

Figure 3. Caustic Cascade 1 (CC1), approximately 10 m downstream from ML2. Fluid can be seen flowing over orange/khaki colored carbonate terraces covered by organic debris from surrounding vegetation.

Additionally, samples were collected from Barlo Mine in Dasol, Pangasinan Province, Luzon, the Philippines. Barlo is a strip mining operation primarily extracting copper ore from a massive sulfide deposit. Most of the rocks in the area are part of a Cretaceous-Paleogene sequence consisting of basalts, quartz keratophyres, shale, and limestone (Bryner 1969). The ore deposit includes pyrite, chalcopyrite, and sphalerite, and is composed of about 8% Cu and 6% Zn (Bryner 1969). This site exhibited bright orange

acid mine runoff from large piles of tailings that drained into a large tailings pond, given the designation "Blue Lagoon". The runoff from which the sample was collected was at a higher elevation near the edge of the tailings pond, and had a wet clay-like consistency with a pH of 4.2. A sterile scoop was used to transfer the upper 2 cm of fine sediment to a WhirlPak bag that was stored at 4°C for culturing analysis, and a second sample was kept frozen for DNA analysis.

1.7 Geologic Setting, Field Site Characterization, and Sample Collection – Yellowstone, WY

Yellowstone National Park has a history that has been shaped by a multitude of geologic processes, the most influential being hotspot volcanism driven by magmatic and partially molten material lying at shallow depths (3-10 km). The Yellowstone caldera is located over a mantle plume that has resulted in three major eruptions since the Cenozoic era (Fournier 1989). Thus, much of the geology is directly influenced by these eruptions and subsequent lava flows that form the underlying bedrock. The area mostly consists of permeable rhyolite through which water circulates and emerges in various springs with a chemical composition partially reflective of elements found in the igneous basement (Fournier 1989). The area was also highly reworked by Pleistocene glaciations; glaciers shaped the geology by eroding much of the surface and leaving large till deposits. The park's numerous geothermal features are dynamic and some are ephemeral, allowing for continuously evolving studies of geology and biogeochemistry.

Sampling was performed at several hydrothermal springs located in various regions of the park. Two sites are located in the Greater Obsidian Pool Area (GOPA); these sites are designated "Obsidian Pool Proper" and "Figure 8 outflow." Many studies have previously characterized springs in this area (Meyer-Dombard et al. 2005; Shock et al. 2005, 2010), which has been described as a gas-dominated system that interacts with the local water table. As such, higher chloride values and very high sulfate values (resulting from sulfide gas interacting with the water) are typical of the area, and the geochemistry in general is

highly variable over time (Table II, Fig. 4). Obsidian Pool is an irregularly shaped feature only a few meters wide in most directions (see Shock et al. 2010 for map of the area). Obsidian Pool is situated in a large topographical depression formed as the feature eroded away part of the surrounding meadow. "Figure 8" Pool is much larger and topographically higher than Obsidian Pool, with approximate dimensions of 10 x 30 m (Shock et al. 2010). Both features express vigorous degassing with lengthy runoff channels that flow independently for tens of meters and ultimately drain into "Goose Lake" located down slope. Sediment from Obsidian Pool was collected from the top 0.5 cm of sediment beneath 1-2 cm of pool fluid. Sediment size is several mm in diameter, black/brown in color, and exhibits a somewhat metallic sheen. The "Figure 8 outflow" sample was taken approximately 30 m downstream of the pool's source, in an area where the fluid was ~10 cm deep. Sediment collected from this site is tan/grey with a silty texture.

Table II. Geochemical data for selected hot springs in Yellowstone National Park. 'nd' refers to no data available. Data provided by Dr. Everett Shock, Arizona State University.

Site	Year	Temp (°C)	pH	Cond. (μS)	Cl^- (mg/L)	SO_4^{-2} (mg/L)	DIC (ppm C)	DOC (ppm C)	DO (mg/L)
Obsidian Pool	2009	79.0	5.18	988	30.71	203.84	nd	5.98	5.8
Obsidian Pool	2010	77.9	4.30	1304	34	256.20	12.1	3	nd
Obsidian Pool	2011	75.9	5.17	1213	26.59	205.96	19.2	5.6	0.57
Log Jam	2007	80.6	7.08	3561	213.1	25.30	nd	nd	nd
Log Jam	2008	80.0	7.30	3466	260.39	21.19	nd	nd	2.1
Log Jam	2009	80.3	7.23	3415	313.73	21.42	65.16	0.2	nd
Figure 8 outflow	2010	43.0	3.63	3291	447.6	386.70	1.5	0.8	1.5
Figure 8 outflow	2011	46.2	3.85	3411	424.9	345.50	4.66	2.4	3.3
Figure 8 outflow	2012	49.8	5.78	4797	nd	nd	1.5	0.9	1.3
Hell's Gate	2009	87.6	4.46	637	0.97	97.35	nd	nd	nd
Hell's Gate	2011	81.6	4.58	437	0.81	77.00	1.4	0.2	0.6

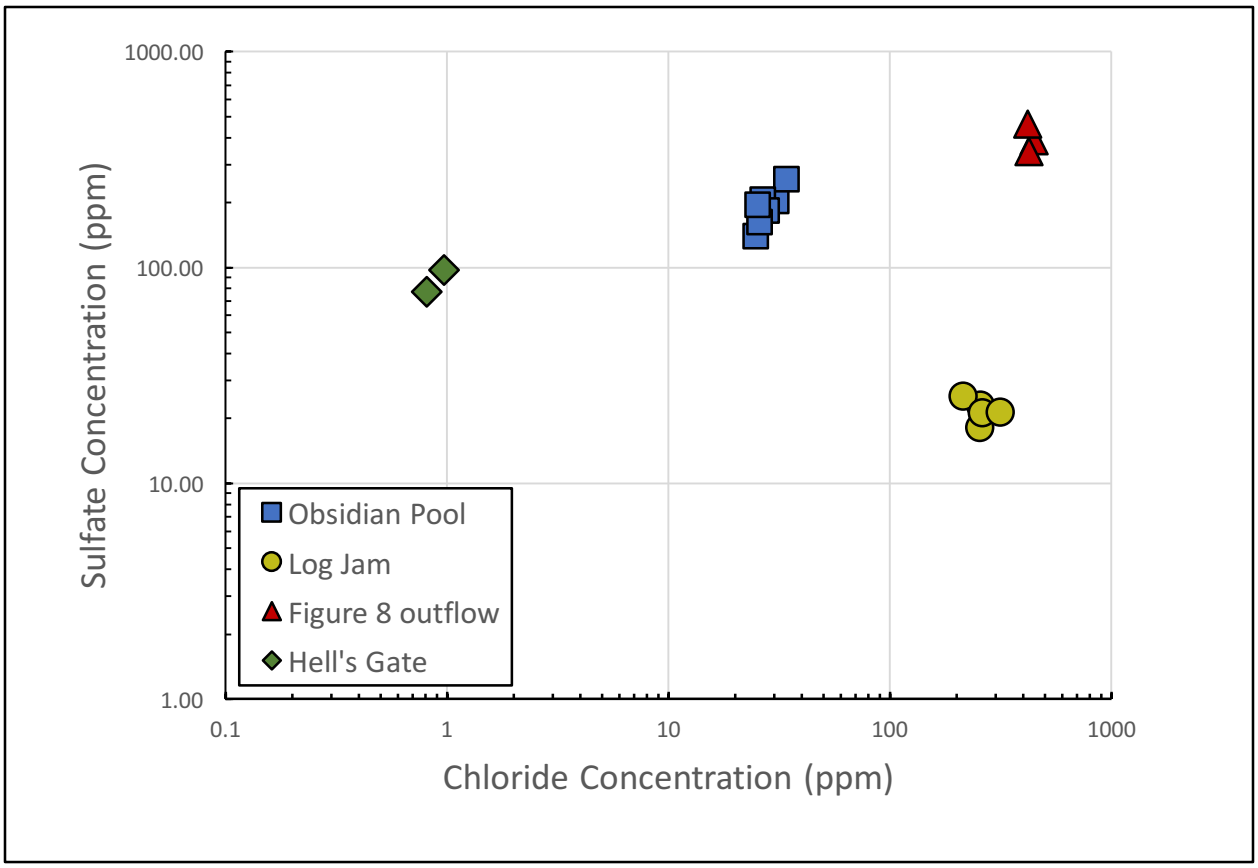

Figure 4. Sulfate and chloride concentrations (in ppm) for select years from four of the five sampling locations (data are not available for site "Wood Chip Beach.")

Samples were also collected from a hot spring called "Log Jam" in the White Creek region of Lower Geyser Basin. The White Creek area is renowned as the general location from which the organism *Thermus aquaticus* was first isolated, a bacterium responsible for revolutionizing PCR technology (Brock and Freeze 1969), and is also where pink streamer biofilms were originally characterized (Reysenbach et al. 1994; Huber et al. 1998; Meyer-Dombard et al. 2011); these streamers are now known to be found in many locations throughout the park and worldwide (Marteinsson et al. 2001; Nakagawa and Fukui 2002; Reysenbach et al. 2005; Meyer-Dombard et al. 2011). The Lower Geyser Basin is a typical alkaline-chloride, hot water dominated system. Chloride concentrations are very high, derived from long-term water-rock interactions, while sulfate concentrations are low due to low sulfide gas presence (Table II, Fig. 4). "Log

Jam" is a nearly circular feature, about 2.5 m in diameter. It is a boiling feature, with very little outgassing and copious amounts of pine tree logs and other detritus that have fallen into the pool from the small slope <1 m away. Sediment samples were taken in the soft, silty area at the edge of the pool where water depth is ~10 cm.

Another sampling site, "Iron Fist," is located in South Rabbit Creek of the Lower Geyser Basin, and is a relatively new formation that exhibited a sudden appearance in 2011. Due to its recent emergence, geochemical data from the specific site is currently limited, although it is possible that the spring was originally part of the feature named "Hell's Gate." Geochemical data for "Hell's Gate" is shown in lieu of "Iron Fist" as a proxy (Table II, Fig. 4) The pool is a large feature, 7 m in diameter in a rhyolite crater that is filled with violently degassing orange fluid. The surrounding sediment is light grey clay. Samples were taken from a small outflow rivulet carved into the soil, which runs about 30 m into the surrounding area. The fluid in the outflow is orange along the entire length of the channel. Samples were collected ~15 m downstream and were silty/clay textured, red/orange at the surface and light grey beneath. The sediment is beneath ~2 cm of fluid.

Sample location, "Wood Chip Beach", is located in the Norris Geyser Basin in the area known as "The Gap". This area has been well studied and is characterized as an acid-sulfate, vapor dominant system that interacts with a hot water dominated system (Jackson et al. 2001; Inskeep and McDermott 2005; Boyd et al. 2007). "Wood Chip Beach" is a large feature, tens of meters in diameter, at the base of a hillside. The sample was collected from a calm "beach" at the edge of the pool on the side opposite of the hill where a collection of wood chips are floating in the water. The sediment consists of clay-sized particles with a pink/tan color. The sampling area exhibited abundant wood chips, and the sample was taken from the upper 1 cm of sediment.

II. Multi-metal Resistant Alkalitolerant Bacteria Isolated from a Serpentinizing Spring in the Zambales Ophiolite, the Philippines

2.1 Introduction

Weathering of serpentinite and ferromagnesium minerals found in ultramafic rocks produces soils containing high concentrations of associated heavy metals such as chromium, nickel, cobalt, magnesium, copper, and zinc (Stoppel and Schlegel 1995; Mengoni et al. 2001; Oze et al. 2004; Pal et al. 2005). Metal concentrations in serpentine soils have been reported to range from a few hundred to thousands of parts per million (Pal et al. 2005; Rajkumar et al. 2008, 2009). Although several metals are required for cellular function in trace amounts, elevated concentrations result in toxicity, primarily through protein denaturation. Whereas metalliferous serpentine soils occur naturally, anthropogenic activities such as burning of fossil fuels, use of fertilizers and pesticides, mining, smelting, steel manufacturing, and municipal wastes are all major sources of environmental metal pollution. As such, metal-contaminated soils and groundwater pose a significant global threat to human health, agriculture, and ecosystems. Alternatively, soils derived from natural weathering of serpentine rocks, such as laterites, are often mined directly due to their economic value. Furthermore, metals in soil are often difficult to remove due to their tendency to bind strongly to organic compounds and their general inability to be transformed to inert forms. Natural processes by which metals in the environment are immobilized or transformed to less toxic states are primarily mediated by microbial activity. Many studies have investigated metal-resistant microbial communities from naturally metalliferous serpentine soils for potential applications in bioremediation (Gadd and Griffiths 1978; Stoppel and Schlegel 1995; Mengoni et al. 2001; Pal et al. 2005; Abou-Shanab et al. 2007; Rajkumar et al. 2009). These microorganisms employ various mechanisms to cope with metallic stressors, such as transforming or removing metals from their surroundings via cellular uptake, adsorption, biomineralization, or production of organic compounds to chelate metal cations. Investigating modes of bacterial metal-resistance from newly discovered serpentinizing environments

may result in discovery of novel strains or mechanisms that may be relevant in biotechnology and bioremediation strategies.

The purpose of this study is to investigate the limits of metal tolerance exhibited by bacteria isolated from natural serpentinizing seeps and acid mine drainage, and to assess their potential for application in biotechnology.

2.2 Materials and Methods

2.2.1 Aqueous geochemistry (ICP-AES)

Surface and groundwater samples were filtered with Millipore Sterivex Sterile Filter Units (Cat. No. SVGPL10RC) into new, certified sampling bottles pre-spiked with trace metal grade nitric acid to stabilize metals in solution; final solutions were ~2% HNO_3. Filtered samples were stored refrigerated, then brought to room temperature and agitated vigorously prior to analysis. Samples were analyzed at the Brown University Environmental Chemistry Facility (http://www.brown.edu/Research/Evchem/) on a Thermo Scientific iCAP 7400 DUO Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES). The instrument was equipped with a Teledyne ASX-560 240 position autosampler, and controlled by Thermo Scientific Qtegra software. The charge injection device (CID86) detector allowed flexible choice of wavelengths. The wavelengths chosen for quantification of each analyte are: K (766.490, radial mode), Mg (279.553, radial mode), Si (251.611, radial mode), Na (589.592, radial mode), Ca (317.933, radial mode), Cr (283.563, axial mode), Fe (259.940, axial mode), Ni (221.647, axial mode), Cu (324.754, axial mode), Zn (213.856, axial mode), Mn (257.610, axial mode), Co (228.616, axial mode). To assess instrument accuracy, two standards were used. QC28 is a 28 element standard purchased from Inorganic Ventures (product IV28, (https://www.inorganicventures.com/productdisplay/quality-control-standard-28-0). Verification Std 1 is a SPEX certified standard.

For the solutions presented to the ICP-AES (diluted or full strength), and given actual calibration ranges, the detection limits for each analyte are effectively: Cr (9 ppb), Fe (4 ppb), Ni (14 ppb), Cu (14 ppb), Zn (14 ppb), Mn (9 ppb), Co (14 ppb).

2.2.2 Cultivation of metal-resistant bacteria from environmental samples

All organisms in this study were cultivated based on aerobic, heterotrophic metabolism on solid culture media. Luria-Bertani agar (LBA) was used as the control medium, and was supplemented with varying concentrations of five metals of interest. The following metal salts were used to adjust media to the appropriate concentrations of the corresponding metal cations: $NiCl_2 \cdot 6H_2O$, $CuCl_2$, $CoCl_2 \cdot 6H_2O$, $ZnCl_2$, and K_2CrO_4. Concentrations of 25, 50, 100, 200, and 400 mg/L of each metal were initially used for culturing directly from environmental samples. The pH of all media was adjusted to 7.2 using 10 N NaOH or 10 M HCl, and the media were subsequently autoclaved at 250 °C for 50 minutes.

Environmental samples were generally in the form of a sediment-fluid slurry; in the case that there was not enough fluid in the sample, approximately 1.0 g of sediment was added to 5 mL of sterile, deionized water to create a slurry. A 100 µL aliquot of each sample was pipetted onto the control and test media. Spread plate technique was used to evenly distribute the sample on the agar surface, and plates were then incubated at 35 °C for up to 2 weeks with growth observations of colony-forming units recorded every 2 days.

2.2.3 Isolation of metal-resistant bacteria

Morphologically distinct colonies from metal-amended plates were selected for isolation. Colonies were transferred via an inoculating loop to fresh media reflective of the corresponding metal and concentration from which the colony originated using the streak plate method. Plates were incubated at 35 °C for up to one week. Following a successful transfer, a single colony was again transferred to a

new plate of LB-medium without metal in order to remove the stressor, promote growth, and prepare stock solutions. The pure isolate was given a specific designation reflective of the sample of origin. Stock cultures of 10% glycerol + yeast-peptone were prepared for each isolate and stored at -80 °C.

2.2.4 DNA extraction from isolated strains and 16S rRNA gene sequencing

Genomic DNA was extracted from cell material of each bacterial strain using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA). The 16S rRNA gene was amplified with 27F and 1492R primers, and purified using QIAquick® PCR Purification Kit (QIAGEN, Germantown, MD, USA). PCR products were verified with agarose gel electrophoresis. Sanger sequencing was performed by DNA Services at the University of Illinois at Chicago, and sequence similarities were compared with GenBank's 16S Bacteria and Archaea database using NCBI's Basic Local Alignment Search Tool (BLAST). Secondarily, sequences were also identified using EzBioCloud's Identify Service (www.ezbiocloud.net/identify), resulting in sequence matches only with valid prokaryotic taxa. Type strain sequences of closely related species were acquired from EzBioCloud's online sequence database (Yoon et al. 2017). Evolutionary analyses were computed using MEGA version 7 (Kumar et al. 2016b). Sequences were aligned using the MUSCLE program (Edgar 2004), and phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) with the Jukes-Cantor model for calculating evolutionary relationships (Jukes and Cantor 1969). Bootstrap resampling analysis was performed with 1000 replicates (Felsenstein 1985).

2.2.5 Examination of individual isolates for cross-metal tolerance and tolerance to alkalinity

As each isolate was obtained from a growth medium containing only one of five metals, resistance to all other metals used in this study was then examined. Cell suspensions were made for each isolate by adding approximately 10 mg of cell material to 500 µL of 0.85% w/v saline solution and vortexing gently

for homogeneity. Ten µL of each cell suspension was pipetted onto the control and metal-amended media to observe growth response and the extent of the isolates' tolerance to metals. A scoring system was implemented for relative growth analysis using a system of 1 (+) to 4 (++++), with 4 indicating abundant growth. If no growth was perceptible, a 0 (-) score was given. Growth score was determined by comparing the spot diameter and opacity relative to the control plate; a smaller diameter and translucence of the spot relative to the control indicated reduced growth and was given lesser scores, while equal diameter and opacity relative to the control was given a score of 4 (++++). Therefore, a score of ++++ indicates no visibly discernible inhibition by the presence of metals. Examples of the scoring system are shown in Fig. 5. For reference diameters of all isolates grown on control media (LBA), refer to Appendix E. In some cases, it was necessary to prepare growth media containing higher concentrations of metal to find the minimum inhibitory concentration (MIC) for several strains. Media with higher metal concentrations were prepared in the same manner as previously described.

Tolerance to alkalinity was determined independently of tolerance to metals. Alkaline pH affects the speciation of metal cations, typically by promoting precipitation of metal hydroxides; metal precipitates reduce the bioavailability of the metal and lower overall toxicity of the solution. Thus, growth response to high pH was tested independently by altering LBA media to pH 8, 9, 10, 11, and 12. The pH was adjusted with 10 N NaOH solution.

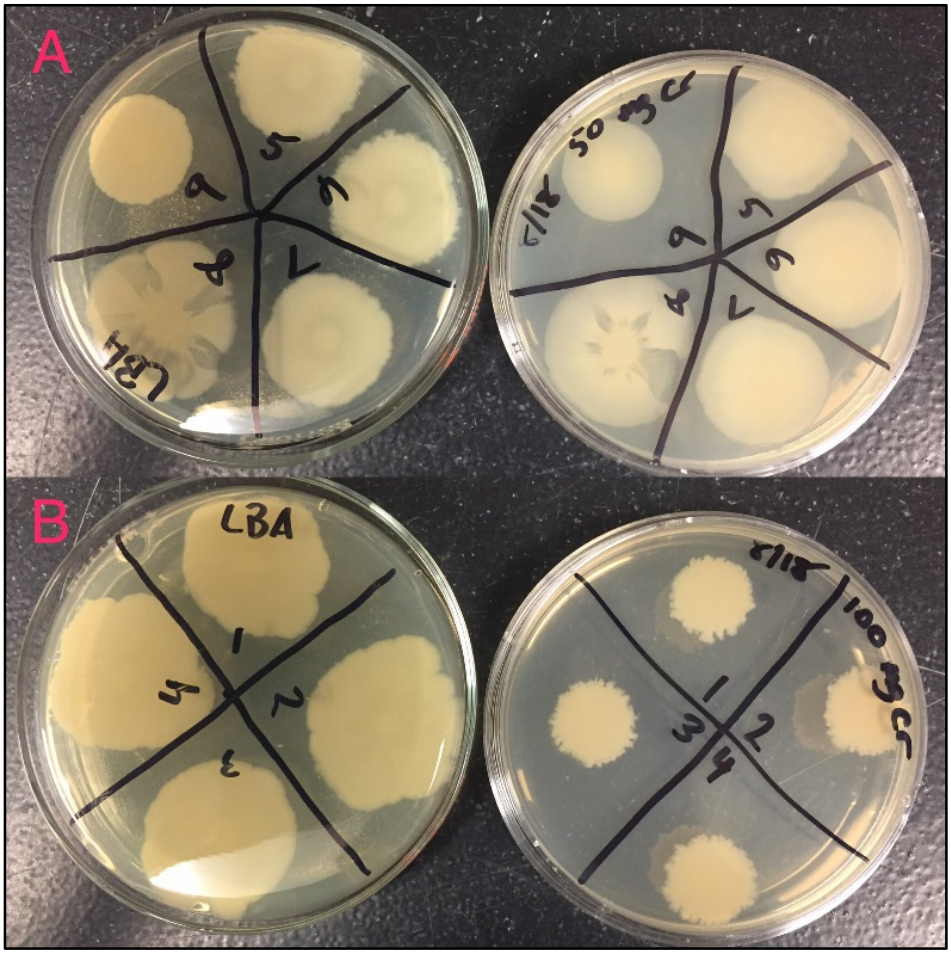

Figure 5. Images comparing bacterial growth on control LBA media (left) and in the presence of metals (right). A) Organisms on right plate are growing in the presence of 50 mg/L Cr and are identical in size and thickness as the control on the left, earning a score of ++++. B) Organisms on the right growing on LBA + 100 mg/L Cr are roughly half the size relative to the control and would be scored ++.

2.2.6 Examination of metal removal efficiency by isolates

Select isolates that displayed tolerance to high concentrations of metals were then examined for capacity to remove metals from a liquid solution. Only Ni, Cu, and Cr were selected for testing metal removal based on abundance of bacterial growth or appearance of precipitates. A yeast-peptone (YP) solution (5.0 g/L yeast extract; 10 g/L peptone) was used as the growth medium to which 200 mg/L of each metal was added. All solutions were adjusted to pH 7.2. Cell suspensions were made for each isolate,

and 10 µL aliquots were inoculated into 10 mL cultures of control and test media. Controls included uninoculated YP without metals, as well as uninoculated YP containing each metal at each concentration. Culture tubes were incubated at 35 °C for two weeks in aerobic conditions. Turbid cultures were centrifuged at 3500 rpm for 30 minutes, followed by filtration with 0.2 µm Whatman filters. Samples were prepared for analysis of Cr, Cu, and Ni by filtering the cultures through a 0.22 µm filter and preserving the samples with 2% nitric acid. Concentrations were determined on an Agilent 5110 ICP-OES, and values were constrained by a continuous calibration standard (numerous compounds spiked with 1 mg/L and potassium at 10 mg/L), a continuous calibration blank (DI water with 2% HNO_3), matrix spikes (YP medium spiked with target elements, 10 mg/L Cr, 10 mg/L Ni, and 10 mg/L mixed Cu standard), field blanks (YP medium), and internal calibration. Margin of error for instrument is ± 10 ppm.

2.2.7 TEM analysis of select isolates

Transmission electron microscopy (TEM) was used to analyze select isolates exhibiting abundant growth or production of precipitates in culture. Isolates were grown in liquid YP media with 200 mg/L Cu for two weeks at 35 °C in aerobic conditions. Cells were recovered with centrifugation at 800 x g for 5 minutes, and culture supernatant was removed. Cells were resuspended in 0.1 M potassium phosphate buffer containing 2.5% glutaraldehyde for 10 minutes. Cells were then centrifuged and resuspended in fresh fixative prior to preparation of thin sections. Ultrathin sections were prepared by Figen Seiler at the Research Resources Center at the University of Illinois at Chicago, and mounted on Ni-coated grids for viewing. Micrographs were taken using a JEOL JEM-1220 TEM at 120 kV.

2.2.8 Identification of mineral precipitates

In one case crystals were observed within bacterial biomass on LB agar medium containing 600 mg/L Ni. The crystals were manually lifted from the biomass and washed 3x in deionized water. Crystals

were then powdered and mounted on zero-background quartz plates. The X-ray data were acquired with a Siemens D5000 automated powder diffractometer in Θ:Θ configuration, using a graphite monochromatized CuKα radiation source (40 kV at 25 mA) and incident and diffracted beam slit sizes of 1.0°. Measurements were made from 18° to 75° with a 0.02° step and 1.0 s count time. Spectral patterns were compared to ICDD PDF-4+ database (2016) using MDI JADE v.9.6.

2.3 Results

2.3.1 Aqueous geochemistry and trace metal concentrations

ICP analysis of environmental fluids from ML2 and CC1 reveals that all metals of interest in this study are below detection limits. In comparison, the Barlo mine drainage sample shows relatively higher concentrations of metals; Zn exists in the highest concentration, while Co and Ni are the lowest (Table III).

Table III. Trace metal data for field sites sampled in 2013. 'bdl' indicates below detection limit (~10 ppb).

Site	Cr (ppm)	Ni (ppm)	Cu (ppm)	Zn (ppm)	Mn (ppm)	Co (ppm)
ML2	bdl	bdl	bdl	bdl	bdl	bdl
CC1	bdl	bdl	bdl	bdl	bdl	bdl
Barlo	bdl	0.039	0.995	3.688	1.028	0.031

2.3.2 Cultivation of metal-resistant organisms from environmental samples

LBA control media exhibited confluent bacterial growth for most plated samples. In general, the number of colony forming units decreased with increasing metal concentration for all metals tested. Interestingly, at the lower concentrations of 25 and 50 mg/L, only Cu-supplemented media showed

discernible inhibitory effects compared with the control (Fig. 6). All other metal-containing media up to 50 mg/L exhibited equal or only slightly reduced numbers of colonies relative to control plates. Greater morphological diversity amongst colonies was exhibited at lower metal concentrations of 25 to 100 mg/L, while concentrations of 200 and 400 mg/L of most metals resulted in limited morphological differences; the exception is Cr-amended plates which exhibited overall more growth relative to other metals. The limited morphological differentiation of colonies at high metal concentrations may indicate a decrease in cultured bacterial diversity, a hypothesis that is later corroborated with 16S rRNA sequence data. Additionally, as metal concentrations increased, organism growth was slowed, especially relative to the LB control media without metals. On control media, abundant growth typically occurred within 2-3 days, while media amended with metals did not exhibit growth until ~7 days.

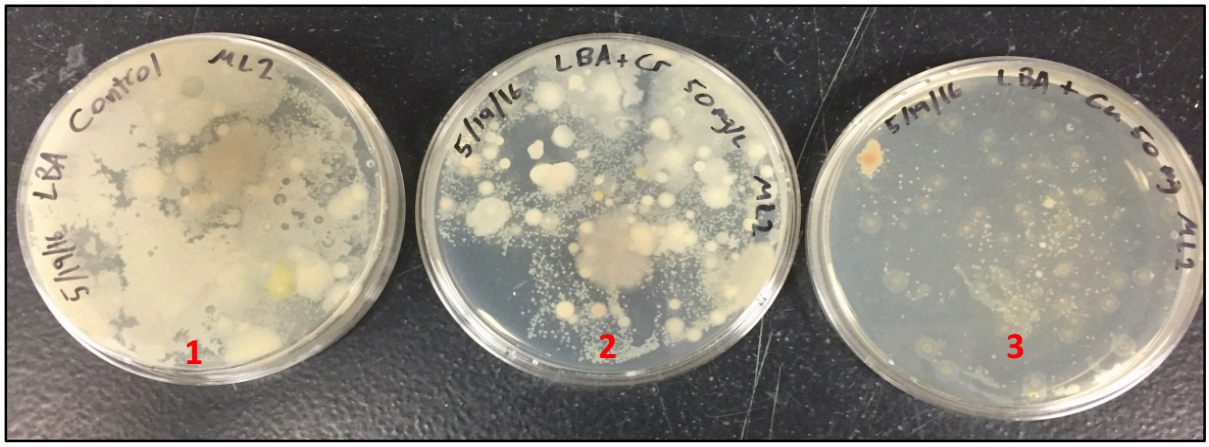

Figure 6. Environmental sample from ML2 plated on 1) LBA control, no metal 2) LBA + 50 mg/L Cr 3) LBA + 50 mg/L Cu; Less growth is observed at 50 mg/L Cu than 50 mg/L Cr; Both metal amended plates show reduced growth relative to the control plate.

2.3.3 Isolate taxonomy

16S rRNA gene sequences assigned isolates to multiple genera including *Bacillus, Pseudomonas*, *Microbacterium*, *Rhodococcus*, and *Streptomyces* (Table IV). Several isolates shared 100% similarity with

16S rRNA sequences of previously described species. The 16S rRNA sequences of ML-1 and ML-8 are identical to *Pseudomonas koreensis* (Kwon et al. 2003) (Fig. 7); isolate ML-16 shares an identical 16S rRNA sequence with *Microbacterium maritypicum* (Takeuchi and Hatano 1998) (Fig. 8); lastly, isolate ML-14 has 100% similarity to *Rhodococcus jialingiae*, also known under the name *Rhodococcus qingshengii* (Wang et al. 2010) (Fig. 9). CC-8 is the only *Streptomyces* organism that was cultured, and the partial 16S sequence is 99% related to *Streptomyces misionensis* (Cercos et al. 1962) (Fig. 10).

Most isolates belong to the genus *Bacillus*. Of these, most are closely related to members of the *Bacillus cereus* group, which currently includes nine other species. These nine species have been shown to have identical 16S sequences, and therefore it has been proposed in prior taxonomic studies that they be considered a single species (Helgason et al. 2000; Ticknor et al. 2001). For this reason, it is impossible to differentiate these organisms based on 16S alone; species differentiation is primarily based on phenotypic and biochemical characteristics, such as substrate utilization. Isolates ML-7, CC-2, BL-6, BL-3, ML-9, CC-3, and ML-6 share 99% or greater sequence similarity with species in the *B. cereus* group (Fig. 11). More diversity is exhibited by the other *Bacillus* isolates. BL-4 shares 98% similarity to *B. stratosphericus* and *B. altitudinis*, while the 16S rRNA sequence of CC-6 is 100% identical to *B. aryabhattai*. ML-4 is 99.7% similar to *B. mesonae*, and CC-7 is 97.4% similar to *B. firmus*.

Table IV. Taxonomic identities of isolated bacterial strains as determined by 16S rRNA gene sequence amplified with the 27F primer; 'Isolation Media' refers to the concentration and corresponding metal in the LB agar from which the organism was isolated.

Strain	Source	Closest Taxon	# of Bases	Seq. Similarity (%)	Isolation Media
BL-3	Barlo Mine Drainage	*Bacillus sp.*	834	99	200 mg/L Cr
BL-4	Barlo Mine Drainage	*Bacillus altitudinis*	867	99	200 mg/L Cr
BL-6	Barlo Mine Drainage	*Bacillus sp.*	880	99	400 mg/L Ni
CC-1	CC1 outflow	*Bacillus sp.*	824	99	100 mg/L Co
CC-2	CC1 outflow	*Bacillus sp.*	915	99	100 mg/L Zn
CC-3	CC1 outflow	*Bacillus sp.*	939	100	200 mg/L Zn
CC-4	CC1 outflow	*Bacillus sp.*	945	99	200 mg/L Ni
CC-6	CC1 outflow	*Bacillus aryabhattai*	1022	100	200 mg/L Cr
CC-7	CC1 outflow	*Bacillus firmus*	745	98	400 mg/L Ni
CC-8	CC1 outflow	*Streptomyces misionensis*	850	99	400 mg/L Ni
ML-1	ML2 source	*Pseudomonas koreensis*	962	100	25 mg/L Cu
ML-4	ML2 source	*Bacillus mesonae*	866	99	50 mg/L Cu
ML-6	ML2 source	*Bacillus sp.*	869	99	100 mg/L Co
ML-7	ML2 source	*Bacillus sp.*	881	100	100 mg/L Zn
ML-8	ML2 source	*Pseudomonas koreensis*	894	100	200 mg/L Cu
ML-9	ML2 source	*Bacillus sp.*	882	99	200 mg/L Zn
ML-10	ML2 source	*Microbacterium maritypicum*	758	99	200 mg/L Zn
ML-14	ML2 source	*Rhodococcus jialingiae*	581	100	400 mg/L Co
ML-15	ML2 source	*Microbacterium sp.*	196	100	400 mg/L Co
ML-16	ML2 source	*Microbacterium maritypicum*	1012	100	400 mg/L Zn

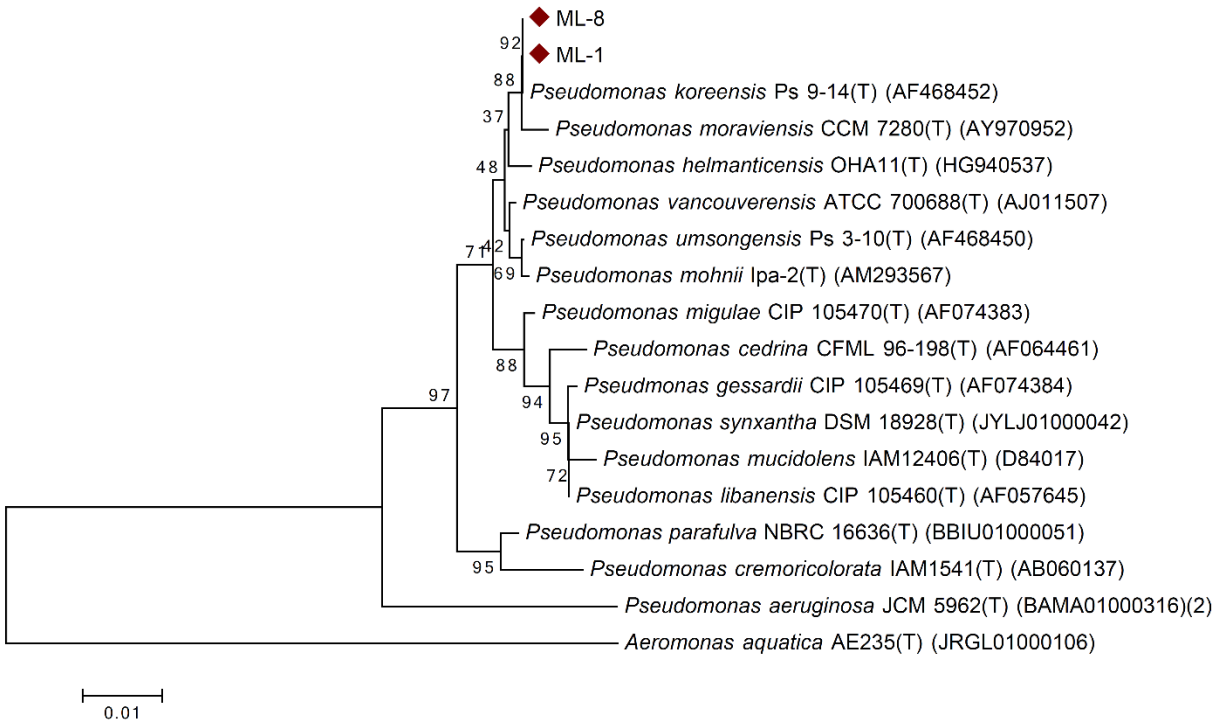

Figure 7. Neighbor-joining tree of *Pseudomonas* isolates demonstrating their phylogenetic position among closely related taxa. Isolates obtained in this study are denoted with red diamonds. Species names are followed by the strain number and accession in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar, 0.01 substitutions per site.

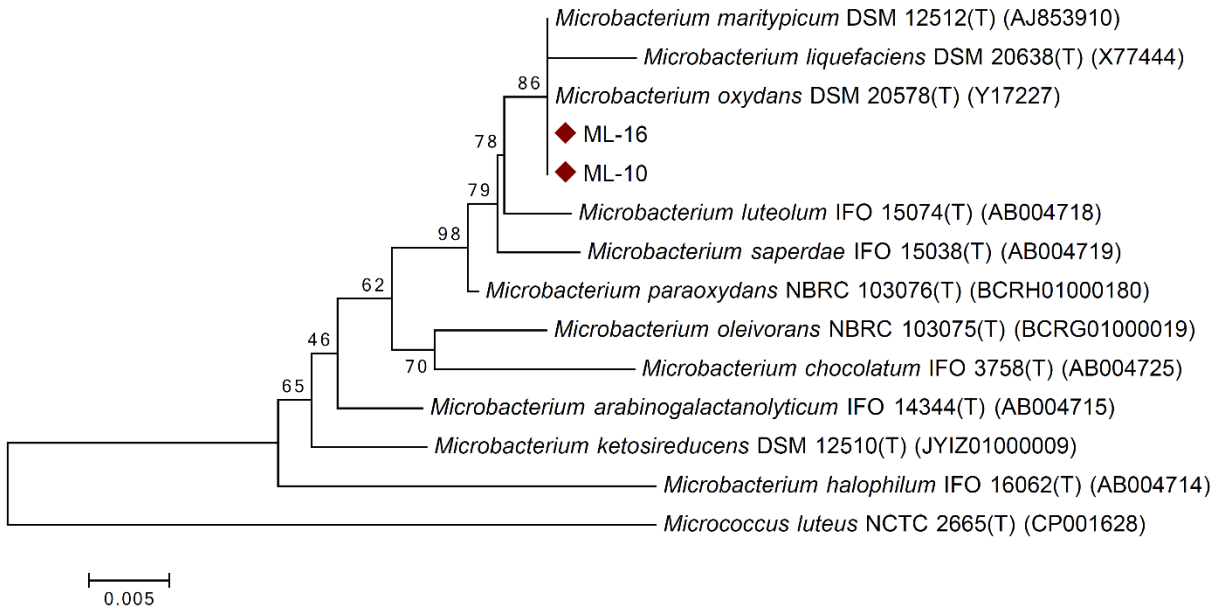

Figure 8. Neighbor-joining tree of *Microbacterium* isolates and their phylogenetic position among closely related taxa. Isolates obtained in this study are denoted with red diamonds. Species names are followed by the strain number and accession in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar, 0.005 substitutions per site.

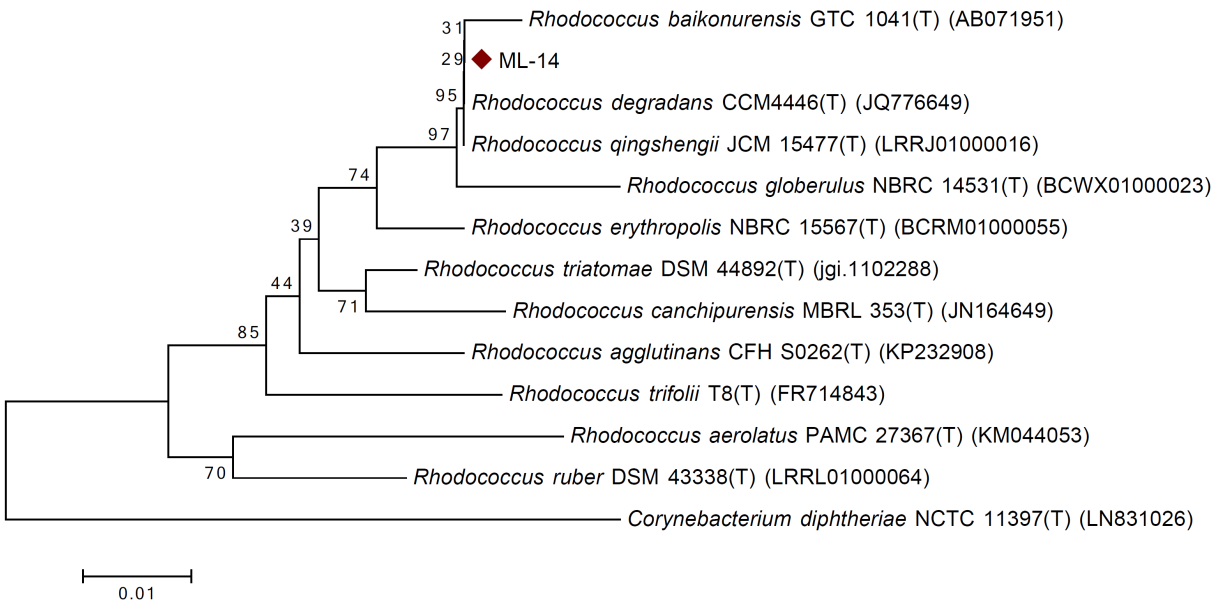

Figure 9. Neighbor-joining tree of *Rhodococcus* isolates demonstrating their phylogenetic position among closely related taxa. Isolates obtained in this study are denoted with red diamonds. Species names are followed by the strain number and accession in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar, 0.01 substitutions per site.

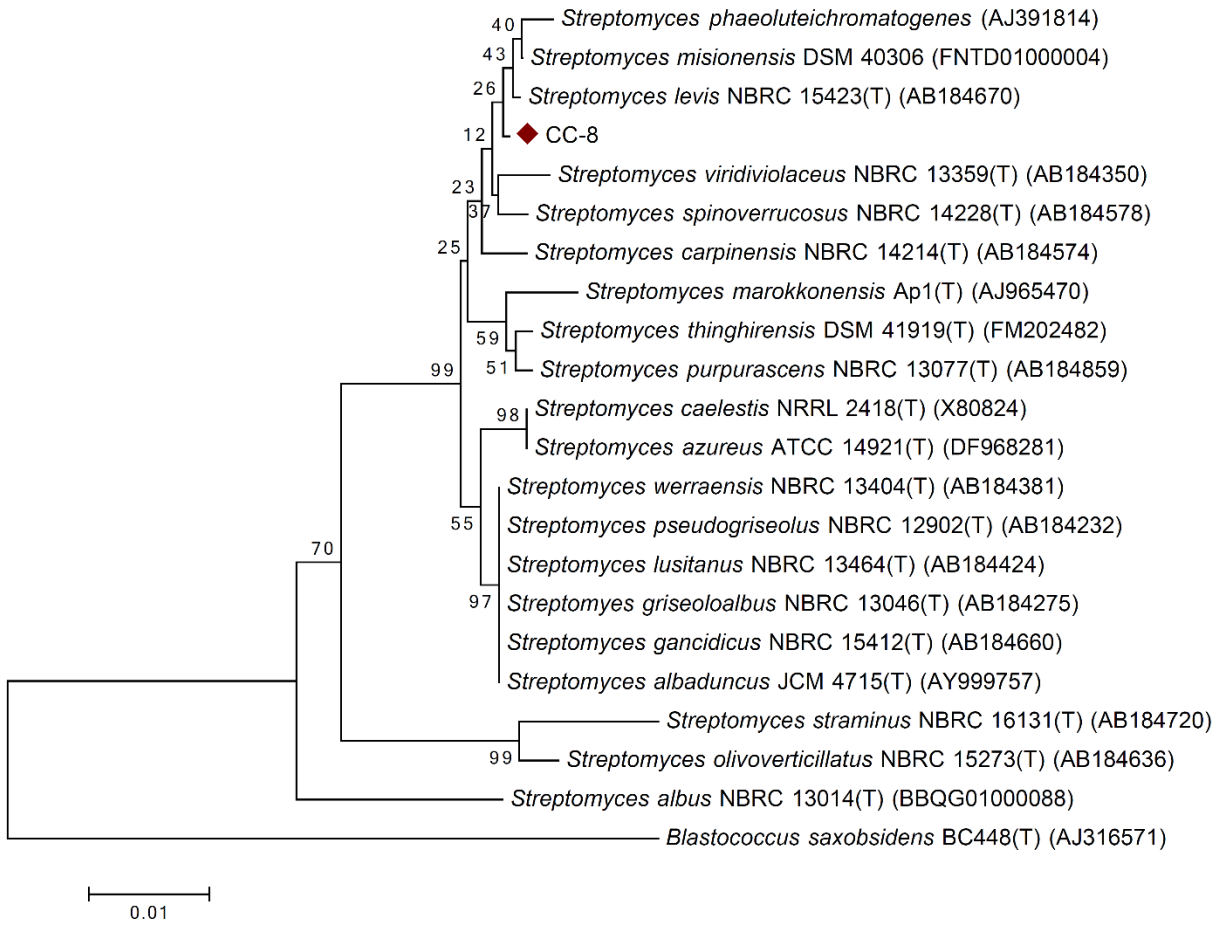

Figure 10. Neighbor-joining tree of isolate CC-8 demonstrating its phylogenetic position among closely related members of the genus *Streptomyces*. The strain isolated from this study is indicated with a red diamond. Species names are followed by the strain number and accession in parentheses. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar, 0.01 substitutions per site.

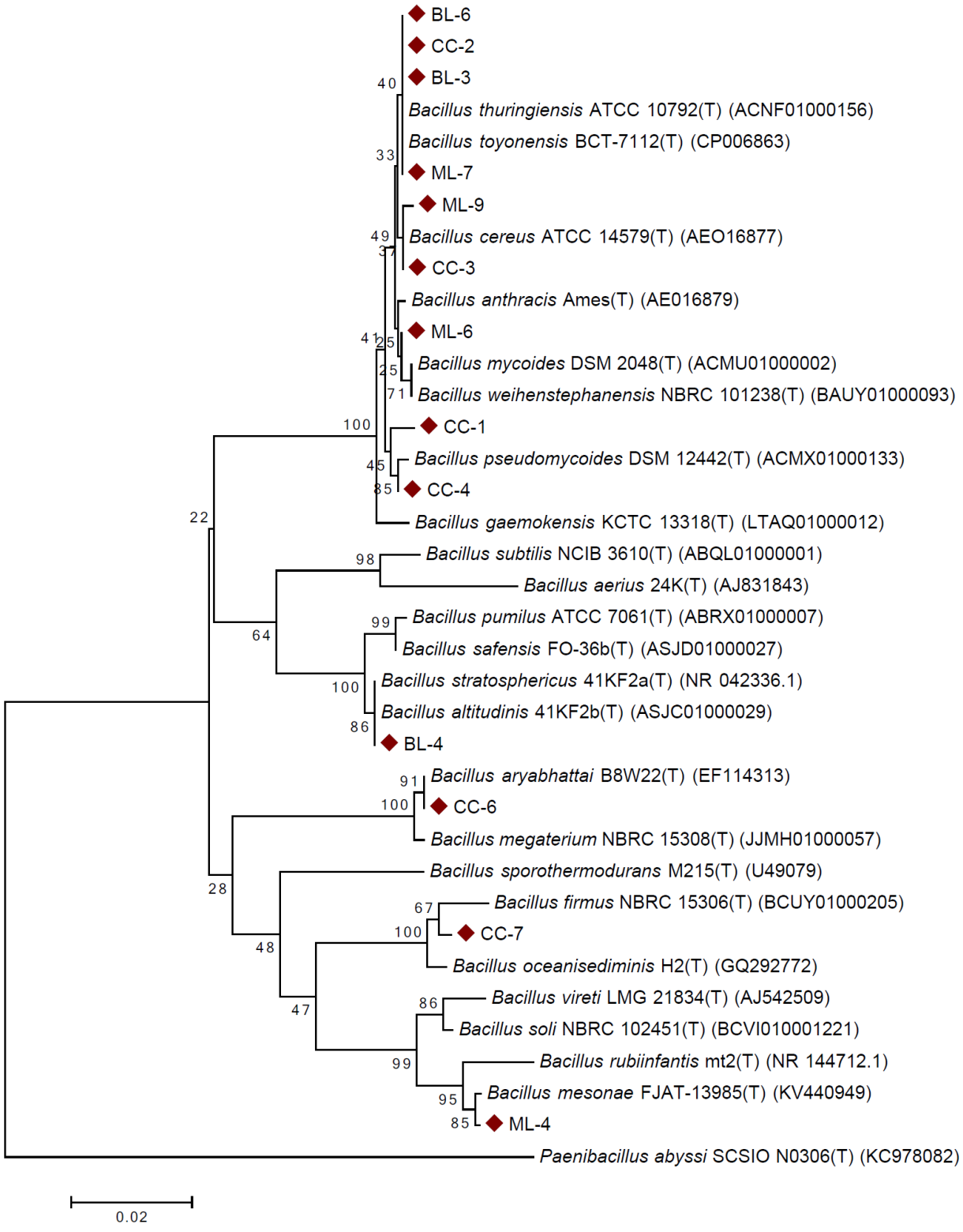

Figure 11. Neighbor-joining tree of *Bacillus* isolates and their phylogenetic relationship with closely related taxa; most of the isolates cluster within the *B. cereus* group in the top-most portion of the tree. Isolates obtained in this study are denoted with red diamonds. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar, 0.02 substitutions per site.

2.3.4 Examination of isolates for cross-metal tolerance

All isolates obtained from media containing one of five metals also show growth in the presence of additional metals used in this study. In general, Cr seemed to be least inhibitory to bacterial growth relative to other metals based on the observation that more isolates could grow at higher concentrations of Cr than any other metal. Close to 75% of the isolates tolerate the maximum concentration of Cr tested (Table V), but many display significantly reduced growth and altered morphology on the agar, evidenced by very thin, translucent growth. The minimum inhibitory concentration of Cr for most isolates is therefore greater than 600 mg/L. Approximately half of the isolates tolerate 400 mg/L Cu, while only one isolate, ML-1, shows growth in the presence of 600 mg/L Cu (Table VI). Thus, 600 mg/L Cu represents the MIC for all isolates, excluding ML-1. For reference diameters of each isolate grown on control media, refer to Appendix E.

Table V. Growth of isolates in the presence of increasing concentrations of Cr. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals. "nd" refers to not determined. The MIC of Cr for most isolates is greater than 600 mg/L.

Strain Designation	Conc. in Isolation Media	LBA	Cr 25	Cr 50	Cr 100	Cr 200	Cr 400	Cr 600
ML-1	25 mg/L Cu	++++	++++	++++	++++	+++	-	-
ML-4	50 mg/L Cu	++++	++++	++++	++++	++	-	-
ML-6	100 mg/L Co	++++	++++	++++	++++	+++	+++	++
ML-7	100 mg/L Zn	++++	++++	++++	++++	+++	+++	++
ML-8	200 mg/L Cu	++++	+++	+++	++	++	+	-
ML-9	200 mg/L Zn	++++	++++	++++	++++	+++	+++	++
ML-10	200 mg/L Zn	++++	++++	++++	++++	+++	+++	nd
ML-14	400 mg/L Co	++++	++++	++++	++++	++++	++++	++
ML-15	400 mg/L Co	++++	++++	++++	++++	+++	+++	++
ML-16	400 mg/L Zn	++++	++++	++++	++++	+++	++	nd
CC-1	100 mg/L Co	++++	++++	++++	++++	+++	++	++
CC-2	100 mg/L Zn	++++	++++	++++	++++	+++	++	++
CC-3	200 mg/L Zn	++++	++++	++++	++++	++++	++	++
CC-4	200 mg/L Ni	++++	++++	++++	++++	+++	++	++
CC-6	200 mg/L Cr	++++	++++	++++	++++	++++	++	++
CC-7	400 mg/L Ni	++++	++++	++++	++++	++++	++	++
CC-8	400 mg/L Ni	++++	++++	++++	++++	++++	++++	nd
BL-3	200 mg/L Cr	++++	++++	++++	++++	+++	++	++
BL-4	200 mg/L Cr	++++	++++	++++	++++	++++	++++	++
BL-6	400 mg/L Ni	++++	++++	++++	++++	++++	++	+

Table VI. Growth of isolates in the presence of increasing concentrations of Cu. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals. The MIC of Cu for most isolates is 600 mg/L.

Strain Designation	Conc. in Isolation Media	LBA	Cu 25	Cu 50	Cu 100	Cu 200	Cu 400	Cu 600
ML-1	25 mg/L Cu	++++	++++	++++	++++	++++	++++	++++
ML-4	50 mg/L Cu	++++	++++	++++	++++	++++	++	-
ML-6	100 mg/L Co	++++	++++	++++	++++	++++	++	-
ML-7	100 mg/L Zn	++++	++++	++++	++++	++++	-	-
ML-8	200 mg/L Cu	++++	++++	++++	++++	++++	+++	-
ML-9	200 mg/L Zn	++++	++++	++++	++++	++++	+++	-
ML-10	200 mg/L Zn	++++	++++	++++	+++	+++	+++	-
ML-14	400 mg/L Co	++++	++++	++++	++++	++++	-	-
ML-15	400 mg/L Co	++++	++++	++++	++++	++++	+++	-
ML-16	400 mg/L Zn	++++	++++	++++	++++	++++	-	-
CC-1	100 mg/L Co	++++	++++	++++	++++	++++	-	-
CC-2	100 mg/L Zn	++++	++++	++++	++++	++++	+++	-
CC-3	200 mg/L Zn	++++	++++	++++	++++	++++	++++	-
CC-4	200 mg/L Ni	++++	++++	++++	++++	-	-	-
CC-6	200 mg/L Cr	++++	++++	++++	++++	++++	-	-
CC-7	400 mg/L Ni	++++	++++	++++	++++	+++	+++	-
CC-8	400 mg/L Ni	++++	++++	++++	++++	+++	+++	-
BL-3	200 mg/L Cr	++++	++++	++++	++++	++++	-	-
BL-4	200 mg/L Cr	++++	++++	++++	++++	-	-	-
BL-6	400 mg/L Ni	++++	++++	++++	+++	+	++	-

Many organisms growing on Cu-amended media display a dark orange-brown coloration (Fig. 12) that typically increases in intensity correlated to Cu concentration. This coloration is only observed on Cu supplemented media, and only by organisms belonging to the *B. cereus* group. While the majority of isolates show growth in the presence of 400 mg/L Co, no organisms are capable of growth at the next highest concentration tested, 600 mg/L; therefore, the MIC of Co lies between 400 and 600 mg/L (Table VII). As with Cu, approximately half of the isolates show growth in the presence of 400 mg/L Ni. Some of these isolates overlap with those displaying tolerance to the same concentration of Cu, though not all. Several isolates show growth at concentrations beyond 600 mg/L Ni as well (Table VIII). ML-1, ML-15, CC-7, and BL-6 can grow up to 600 mg/L Ni, and ML-15 and BL-6 also grow at 800 and 1000 mg/L Ni. Media

with concentrations exceeding 1000 mg/L were not prepared, and therefore the MIC for ML-15 and BL-6 was not determined. Lastly, approximately half of the isolates are capable of growth at 400 mg/L Zn, and eight of those strains also grow at 600 mg/L Zn (Table IX). The MIC of Zn for these organisms was not determined.

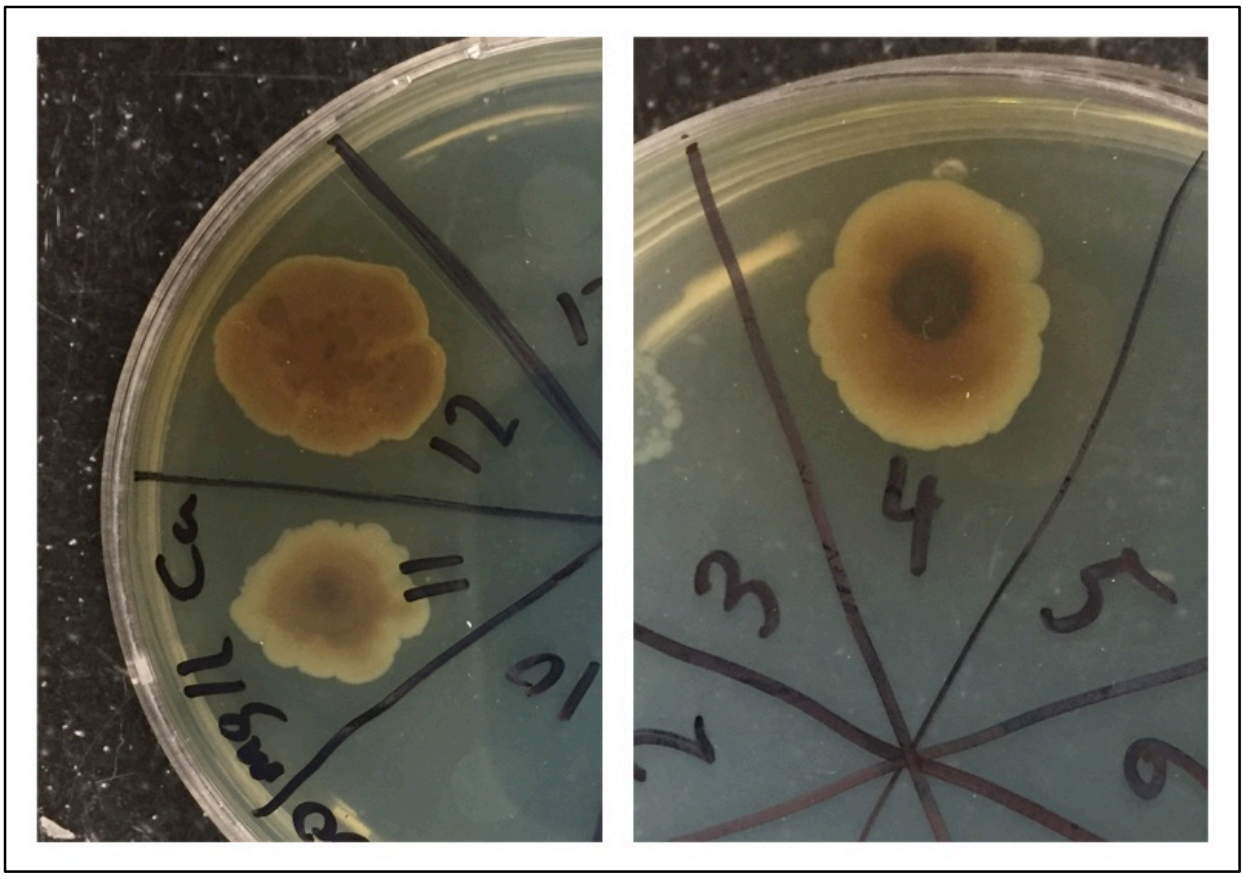

Figure 12. Bacteria exhibiting dark brown coloration on media containing 400 mg/L Cu. The organism in the left image is isolate CC-3, and the organism in the right image is isolate ML-6.

Table VII. Growth of isolates in the presence of increasing concentrations of Co. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals. The MIC for most strains is 600 mg/L.

Strain Designation	Conc. in Isolation Media	LBA	Co 25	Co 50	Co 100	Co 200	Co 400	Co 600
ML-1	25 mg/L Cu	++++	++++	++++	+++	+++	-	-
ML-4	50 mg/L Cu	++++	++++	++++	++++	++++	+++	-
ML-6	100 mg/L Co	++++	++++	++++	+++	+++	+	-
ML-7	100 mg/L Zn	++++	++++	++++	+++	+++	+	-
ML-8	200 mg/L Cu	++++	+++	+++	-	-	-	-
ML-9	200 mg/L Zn	++++	++++	++++	+++	+++	+++	-
ML-10	200 mg/L Zn	++++	++++	++++	+++	+++	+++	-
ML-14	400 mg/L Co	++++	++++	++++	++++	++++	-	-
ML-15	400 mg/L Co	++++	++++	++++	++++	++++	++	-
ML-16	400 mg/L Zn	++++	++++	++++	++++	++++	++	-
CC-1	100 mg/L Co	++++	++++	++++	+++	+++	+++	-
CC-2	100 mg/L Zn	++++	++++	++++	+++	+++	+++	-
CC-3	200 mg/L Zn	++++	++++	++++	+++	+++	+++	-
CC-4	200 mg/L Ni	++++	++++	++++	++++	+++	+++	-
CC-6	200 mg/L Cr	++++	++++	++++	++++	+++	+++	-
CC-7	400 mg/L Ni	++++	++++	++++	++	++	++	-
CC-8	400 mg/L Ni	++++	++++	++++	+++	++	-	-
BL-3	200 mg/L Cr	++++	++++	++++	+++	+++	++	-
BL-4	200 mg/L Cr	++++	++++	++++	++	++	++	-
BL-6	400 mg/L Ni	++++	++++	++++	++++	+++	+++	-

Table VIII. Growth of isolates in the presence of increasing concentrations of Ni. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals. "nd" refers to not determined. The MIC of Ni for most isolates is 600 mg/L.

Strain Designation	Conc. in Isolation Media	LBA	Ni 25	Ni 50	Ni 100	Ni 200	Ni 400	Ni 600	Ni 800	Ni 1000
ML-1	25 mg/L Cu	++++	++++	++++	++++	+++	+++	++	-	-
ML-4	50 mg/L Cu	++++	++++	++++	++++	+++	++	-	-	-
ML-6	100 mg/L Co	++++	++++	++++	++++	+++	++	-	-	-
ML-7	100 mg/L Zn	++++	++++	++++	++++	++++	-	-	-	-
ML-8	200 mg/L Cu	++++	++++	++++	++++	+++	-	-	-	-
ML-9	200 mg/L Zn	++++	++++	++++	++++	+++	-	-	-	-
ML-10	200 mg/L Zn	++++	++++	++++	++++	++++	-	-	-	-
ML-14	400 mg/L Co	++++	++++	++++	++++	+++	-	-	-	-
ML-15	400 mg/L Co	++++	++++	++++	++++	++++	++++	++++	++	+
ML-16	400 mg/L Zn	++++	++++	++++	++++	++++	++++	-	-	-
CC-1	100 mg/L Co	++++	++++	++++	++++	++++	++	-	-	-
CC-2	100 mg/L Zn	++++	++++	++++	++++	+++	-	-	-	-
CC-3	200 mg/L Zn	++++	++++	++++	++++	++++	++++	-	-	-
CC-4	200 mg/L Ni	++++	++++	++++	+++	++	-	-	-	-
CC-6	200 mg/L Cr	++++	++++	++++	++++	+++	-	-	-	-
CC-7	400 mg/L Ni	++++	++++	++++	++++	++++	++++	+++	-	-
CC-8	400 mg/L Ni	++++	++++	++++	++++	++++	++++	nd	nd	nd
BL-3	200 mg/L Cr	++++	++++	++++	++++	+++	++	-	-	-
BL-4	200 mg/L Cr	++++	++++	++++	++++	++	-	-	-	-
BL-6	400 mg/L Ni	++++	++++	++++	++++	++++	++++	++++	++	+

Table IX. Growth of isolates in the presence of increasing concentrations of Zn. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals. The MIC for most isolates is 400 mg/L.

Strain Designation	Conc. in Isolation Media	LBA	Zn 25	Zn 50	Zn 100	Zn 200	Zn 400	Zn 600
ML-1	25 mg/L Cu	++++	++++	+++	+	-	-	-
ML-4	50 mg/L Cu	++++	++++	++++	-	-	-	-
ML-6	100 mg/L Co	++++	+++	+++	+++	++	+	+
ML-7	100 mg/L Zn	++++	++++	++++	++++	++	+	+
ML-8	200 mg/L Cu	++++	++++	+++	+++	++	-	-
ML-9	200 mg/L Zn	++++	++++	++++	+++	++	++	+++
ML-10	200 mg/L Zn	++++	++++	++++	++++	++	++	-
ML-14	400 mg/L Co	++++	+++	++	+	+	-	-
ML-15	400 mg/L Co	++++	++++	++++	++++	++++	++	++
ML-16	400 mg/L Zn	++++	++++	++++	++++	++++	+	-
CC-1	100 mg/L Co	++++	++++	++++	+++	++	-	-
CC-2	100 mg/L Zn	++++	++++	++++	+++	++	-	-
CC-3	200 mg/L Zn	++++	++++	++++	+++	++	++	++
CC-4	200 mg/L Ni	++++	++++	+++	+++	++	-	-
CC-6	200 mg/L Cr	++++	+++	-	-	-	-	-
CC-7	400 mg/L Ni	++++	-	-	-	-	-	-
CC-8	400 mg/L Ni	++++	-	-	-	-	-	-
BL-3	200 mg/L Cr	++++	++++	++++	+++	++	++	++
BL-4	200 mg/L Cr	++++	++++	++++	++++	++++	+	+
BL-6	400 mg/L Ni	++++	++++	++++	+++	++	+	+

2.3.5 ML-15 and associated Ni-mineral identification

Multiple green crystalline precipitates (1-2 mm) were observed associated with bacterial biomass of isolate ML-15 on LBA plates supplemented with 600 mg/L Ni (Fig. 13). The crystals are not observed at lesser concentrations of Ni, in the presence of any other metal, or associated with any other organism. The observation was initially noted approximately 3 months post inoculation, after the culture plate was stored in a 15°C refrigerator. Thus, the exact length of time needed for growth of the crystals was initially not determined.

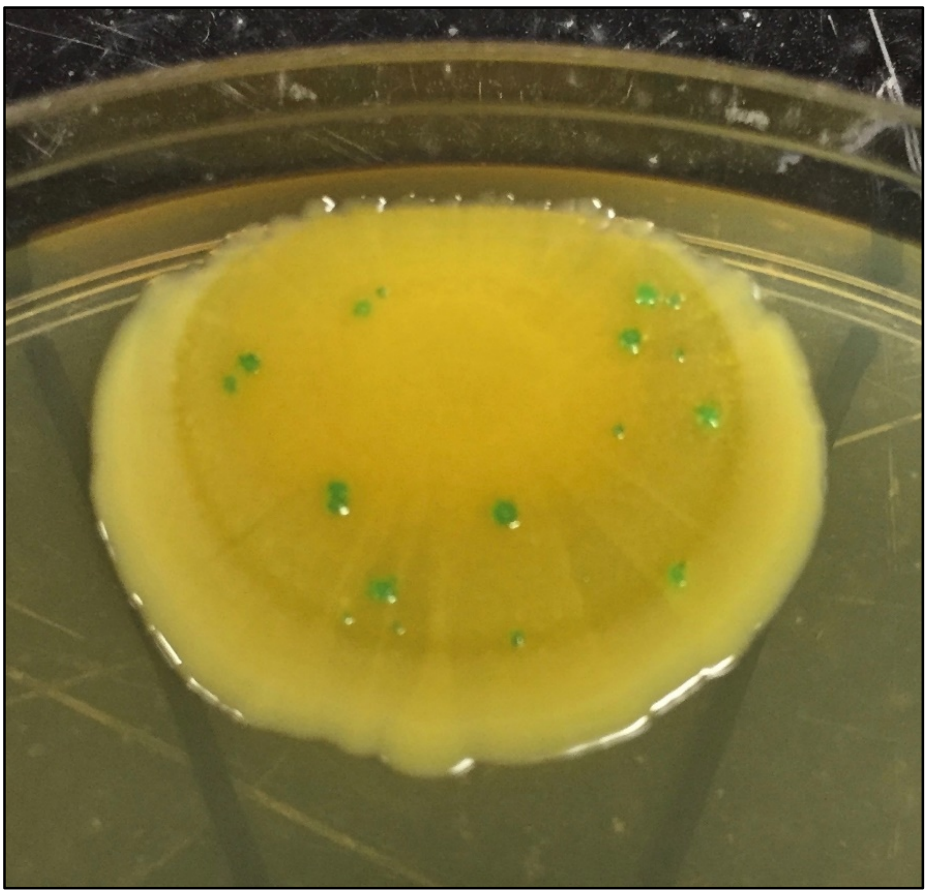

Figure 13. Green crystals (~1-2 mm) observed in association with bacterial growth of strain ML-15, *Microbacterium* sp.

The experiment was replicated with concentrations of 800 and 1000 mg/L Ni, and after 2 months of incubation at 25°C, small (~1-2 mm) green crystals are observed to form at these concentrations as well. Crystal formation time at 25°C can therefore be approximated as 2 months. It is important to note that crystals are observed only in the field of cellular growth of the organism, and are not present elsewhere on the agar surface. Individual crystals are half submerged within the agar, and could be plucked from the media with a sterile inoculating loop. Phase contrast microscopy indicated crystallinity (Fig. 14), and subsequent petrographic analysis with a polarizing lens enabled microscope showed crystal extinction with rotation of the stage (Fig. 15). Having confirmed the crystalline nature of the mineral, crystals were

next analyzed with X-ray powder diffraction. Based on the diffraction pattern, it can be concluded that the mineral is most likely a Ni-chloride hydrate (Fig. 16).

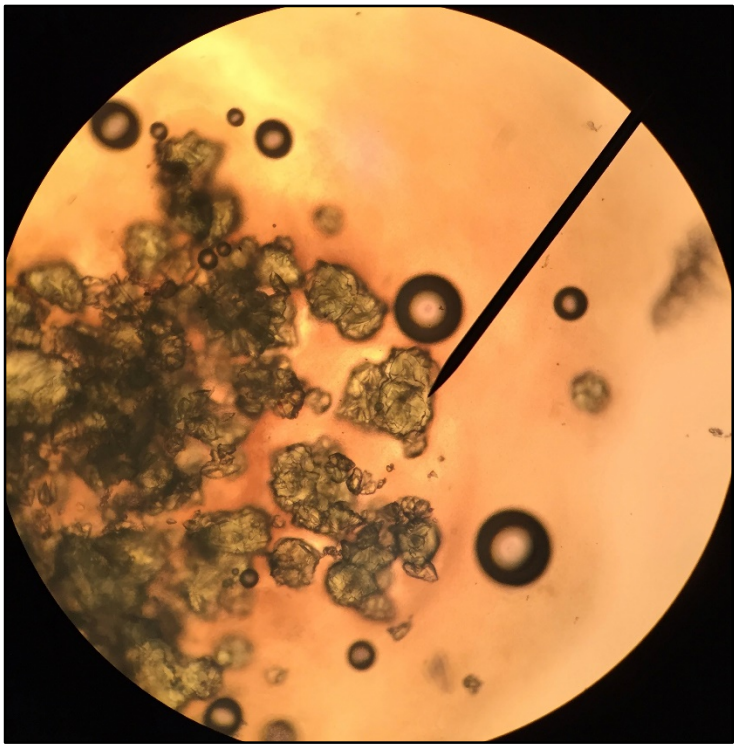

Figure 14. Phase contrast image at 100x magnification of crushed green crystals from ML-15 culture. Mineral grains indicate crystallinity.

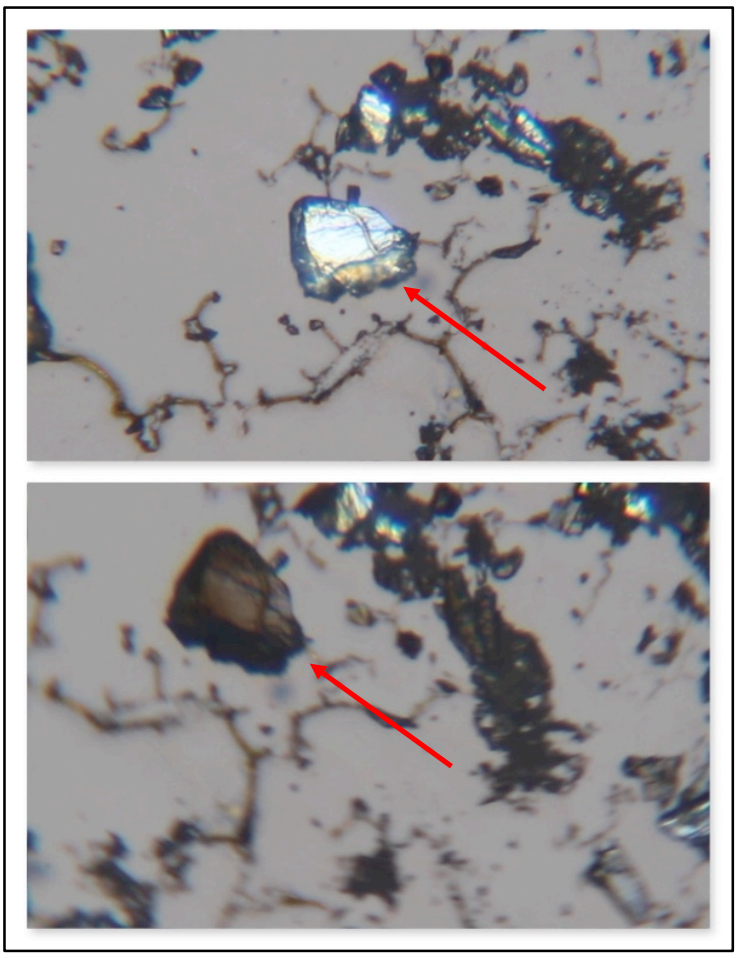

Figure 15. Red arrows indicate a single crystal produced by ML-15 viewed under a polarizing lens; top image shows plane polarized light passing through crystal structure, while the bottom image shows extinction with stage rotation.

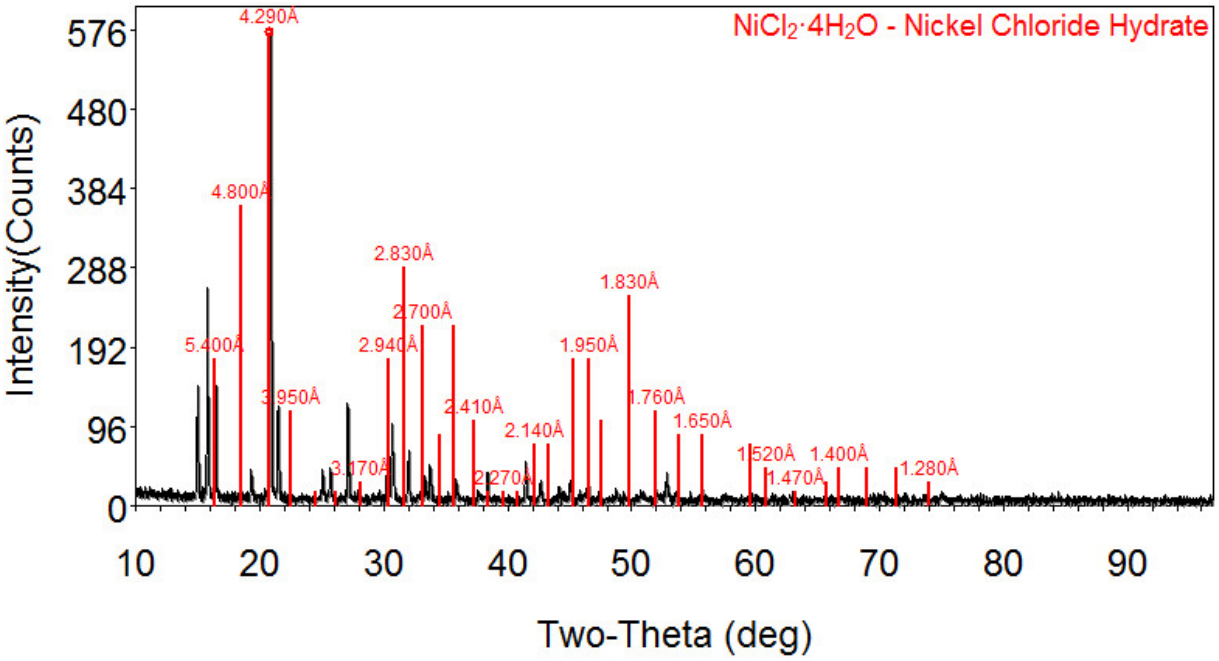

Figure 16. Powder diffraction pattern produced by X-ray analysis of green crystals associated with ML-15 growth on 600 mg/L Ni media. The name of the mineral with the closest matching pattern is displayed in the upper right corner.

2.3.6 Production of a secondary metabolite by isolate CC-8, Streptomyces misionensis

Isolate CC-8 was initially selected from isolation media based on the observation of a dark brown substance diffused into the agar surrounding the colony on 400 mg/L Ni media. Upon examination of tolerance to other metals, it is observed that production of the colored substance most consistently occurs in the presence of Ni (Fig. 17,18), and is only occasionally observed in the presence of Cu and Co. Color intensity increases with increasing concentrations of Cu and Ni. The coloration is not present on control LBA plates or media with added Cr and Zn.

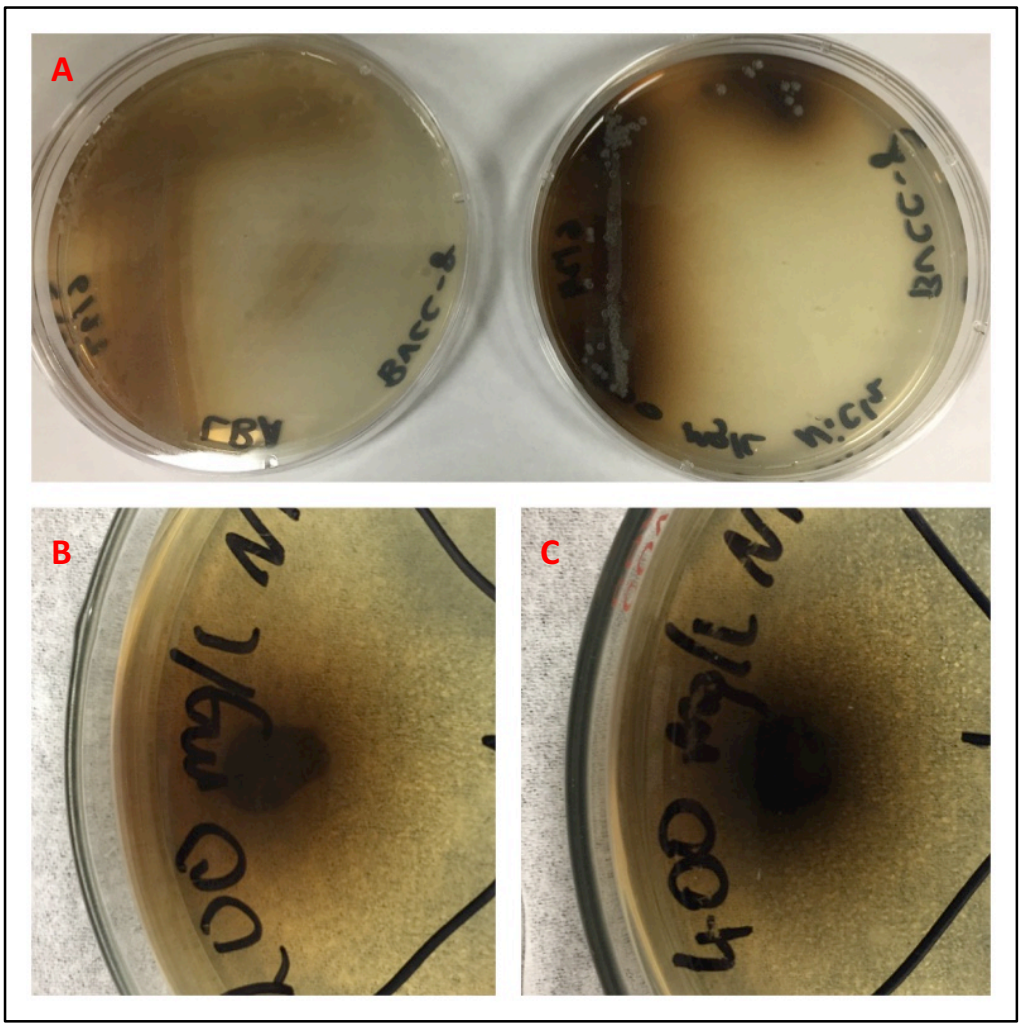

Figure 17. Comparisons of CC-8 growing on control and Ni-supplemented media; Dark coloration surrounding bacterial growth only occurs in the presence of metal and increases with metal concentration A) LBA control (left plate) vs. 200 mg/L Ni (right plate); B) 200 mg/L Ni; C) 400 mg/L Ni.

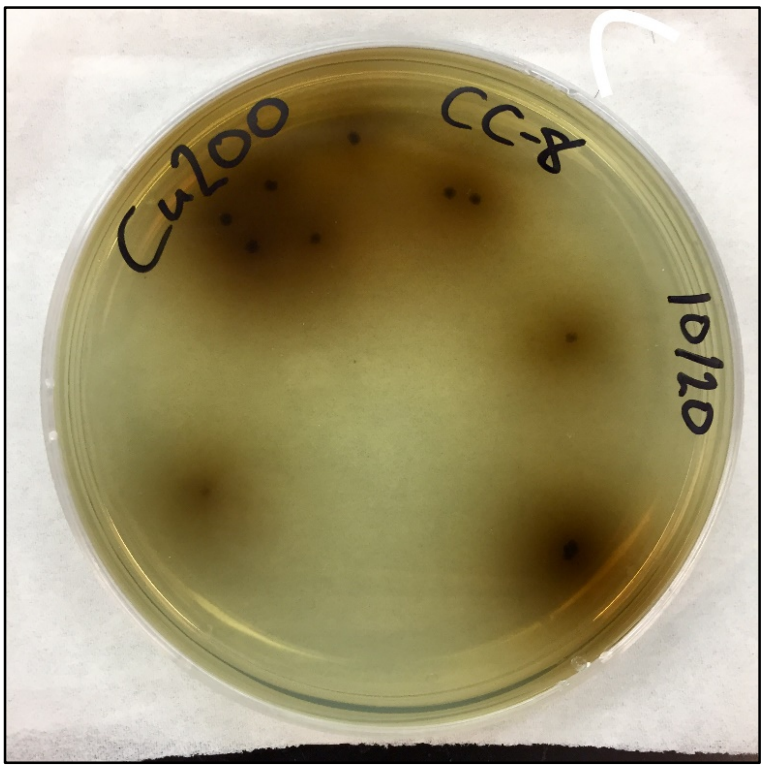

Figure 18. Dark brown coloration surrounding colonies of isolate CC-8 on LBA + 200 mg/L Cu.

2.3.7 Determination of metal removal capacity by isolates

Select organisms showing high tolerance to metals on solid media were chosen for transfer to liquid cultures modified with 200 mg/L of one of three metals – Cr, Ni, or Cu. After two weeks of incubation, several cultures showed visual changes. Notably, several Cu-containing cultures showed apparent precipitates in the form of a rusty brown powder on the bottom surface of the test tube, seemingly mixed with cell material. In comparison, Ni-containing cultures showed abundant cell growth but no discernible color changes or precipitates. Cultures growing in the presence of Cr generally exhibited turbidity, and some cultures showed a marked change in color. The Cr-supplemented YP media is initially bright yellow in color, but post incubation with certain isolates became a dull purple to colorless.

Representative cultures for each metal were selected for ICP analysis based on relative amounts of precipitate, growth, or color change. Of the analyzed samples, organisms growing in Cu show significant removal of Cu from solution (Table X). BL-3 exhibits the greatest potential for Cu removal, reducing the aqueous Cu concentration by approximately 44%. The other three isolates are capable of removing between 4% and 19% of Cu. Analysis of cultures grown in the presence of Cr did not show significant reduction in metal concentration, and were within the margin of error for the instrument (±10 ppm). Most isolates growing in the presence of Ni show minimal or no removal of the metal from solution. However, it is worth noting that ML-1 removed nearly the same amount of Ni as Cu (~9%), potentially implying a correlation with this organism's metal removal capacity.

Table X. Metal removal capacity of each isolate is represented as a percentage of total metal concentration; Most isolates growing in Cr and Ni media showed minimal metal removal, while more significant changes can be seen with BL-3 and ML-15 grown in Cu media. Asterisk indicates a potential instrumental error.

Isolate	Metal	Initial Concentration	Final Concentration	% Removal
CC-7	Cr	209.84	206.46	1.6
ML-7	Cr	209.84	205.02	2.3
ML-9	Cr	209.84	205.37	2.1
BL-6	Ni	211.41	204.1	3.5
CC-8	Ni	211.41	213.39*	n/a
ML-1	Ni	211.41	191.65	9.4
ML-15	Ni	211.41	210.37	0.5
BL-3	Cu	196.74	110.44	43.9
ML-1	Cu	196.74	179.59	8.7
ML-8	Cu	196.74	188.58	4.2
ML-15	Cu	196.74	160.59	18.4

2.3.8 TEM analysis of isolate BL-3

TEM results reveal the accumulation of Cu within cell membranes of strain BL-3 (Fig 19), previously noted as removing ~40% of Cu from liquid culture media. Electron-dense regions in the membrane correlate with EDS data showing a high concentration of Cu signals within the cell membrane and cytosol of bacterial cells grown in the presence of 200 mg/L Cu. These data confirm metal removal and sequestration by bacterial cells.

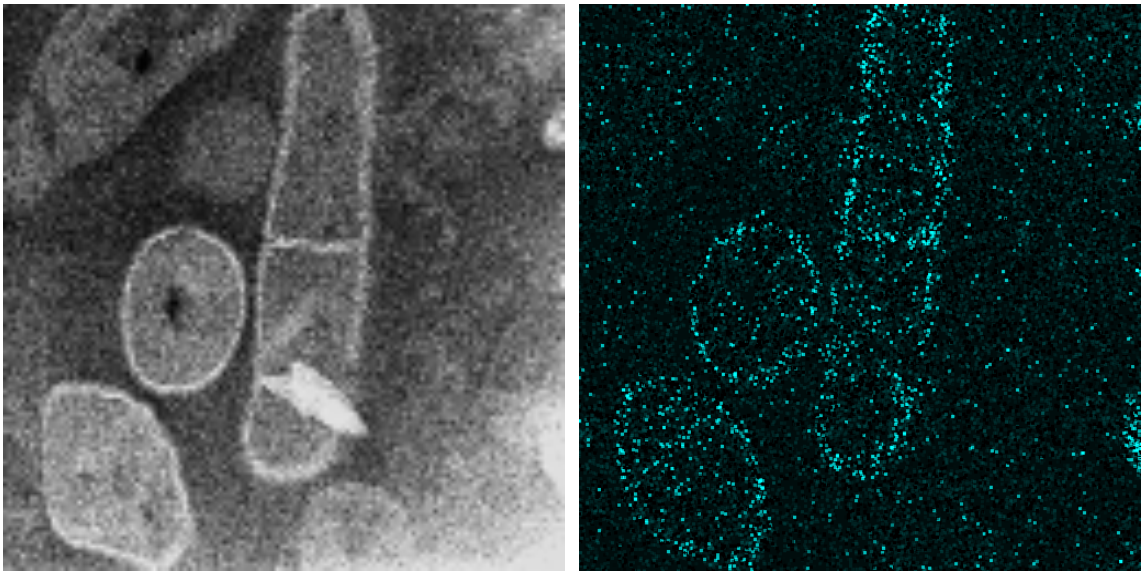

Figure 19. Left - Electron micrograph of cells of strain BL-3, Bacillus sp., grown in the presence of 200 ppm Cu; white areas seen in the cell membranes indicate electron-dense regions. Right – Cu signals as determined by Energy Dispersive Spectroscopy (EDS), showing higher concentration of Cu signals along the perimeter of and within cells.

2.3.9 Tolerance of isolates to alkalinity

All isolates are capable of growth at up to pH 10 on solid media, and most can tolerate the maximum pH tested (Table XI). The tolerance of all strains to pH 10 is worth noting, as this value is near the pH of the serpentinizing seep, recorded as 10.23 – 10.83. Above pH 10, growth of ML-8 and CC-8 was completely inhibited and several other strains showed decreased growth relative to the control. These results show that despite the neutral pH of the metal-amended media on which they were isolated, most strains show tolerance to a pH value greater than or equal to the environment of origin.

Table XI. Growth of isolates at varying pH; Control media was adjusted to pH 7; Most isolates show growth up to pH 12, including those obtained from an acidic site, indicated with asterisks.

Strain	Identity	Source	Source pH	Control	pH 8	pH 9	pH 10	pH 11	pH 12
ML-1	*Pseudomonas sp.*	ML2 source	10.83	++++	++++	++++	++++	++	+
ML-4	*Bacillus sp.*	ML2 source	10.83	++++	++++	++++	++++	++	++
ML-6	*Bacillus sp.*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
ML-7	*Bacillus sp.*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
ML-8	*Pseudomonas sp.*	ML2 source	10.83	++++	++++	++++	++++	-	-
ML-9	*Bacillus sp.*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
ML-10	*Microbacterium sp.*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
ML-14	*R. jialingiae*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
ML-15	*Microbacterium sp.*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
ML-16	*Microbacterium sp.*	ML2 source	10.83	++++	++++	++++	++++	++++	++++
CC-1	*Bacillus sp.*	CC1 outflow	10.23	++++	++++	++++	++++	++++	++++
CC-2	*Bacillus sp.*	CC1 outflow	10.23	++++	++++	++++	++++	+++	+++
CC-3	*Bacillus sp.*	CC1 outflow	10.23	++++	++++	++++	++++	+++	+++
CC-4	*Bacillus sp.*	CC1 outflow	10.23	++++	++++	++++	++++	+++	+++
CC-6	*Bacillus sp.*	CC1 outflow	10.23	++++	++++	++++	++++	+	+
CC-7	*Bacillus sp.*	CC1 outflow	10.23	++++	++++	++++	++++	++++	++++
CC-8	*S. misionensis*	CC1 outflow	10.23	++++	++++	++++	+	-	-
BL-3*	*Bacillus sp.*	Barlo Mine	4.2	++++	++++	++++	++++	++++	++++
BL-4*	*Bacillus sp.*	Barlo Mine	4.2	++++	++++	++++	++++	++++	++++
BL-6*	*Bacillus sp.*	Barlo Mine	4.2	++++	++++	++++	++++	++++	++++

2.4 Discussion

2.4.1 Environmental fluid chemistry and metal-tolerance

Fluids from Manleluag serpentinizing seep had extremely low to below detectable concentrations of metals relevant to this study. However, it has been shown that organisms displaying tolerance to high concentrations of metals can be cultured from this environment. As microorganisms typically develop adaptations to resist harsh conditions in their environment, an alternate explanation is needed for the observed tolerance to metals. A likely explanation may be that metals in this environment would precipitate out of solution at such alkaline pH, and thereby accumulate in sediments. As all isolates in this study were cultured from sediment slurries from the spring and its outflow, it is possible the sediments may be laden with metal compounds. While mineral forms of these metals would be far less toxic than mobilized aqueous species, they still represent a potential source of toxicity for organisms living in sediments of the spring. An alternate hypothesis is that these adaptations would have been developed through prolonged exposure to metal toxicity as fluid harboring these microorganisms circulated through the subsurface. If these organisms represent subsurface microbiota, the presence of ultramafic material in the subsurface would undoubtedly result in microbe-metal contact, as well as potential leaching and re-precipitation of mineral-derived metals. Similarly, surface weathering of serpentinite rocks in the area may also cause an influx of metal leachates into springs and outflows, thereby driving microbial adaptation as well. Considering the alkalinity of the environment, these metals would likely be bound in mineral, or possibly organic complexes. Thus, the inability of several isolates to remove metal ions from aqueous solution may be explained by the low natural concentrations of these metals. Furthermore, abundant strain growth on solid media containing high metal concentrations potentially supports the hypothesis that these organisms may be more adapted to coping with metals that are bound to organics.

It is also necessary to consider the formation of organometallic complexes in relation to both environmental chemistry and laboratory media preparation. It has been demonstrated that metal ions

tend to have a high affinity for organic ligands, resulting in reduction of metal bioavailability and overall toxicity (Ramamorthy and Kushner 1975; Zevenhuizen et al. 1979). Organic compounds in commonly used microbial growth media, including those used in this study, have been reported to have high binding capacities for metals. This issue is largely unavoidable when designing experiments, as microorganisms require organics for sustenance. However, the binding capacity is directly related to the concentrations of available metal ions and organic substrate; thus, beyond a certain concentration, organic binding sites are saturated and free ions would be present in solution. This is corroborated by a study conducted by Ramamoorthy and Kushner (1975). The study demonstrated that in one microbial culture medium, free Hg^{2+} is not present until concentrations exceed 160 ppm (mg/L); however, it was found that even concentrations of 10 ppm Hg^{2+} were inhibitory to aquatic bacterial organisms, although no free ions were present at that concentration. Therefore, it is supported that inhibitory effects by organometallic complexes, although lessened, still occur to a significant degree. This is especially true when considering the high concentrations used in the present study. One likely explanation for persisting toxicity is that bacterial cells must still metabolize metal-bound organics, and thus are required to cope with metals that enter the cytosol bound to their nutrient source. Thus, methods used for testing metal tolerance in this study are justified, both in terms of the natural environment and experimental design.

2.4.2 Metal removal capacity – ICP and TEM analyses

The data show that removal of Cr from aqueous solution does not occur in the analyzed cultures, and removal of Ni was very minimal (<4%) in all but one sample. Thus, speculation is required to clarify the mechanism of metal tolerance utilized by these microorganisms. If these isolates are not accumulating, adsorbing, or otherwise biomineralizing metals, the only reasonable assumption is that they possess a highly efficient efflux pump to expel toxic metals from cells. This hypothesis is strongly

supported by the observed abundant cellular growth in conjunction with completely unchanged concentrations of metals in the culture media.

ML-1 potentially represents an exception. It was demonstrated that this organism reduced both the Ni and Cu concentrations by roughly 9%. Though more data points are needed for confirmation, this potentially suggests a correlation between the results, especially when considering that metal concentrations in other cultures were typically only reduced by approximately 2%, a difference that is more likely attributed to instrumental error. It may therefore be postulated that ML-1 utilizes a nonspecific metal removal mechanism, such as passive cellular adsorption.

All examined isolates show some capacity to remove Cu from solution. Based on the observation of rust-brown precipitates collected on the bottom surface of test tubes, it is probable that Cu is being removed through biomineralization. An Eh/pH diagram of Cu^{2+} at 200 mg/L supports this possibility (Fig. 20). The diagram shows that at higher oxidation states and circumneutral pH, Cu exists in aqueous phases; furthermore, there is no phase change observed with an increase in pH as exhibited by cultures that alkalified media to a final pH of ~9. However, as the solution becomes more reducing, as would be typical during bacterial growth, the mineral phase cuprite is observed followed by elemental Cu^0 under the most reducing conditions. Thus, it can be reasonably assumed that any mineral precipitation is likely facilitated by microbial activity.

Electron microscopy analysis of cells grown in the presence of 200 mg/L Cu confirms these assumptions of biologically facilitated metal removal. Electron micrographs reveal electron-dense areas along the perimeter of cells of BL-3, and EDS confirms that the areas correspond to an accumulation of Cu. The oxidation state of the Cu could not immediately be determined, and thus requires further analysis to elucidate the type of metal transformation taking place.

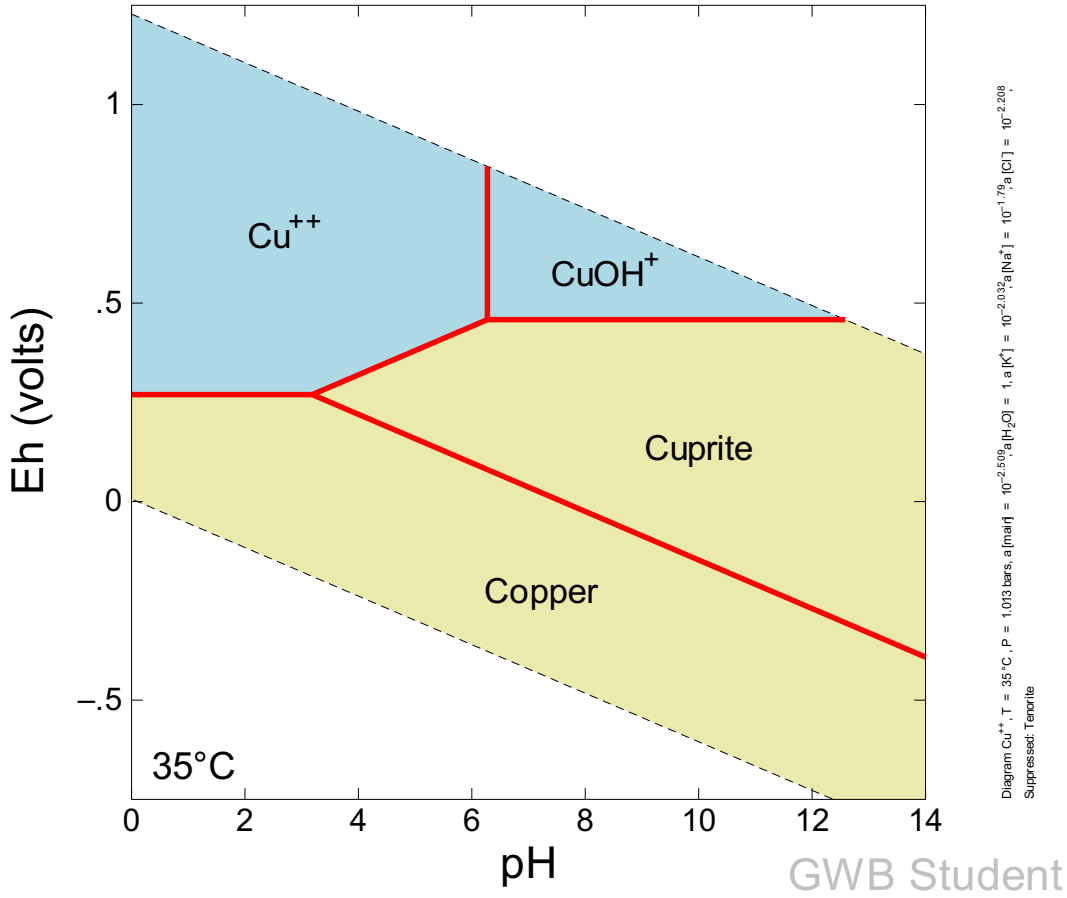

Figure 20. Eh/pH diagram of Cu speciation at 200 mg/L Cu, the concentration in culture media. As the solution becomes more reduced, cuprite is the first mineral phase, followed by copper in more reducing conditions. (The Geochemist's Workbench, Ed. 11.0)

2.4.3 Bacillus sp. and interactions with metals

The genus *Bacillus* consists of a highly diverse group of bacteria commonly found in extreme environments ranging from hot springs to spacecraft assembly clean rooms (La Duc et al. 2007). Therefore, it is no surprise that the majority of metal-tolerant isolates in this investigation are *Bacillus* species. Seven isolates obtained from this study have 16S rRNA sequences that are 99% similar to members of the *B. cereus* group, which includes nine other related species. Most isolates were capable of tolerating at least 200 mg/L concentrations of each metal that was tested. One of these strains, BL-6, could grow at 1000

mg/L Ni, the highest concentration prepared. Another isolate, BL-3 exhibited the largest capacity to remove metal ions from aqueous solution, reducing the Cu concentration in growth media by roughly half. TEM analysis of BL-3 cells grown in the presence of 200 mg/L Cu confirmed removal through accumulation of Cu in the membrane. These results demonstrate the potential for these isolates, and other organisms within this group, to be used in industrial and environmental applications.

The *B. cereus* group consists of many organisms previously cited as having a high tolerance to metals (Wang et al. 2015; Chen et al. 2016; Kumar et al. 2016a; Nayak et al. 2016; Wu et al. 2016). The results from this study further support findings that *Bacillus* species are commonly found in environments associated with physicochemical extremes, and so have adapted a uniquely robust set of mechanisms to enable them to cope with those conditions. These mechanisms have potential for applications in bioremediation and leaching of valuable metals from wastes. Studies have shown that strains of *B. cereus* can biomineralize Pb from mine tailings (Chen et al. 2016), remove Cd ions from polluted water (Wu et al. 2016), and oxidize arsenite to arsenate (Naureen and Rehman 2016). In addition, *B. thuringiensis* strains have previously been documented in bioremediation studies as being capable of removing metals like Cu, Co, Ni, and Ag from solution (Kumar et al. 2016a; Nayak et al. 2016). A recent study found that a strain of *B. toyonensis*, another member of the *B. cereus* group, cultured from marine sediments in South Africa produced a bioflocculant (Okaiyeto et al. 2015); bioflocculants are organic molecules produced by microorganisms that are capable of flocculating various contaminants, and therefore have a potentially wide range of applications, including heavy metal removal. Although strains from this study were not specifically examined for bioflocculant production, this prior discovery suggests one potential mechanism of metal tolerance implemented by members of this group.

Other *Bacillus* species were also represented by isolates obtained from this study. Interestingly, BL-4's closest relatives, *B. stratosphericus* and *B. altitudinis*, are organisms initially isolated from high altitudes between 24 and 41 km and can tolerate high levels of UV radiation (Shivaji et al. 2006), though

there is no prior evidence for metal tolerance. CC-7 shares 99% of its 16S rRNA gene sequence with *B. firmus*, a species known to display resistance to metal toxicity. A previous study found that a *B. firmus* strain produced a polysaccharide capable of binding and removing metal ions from aqueous solution (Salehizadeh and Shojaosadati 2003). Another strain of *B. firmus* isolated from tannery effluent has been shown to reduce hexavalent Cr and oxidize As (Bachate et al. 2013). CC-6 shares 100% 16S rRNA gene sequence similarity with *Bacillus aryabhattai*, an organism incidentally also isolated from the upper atmosphere, and since been found in environments ranging from fish guts to soils and waste water (Arora et al. 2016; Dey 2016). More notably however, *B. aryabhattai* is well-known as a multi-tasking bacterium, having been described in previous studies as displaying a range of biotechnological applications including As bioremediation, conversion of lignocellulosic waste into valuable resources, treatment of waste dyes in the textile industry, and production of the aromatic compound vanillin (Paz et al. 2016a, b; Singh et al. 2016). Additionally, strains of this species have been shown to tolerate high concentrations of Cu and accumulate hexavalent Cr from water (Verma et al. 2014; Mesa et al. 2015). Considering the genetic homology and demonstrated tolerance to Cu and other metals, isolate CC-6 warrants further examination to thoroughly elucidate its potential utility.

2.4.4 Microbacterium sp. and Ni-biomineralization

The mineral precipitate on the agar media is interesting for many reasons. XRD analysis determined that the mineral may be a Ni-chloride hydrate. This is possibly a compound identical or related to the metal salt used in the media preparation, $NiCl_2 \cdot 6H_2O$. Although there are examples of *Pseudomonas* species capable of reducing aqueous Ni^{2+} to elemental Ni^0 (Zhan et al. 2012), and even *Microbacterium* capable of synthesizing NiO nanoparticles from $NiSO_4$ (Sathyavathi et al. 2014), there are no published examples of biomineralization of a $NiCl_2$ mineral. Furthermore, the occurrence of this mineral on solid media is intriguing, as most studies describe metal biomineralization in aqueous conditions. It may be that

the mineral precipitation is not directly facilitated by the bacterium, but by conditions created by bacterial growth, such as an increase in alkalinity. However, this does not provide an adequate explanation as many isolates were observed to increase alkalinity when final pH was measured in metal-supplemented liquid media (refer to Appendix B). CC-7 and BL-6 exhibited abundant growth at 600 mg/L Ni, and both were noted to increase alkalinity in liquid culture; yet, no crystals were associated with either organism. Hence, it is more likely that isolate ML-15 is in some way involved in the synthesis of the mineral. Even more confounding is that ML-15 exhibits the least metal removal capacity of all tested cultures, indicating that intracellular accumulation and precipitation is not likely. Based on the ICP data, one hypothesis may be postulated. As the final concentration of Ni in liquid culture with ML-15 was virtually unchanged, the organism may have a highly efficient efflux pump for unwanted intracellular Ni. Additionally, the presence of Cl^- in growth media has been largely ignored, but is present in large quantities, as there are two Cl^- ions present for every one metal ion in the salts used for media preparation. During growth, isolate ML-15 was certainly ingesting nutrient-metal complexes from the growth media, and may have been subsequently expelling Ni^{2+} and possibly Cl^-. Millions of cells exhibiting this behavior in a small area on a dry surface may have led to the concentration of Ni^{2+} and Cl^-, and eventually to the crystallization of Ni-chloride, possibly with cells as nucleation points. Further investigation is needed to confirm this hypothesis.

2.4.5 Streptomyces misionensis and secondary metabolite production

Isolate CC-8 displays the unique characteristic of releasing a brown, water-soluble substance that increases in response to metal concentration, but is not produced in response to every metal tested. The most abundant production of this substance is observed in the presence of Ni, followed closely by Cu. Though the molecular character of the substance could not be determined in this study, literature regarding *Streptomyces* organisms offers a likely explanation. These bacteria are renowned for the production of antibiotics, but many *Streptomyces* species are also capable of producing melanin, an

organic pigment composed of chains of phenolic compounds, in response to stressors such as metals (Raytapadar et al. 1995; Plonka and Grabacka 2006; Haferburg and Kothe 2007). Melanin has a strong affinity for metal cations, and is probably the mechanism of metal tolerance utilized by members of this genus. The mechanism acts in the same way that formation of organometallic complexes binds free metal cations and reduces toxicity. This also explains the increase in CC-8's pigment production with increasing metal concentration, as more melanin would be required to bind a greater quantity of toxic metal cations. Another piece of supporting evidence is that the concentration of Ni in CC-8 culture was unchanged (data suggests a slight increase in Ni concentration after growth of the culture, but this is likely a result of instrumental error). Melanin is a soluble compound, and thus binding of metals by this pigment would not remove cations from solution, explaining the constant concentration. Additionally, melanin is also known to act as an electron donor or acceptor, so melanogenesis may also be involved in microbial metabolism in some cases (Plonka and Grabacka 2006).

Considering the evidence, it may be reasonably concluded that this organism is synthesizing extracellular melanin as a defense mechanism against metal toxicity. Specifically, a brown or black melanin is categorized as a eumelanin, produced via the oxidation of tyrosine (Plonka and Grabacka 2006). Confirmation of the production of eumelanin by CC-8 may be achieved through the use of high performance liquid chromatography (HPLC), and must be considered for future work.

2.4.6 Tolerance to alkalinity

All isolates exhibit growth at the highly alkaline pH of the natural serpentinizing environment (10.8). Additionally, most organisms show robust growth up to pH 12. These results suggest that the isolates obtained in this study do represent a subset of the natural microbial community, and the majority are well-suited to survive extreme alkalinity. It is interesting to note that CC-8 and ML-8 exhibit a sharp cessation of growth beyond pH 10, while ML-1, ML-4, and CC-6 all show diminished growth at pH 11 and

12. This suggests that these isolates may be narrowly adapted to the conditions of this environment and, while most can tolerate higher pH, it negatively impacts growth of others.

2.5 Conclusion

Organisms isolated in this investigation represent diverse taxa that consist of many known extremophiles, especially those belonging to the genus *Bacillus*. 16S rRNA gene sequence analysis determined that several species that are closely related to the isolates have been previously documented as metal-tolerant and interact with metals using a variety of mechanisms, including intracellular metal sequestration and biomineralization. The isolates in this study also display a diversity of metal tolerance mechanisms. Considering the ICP data, it can be presumed that most of the strains likely use an efflux pump to regulate intracellular metal concentrations. However, in the presence of Cu, several strains show the capacity to biomineralize Cu minerals from aqueous solution, a process with strong implications in bioremediation of metal contaminated environments and biomining valuable metals from mine tailings. Isolate ML-15, a *Microbacterium* sp., demonstrated the novel ability to biomineralize a Ni-chloride mineral from solid agar media, a feat that is unprecedented to the best of the author's knowledge. This process not only has implications in bioremediation, but possibly in production of chemical reagents for industrial use as well. Lastly, isolate CC-8, a strain closely related to *Streptomyces misionensis*, produced the secondary metabolite melanin to chelate metals and reduce toxic stress; microbial melanin may play an important role in studying organometallic complexes and their mobility through the environment. Additionally, the type strain of *S. misionensis* was originally described as producing misionin, an antibiotic active against phytopathogenic fungi. As many *Streptoymyces* organisms are renowned for the production of secondary metabolites, especially in the presence of metals, isolate CC-8 warrants further examination for production of antibiotics in addition to melanin.

The results demonstrate that alkalitolerant, multi-metal resistant bacteria are present and can be cultured from natural serpentinizing environments. Although trace metal concentrations in source fluids were generally determined to be too low for detection, there are potential alternate sources of metal stress. These include subsurface fluid-rock interactions or surface weathering of ultramafic material and subsequent deposition into serpentinizing springs and outflow channels of the Manleluag seep. As the metals used in this study are commonly associated with serpentine environments, the metal-resistant bacterial isolates have implications in understanding the local serpentinizing ecosystem and in biotechnology.

III. Multi-metal Resistant Bacteria Isolated from Hydrothermal Springs, Yellowstone National Park, WY

3.1 Introduction

Hydrothermal environments are characterized by numerous physicochemical extremes that seemingly make them inhospitable to life. Yet, microorganisms have adapted to tolerate such conditions through the development of various structural and metabolic adaptations. Because of the geochemical and physical extremes, the survival mechanisms of hydrothermal communities are appropriately robust and efficient. The functions of such microorganisms are of significant interest from a biogeochemical standpoint, but are also particularly useful in biotechnology.

Hot springs are commonly characterized by acidic conditions, increasing the solubility of metal ions and thereby leaching elements from the rock. Many organisms in hydrothermal environments have adapted to take advantage of metals by using them as electron acceptors for metabolism, while others accumulate and precipitate toxic metals in the cell periplasm (Lovley and Coates 2000; Rathgeber et al. 2002; Ghosh et al. 2003; Meyer-Dombard et al. 2012). Organisms displaying the ability to biomineralize or otherwise sequester metals at high temperatures offer new potential strategies for removing highly toxic elements like uranium and chromium from contaminated water. In addition, metal-extracting bioreactors in the biomining industry operate at high temperatures and extreme acidity. Thus, hydrothermal environments are ideal sites in which to study microbial metal tolerance in relation to applications in bioremediation and biometallurgy.

The purpose of this study is to enumerate heterotrophic, metal tolerant bacteria from hydrothermal springs in Yellowstone National Park, and to isolate pure strains for further investigation of metal tolerance mechanisms for potential applications in biotechnology.

3.2 Materials and methods

3.2.1 Study sites

Samples were collected from the outflows of two hydrothermal springs in Yellowstone National Park (YNP). The first is named Iron Fist Outflow in South Rabbit Creek and the second is named Figure 8 outflow in located in the Greater Obsidian Pool Area. Samples were collected and stored as described in Chapter I section 1.6.

All methods and analyses performed in this investigation are identical to those described in "Multi-metal resistant, alkalitolerant bacteria isolated from a serpentinizing seep." For all culturing methods and analyses performed, refer to Chapter II section 2.1. For microbial community analysis of environmental samples, refer to Appendix D.

3.3 Results

3.3.1 ICP data for metals of interest in environmental samples

In general, previous geochemical data shows that all metals relevant to this study exist in extremely low concentrations in environmental samples from Figure 8 and Hell's Gate, a proxy for Iron Fist outflow (see Chapter I, section 1.6). For select years from 2009-2011, Zn occurred in the highest concentrations at both sites, while Co generally exhibits the lowest concentration (Table XII).

Table XII. Trace metal data from Figure 8 and "Hell's Gate," a proxy for Iron Fist outflow for 2009-2011. Data provided by Dr. Everett Shock, Arizona State University.

Site	Year	Cr (ppb)	Ni (ppb)	Co (ppb)	Cu (ppb)	Zn (ppb)
Figure 8	2010	0.29	0.34	bdl	0.105	10.6
Figure 8	2011	0.79	0.9	0.25	0.15	21
Hell's Gate	2009	bdl	0.18	0.062	0.58	9.03
Hell's Gate	2011	0.02	0.103	0.048	0.164	23.9

3.3.2 Cultivation of metal-tolerant bacteria from environmental samples

In general, a greater number of metal-tolerant organisms were cultured from Iron Fist outflow than Figure 8. Additionally, on control media with no metals, Iron Fist exhibited a confluent lawn of bacterial growth, while Figure 8 showed four large, morphologically similar colonies. This trend was consistent for both sites at concentrations of metals below 100 mg/L of metals. The exception was media containing Cu; at the lowest concentration tested, no growth was observed from Figure 8. In comparison, Iron Fist exhibited reduced colony forming units at 25 mg/L Cu relative to the control media, but colonies were large, approximately 5-10 mm in diameter (Fig. 21). All concentrations of Cr resulted in greater than 300 densely-packed colonies from Iron Fist, but only an average of 5 colonies from Figure 8. Interestingly, the number of colonies from both sites generally did not decrease with increasing concentration of Cr; however, the colony diameter gradually decreased with increasing Cr concentration. The same phenomenon was also observed on Co media, the difference being that no growth from either site was observed beyond a concentration of 100 mg/L Co (Fig. 22). Iron Fist also exhibited growth of organisms up to a maximum of 200 mg/L Ni and 400 mg/L Zn. In contrast, Figure 8 did not show bacterial growth at any concentration of Zn, and only on media containing up to a maximum of 100 mg/L Ni.

It is important to note that morphological differentiation in colonies was extremely limited in the presence of all metals, as well as control media. This consideration is significant in relation to the taxonomic identities of the selected isolates described in the next section.

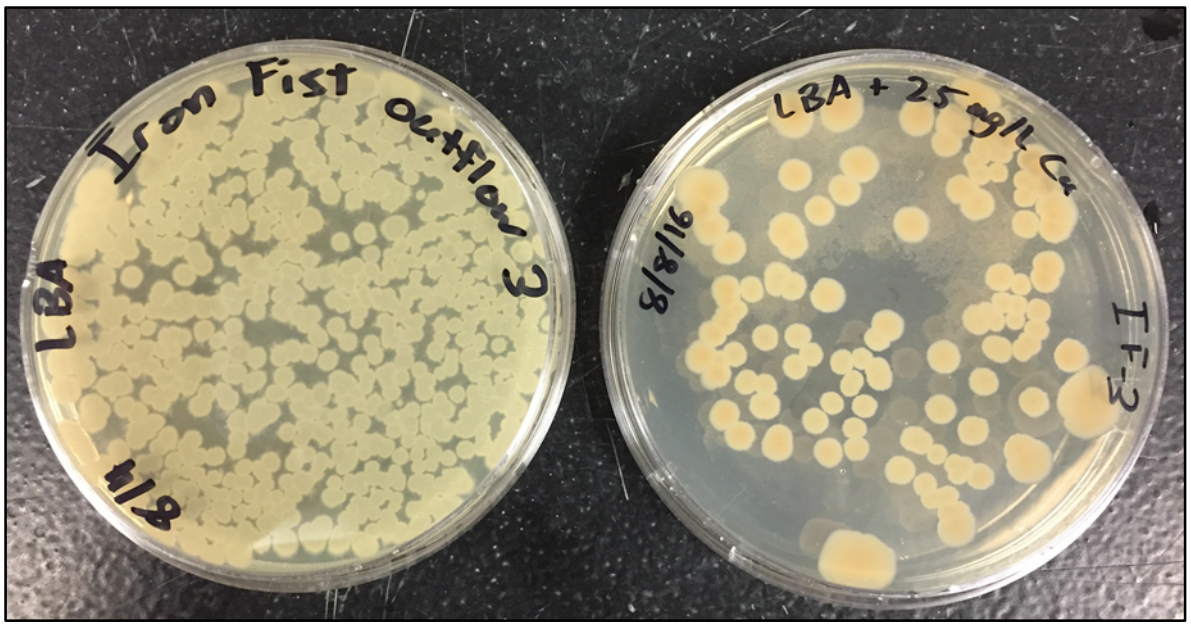

Figure 21. Comparison of bacterial growth from Iron Fist outflow on LBA control (left) vs. LBA + 25 mg/L Cu (right).

Figure 22. Comparison of bacterial growth from Iron Fist outflow in the presence of increasing concentrations of Co. Plates from left to right: LBA + 25 mg/L Co; LBA + 50 mg/L Co; LBA + 100 mg/L Co.

3.3.3 Isolate Taxonomy

The low morphological diversity of organisms growing on metal-amended media is explained by 16S analysis of eight pure isolates. Seven of the eight strains share 100% 16S rRNA gene sequence with a single species, *Bacillus wiedmannii*, a member of the *Bacillus cereus* group. The remaining isolate is also closely related, but its 16S rRNA gene sequence is more similar to *Bacillus cereus* (Table XIII).

Table XIII. Isolate identities determined by 16S sequence. 'Isolation Media' indicates the concentration of metal and metal type in the media from which the strain was isolated.

Strain	Source	Closest Taxon	# of Bases	Seq. Similarity (%)	Isolation Media
FE-1	Figure 8 Outflow	*Bacillus wiedmannii*	586	100	50 mg/L Co
FE-2	Figure 8 Outflow	*Bacillus wiedmannii*	943	100	100 mg/L Co
FE-3	Figure 8 Outflow	*Bacillus cereus*	670	100	100 mg/L Ni
IF-1	Iron Fist Outflow	*Bacillus wiedmannii*	913	100	400 mg/L Cr
IF-3	Iron Fist Outflow	*Bacillus wiedmannii*	836	100	100 mg/L Co
IF-5	Iron Fist Outflow	*Bacillus wiedmannii*	691	99	200 mg/L Ni
IF-6	Iron Fist Outflow	*Bacillus wiedmannii*	570	100	25 mg/L Cu
IF-8	Iron Fist Outflow	*Bacillus wiedmannii*	846	100	200 mg/L Zn

3.3.4 Examination of isolates for cross metal tolerance

All isolates show marked tolerance to each of the five metals examined in this study. Although virtually all isolates had identical 16S rRNA gene sequences, each displayed subtle differences in specific metal tolerance. Every strain grew at 600 mg/L Cr, the highest concentration tested (Table XIV). Thus, the minimum inhibitory concentration (MIC) of Cr was not determined in this investigation. Cu appears to have the greatest inhibitory effect on organism growth (Table XV); the MIC of Cu for seven of the eight strains is 400 mg/L. In the presence of Ni and Co, roughly half of the isolates displayed tolerance to 400 mg/L (Table XVI, XVII). All strains exhibit growth at 400 mg/L Zn (Table XVIII). Generally, 600 mg/L

represents the MIC for all strains in the presence of all metals tested, excluding Cr. For reference diameters of each isolate grown on LBA, refer to Appendix E.

Table XIV. Growth of isolates in the presence of increasing concentrations of Cr. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals.

Strain	Conc. in Isolation Media	LBA	Cr 25	Cr 50	Cr 100	Cr 200	Cr 400	Cr 600
FE-1	50 mg/L Co	++++	++++	++++	++++	++++	+++	++
FE-2	100 mg/L Co	++++	++++	++++	++++	++++	+++	++
FE-3	100 mg/L Ni	++++	++++	++++	++++	++++	+++	++
IF-1	400 mg/L Cr	++++	++++	++++	+++	+++	++	++
IF-3	100 mg/L Co	++++	++++	++++	+++	+++	++	++
IF-5	200 mg/L Ni	++++	++++	++++	++++	++++	+++	++
IF-6	25 mg/L Cu	++++	++++	++++	++++	+++	+++	++
IF-8	200 mg/L Zn	++++	++++	++++	++++	+++	+++	++

Table XV. Growth of isolates in the presence of increasing concentrations of Cu. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals.

Strain	Conc. in Isolation Media	LBA	Cu 25	Cu 50	Cu 100	Cu 200	Cu 400	Cu 600
FE-1	50 mg/L Co	++++	++++	++++	++++	++++	-	-
FE-2	100 mg/L Co	++++	++++	++++	++++	-	-	-
FE-3	100 mg/L Ni	++++	++++	++++	++++	-	-	-
IF-1	400 mg/L Cr	++++	++++	++++	++++	++++	-	-
IF-3	100 mg/L Co	++++	++++	++++	++++	++++	+++	-
IF-5	200 mg/L Ni	++++	++++	++++	++++	-	-	-
IF-6	25 mg/L Cu	++++	++++	++++	++++	++++	-	-
IF-8	200 mg/L Zn	++++	++++	++++	++++	++++	-	-

Table XVI. Growth of isolates in the presence of increasing concentrations of Co. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals.

Strain	Conc. in Isolation Media	LBA	Co 25	Co 50	Co 100	Co 200	Co 400	Co 600
FE-1	50 mg/L Co	++++	++++	++++	++	+	-	-
FE-2	100 mg/L Co	++++	++++	++++	++	+	-	-
FE-3	100 mg/L Ni	++++	++++	++++	+++	+	-	-
IF-1	400 mg/L Cr	++++	++++	++++	++	+	-	-
IF-3	100 mg/L Co	++++	++++	++++	++	+	-	-
IF-5	200 mg/L Ni	++++	++++	++++	++	++	+	-
IF-6	25 mg/L Cu	++++	++++	++++	+++	+++	+++	-
IF-8	200 mg/L Zn	++++	++++	++++	+++	+++	+++	-

Table XVII. Growth of isolates in the presence of increasing concentrations of Ni. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals.

Strain	Conc. in Isolation Media	LBA	Ni 25	Ni 50	Ni 100	Ni 200	Ni 400	Ni 600
FE-1	50 mg/L Co	++++	++++	++++	++++	+++	-	-
FE-2	100 mg/L Co	++++	++++	++++	++++	+++	-	-
FE-3	100 mg/L Ni	++++	++++	++++	++++	+++	-	-
IF-1	400 mg/L Cr	++++	++++	++++	++++	+++	++	-
IF-3	100 mg/L Co	++++	++++	++++	++++	+++	++	-
IF-5	200 mg/L Ni	++++	++++	++++	++++	+++	++	-
IF-6	25 mg/L Cu	++++	++++	++++	++++	+++	++	-
IF-8	200 mg/L Zn	++++	++++	++++	++++	+++	++	-

Table XVIII. Growth of isolates in the presence of increasing concentrations of Zn. Concentrations are in mg/L. Growth score is relative to observed growth on LBA without metals.

Strain	Conc. in Isolation Media	LBA	Zn 25	Zn 50	Zn 100	Zn 200	Zn 400	Zn 600
FE-1	50 mg/L Co	++++	++++	++++	+++	+++	++	-
FE-2	100 mg/L Co	++++	++++	++++	+++	+++	++	-
FE-3	100 mg/L Ni	++++	++++	++++	++++	+++	++	-
IF-1	400 mg/L Cr	++++	++++	++++	+++	++	++	-
IF-3	100 mg/L Co	++++	++++	++++	+++	++	++	-
IF-5	200 mg/L Ni	++++	++++	++++	+++	++	++	-
IF-6	25 mg/L Cu	++++	++++	++++	+++	++	++	-
IF-8	200 mg/L Zn	++++	++++	++++	+++	++	++	-

3.3.5 Morphological variation in the presence of metals

Many strains exhibit various coloration in response to the presence of Ni and Cu. Furthermore, the color typically intensifies with increasing metal concentration. On plates supplemented with Cu, strains exhibit an orange-brown color that darkens with increasing Cu concentration (Fig. 23); no coloration is observed on control media without metal, or in the presence of any other metals like Zn or Cr. The same organisms also display a dark green coloration in the presence of one specific concentration of Ni, at 200 mg/L; interestingly, this is not observed in the presence of any other concentration of Ni (Fig. 24,25). Phase contrast microscopy showed that cells growing in the presence of 200 mg/L Ni are associated with numerous dark particles several times the size of an average cell, and are not present when viewing cells grown on LBA without Ni (Fig. 26).

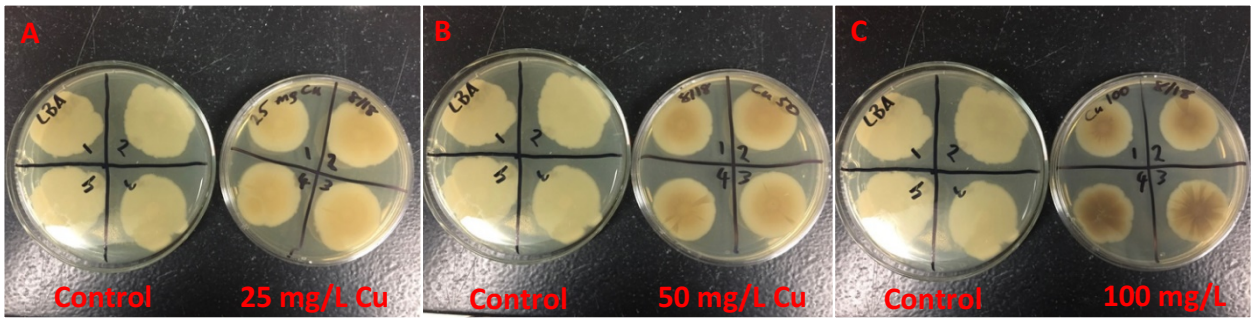

Figure 23. Growth of four *B. wiedmannii* strains on LBA control (left plate in A, B, and C) vs increasing concentration of Cu (right plate in A, B, and C). The orange-brown pigmentation darkened with increasing Cu concentration.

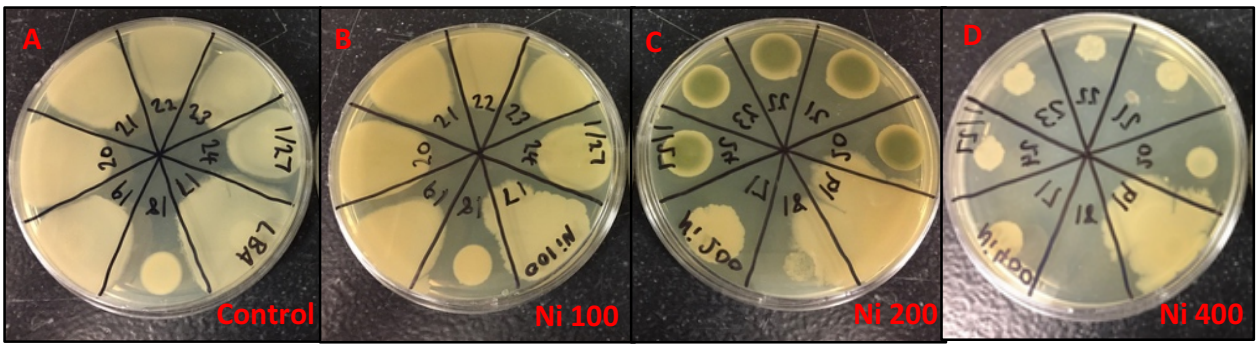

Figure 24. Growth of five *B. wiedmannii* strains (numbered 20-24 in the top half of the Petri plate) on various media A) LBA control; B) 100 mg/L Ni; C) 200 mg/L Ni; D) 400 mg/L Ni. Green pigmentation was observed only on media with 200 mg/L Ni.

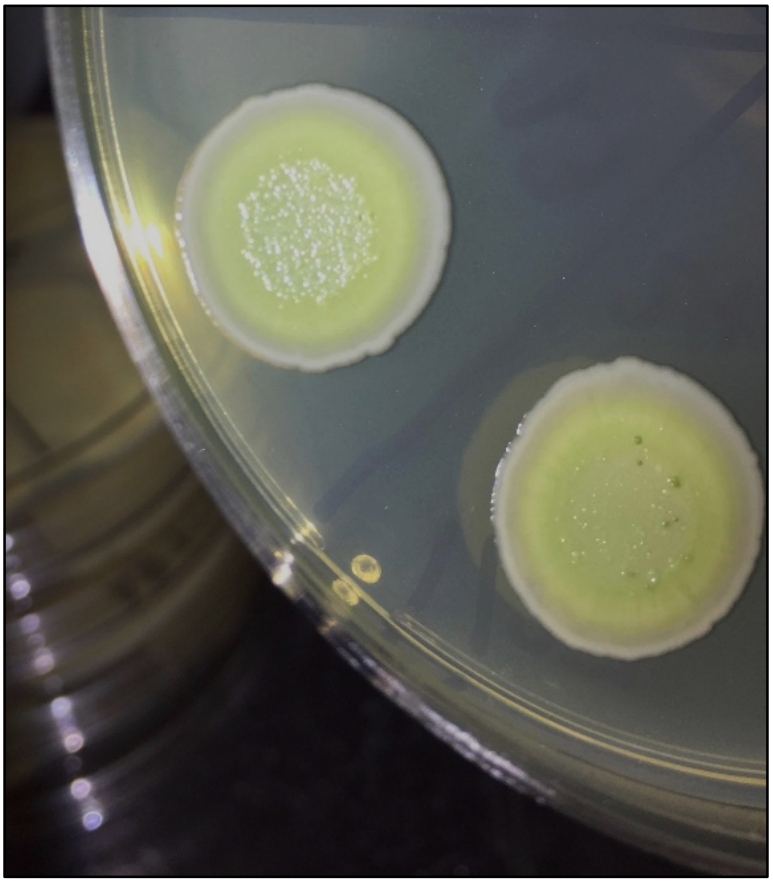

Figure 25. Green pigmentation exhibited by two *B. wiedmannii* isolates growing in the presence of 200 mg/L Ni

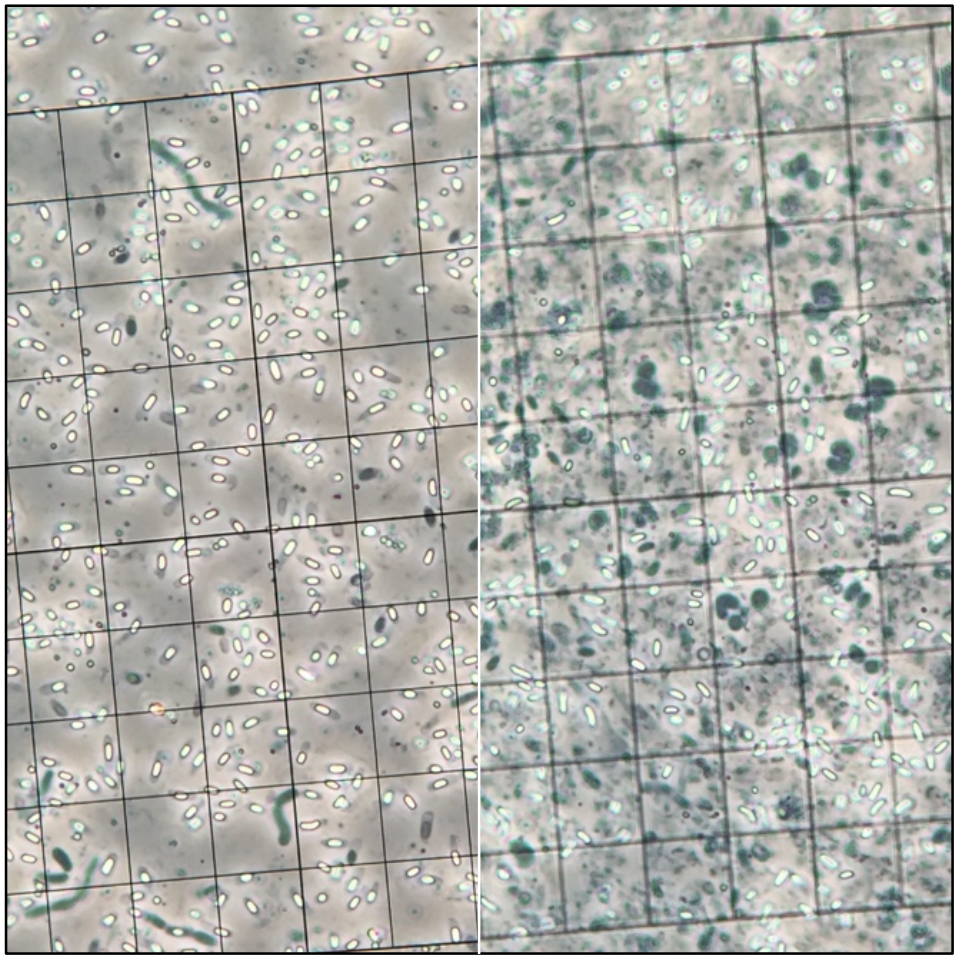

Figure 26. Phase contrast images of microbial biomass from IF-5, a *B. wiedmannii* isolate, grown on LBA (left) vs in the presence of 200 mg/L Ni (right). Cells grown on Ni-supplemented media are associated with numerous refractive particles not observed with cells grown on LBA.

3.3.6 Metal removal capacity of isolate IF-3

B. wiedmannii strain, IF-3, was examined for ability to biomineralize or otherwise remove Ni from aqueous solution. ICP analysis of culture filtrate determined that there was no significant decrease in metal concentration facilitated by IF-3, and no observable precipitates were noted in culture media. Initial Ni concentration in media was measured to be 211.41 mg/L; post-growth of IF-3, the concentration was

measured to be 207.94 mg/L Ni. This results in a 1.7% total reduction in Ni concentration, and is well within the margin of error for the instrument.

3.4 Discussion

A significant dichotomy between growth of organisms from Iron Fist and Figure 8 is evident from the observed results. In all cases, even when no metals were present, a greater number of bacteria could be cultivated from Iron Fist than Figure 8 with the given culturing conditions. Furthermore, colonies observed on all media, especially those containing metals, appeared morphologically identical. In fact, it has been previously shown that using elevated trace metal concentrations to culture thermophiles from hot springs in Yellowstone results in a limited diversity of organisms (Meyer-Dombard et al. 2012). Considering that the concentrations used in the present study are far beyond typical levels used in cultivation, and that organisms were cultured in mesophilic conditions, an even lower bacterial diversity may be expected. Unsurprisingly, 16S rRNA gene sequence analysis confirmed that three isolates from Figure 8 and five isolates from Iron Fist all share nearly identical sequences and essentially belong to one species. However, between different strains of the same species, considerable variability is observed in metal tolerance. This demonstrates that these organisms may be more diverse than is evidenced by 16S rRNA gene sequencing alone. A recent study found that a psychrotolerant bacterium isolated from raw milk collected from a dairy processing plant was closely related to the *Bacillus cereus* group based on 16S rRNA sequence; however, significant differences in phenotype and whole genome analysis warranted the classification of this organism as a new species within the *B. cereus* group named *Bacillus wiedmannii* (Miller et al. 2016). This is the same organism with which seven of eight isolates share 100% sequence similarity, the eighth being more closely related to *B. cereus*. Considering the stark difference in environment of origin alone, it is a reasonable assumption that phenotypic characteristics and whole genome analysis may very well distinguish one or more of these isolates as novel species. This study marks the first isolation of metal-tolerant *B. wiedmannii* strains from an acidic hydrothermal spring.

Various coloration exhibited by isolates in the presence of metals is particularly intriguing. On Cu-containing media, the positive correlation between intensity of the brown color and Cu concentration suggests potential intracellular accumulation or biomineralization of Cu minerals in cells. The only prior example of such a process by organisms grown on solid media is from a study published in 1993 involving the intracellular accumulation of Cu by *Pseudomonas syringae* (Cooksey 1993). However, in this study intracellular Cu accumulation resulted in blue pigmentation by *P. syringae*, rather than brown. In addition, the investigators added Cu in the form of $CuSO_4$, and thus Cu mineralization may have been coupled to SO_4^{2-} reduction, which contrasts with methods used in this study. There are many examples of sulfate-reducing bacteria capable of precipitating aqueous Cu as sulfide minerals (Panchanadikar and Kar 1993; Jalali 2000; White and Gadd 2000; Dave et al. 2010), though this does not apply to the present study as no sulfate was introduced into the media. Thus, it can only be presently speculated that intracellular Cu accumulation and precipitation may be occurring in these organisms. This may be confirmed through the use of Energy Dispersive X-ray Spectroscopy (EDXA) and Transmission Electron Microscopy (TEM).

Similarly, green coloration by *Bacillus* organisms growing on Ni-supplemented media is so far undocumented to the best of the author's knowledge. Furthermore, it is anomalous that the color is only exhibited at a specific concentration of Ni. One possibility is suggested considering the overwhelming presence of Ni and the specific color of the biomass. Ni-oxide minerals are green in color and have even shown to be synthesized by metal-tolerant *Microbacterium* sp. (Sathyavathi et al. 2014). However, the study in question found NiO biosynthesis in liquid media supplemented with 2000 mg/L $NiSO_4$, and is therefore an insufficient explanation of the present results. Phase contrast images showing the presence of relatively large particles associated with cells may be considered further support for the possible extracellular precipitation of Ni-associated minerals. The particles are two to three times the size of an average cell and may indicate mineral-encrusted cells or aggregates of a Ni mineral formed by expulsion of Ni from the cytoplasm. Further support for this hypothesis may be provided by a previous study

describing the secretion of Se from cells of *Pseudoalteromonas* grown on Se-supplemented agar media (Rathgeber et al. 2002). In this study, the Se globules appeared as light-refractive particles visible in phase contrast microscopy, and bear resemblance to results shown here. As with the potential Cu precipitates, EDXA and SEM may confirm this hypothesis.

The remarkable degree of metal tolerance exhibited by organisms from these hydrothermal environments suggests a high degree of adaptation. However, metal concentrations in such springs are orders of magnitude less than even the lowest concentrations used in this study (refer to Table XII), and so another explanation must be proposed. Typical trace metal concentrations in various springs in Yellowstone are known to vary widely depending on pH and temperature and may range from a few hundred to thousands of micrograms per liter (Spear et al. 2005), bordering on the lower end of concentrations used in this study. Therefore, it is expected that these organisms would able to tolerate relatively low concentrations of these metals at any given time in their natural habitat. However, it may be possible that the tolerance mechanism used by these microorganisms functions successfully until the concentration exceeds the efficiency of the mechanism, and thus results in organism death. For example, ICP analysis of culture filtrate from Ni-supplemented media inoculated with strain IF-3 showed no change in Ni concentration. This suggests the use of an ion pump that functions efficiently at concentrations around 200 mg/L, but beyond 400 mg/L, IF-3 is completely inhibited from growth. It is reasonable then to assume that an efflux pump regulating low concentrations of intracellular trace metals would become overburdened at the highest concentrations used in this study. Similarly, the exhibited tolerance to multiple metals may further indicate non-specificity in the expulsion of these ions via an efflux pump, especially when considering that these metals are all divalent cations.

3.5 Conclusion

The results show that highly metal tolerant organisms exist and can be cultured from hydrothermal springs in Yellowstone National Park. Microbial diversity, however, is very limited with respect to the implemented culturing conditions. All organisms cultivated were highly related, and all but one belong to the species *Bacillus wiedmannii*. This study represents the first isolation of metal-tolerant *B. wiedmannii* strains from acidic hot spring environments. Furthermore, these organisms may represent potentially useful strains in biotechnology when considering the possibility of intracellular accumulation and biomineralization of metal complexes. However, further analysis must be performed to confirm the nature of the proposed mineral precipitates, and elucidate any industrial potential exhibited by these organisms.

IV. Cellulolytic Bacterial Communities from an Alkaline Serpentinizing Spring in the Zambales Ophiolite, the Philippines

4.1 Introduction

Most primary production on Earth results in the synthesis of cellulose, making it one of the most important organic molecules on the planet. The base structure of cellulose consists of thousands of glucose molecules connected by β-1,4 glycosidic bonds arranged in a linear chain. This structural homogeneity allows for crystallization through hydrogen bonding between multiple chains, and results in the formation of tightly packed microfibrils which can then twist into rope-like structures for additional rigidity. This structure causes cellulose to be highly resistant to degradation, and may even allow for the preservation of cellulosic biomass for millions of years in optimal conditions (Wilson 2011). Whereas the general composition of cellulose is simple, heterogeneity in chemical components results in a suite of related compounds. The most common heteropolymers of cellulose are hemicellulose and lignin, which are composed of multiple types of sugars arranged with a higher degree of structural complexity. Hemicellulose is the second most abundant organic compound following cellulose, and is composed of up to five different sugar monomers with a branching structure. Lignin has a highly heterogeneous composition and complex structure, making it the most recalcitrant compound in the cellulose family.

Considering that cellulose is the most abundant organic compound, it follows that the microorganisms capable of degrading this substrate play a critical role in global carbon cycling. Bacteria have evolved to utilize enzymes known as cellulases to catalyze the breakdown of cellulose, but no single enzyme is sufficient for complete degradation. Cellulolytic bacteria have developed a suite of enzymes that synergistically deconstruct cellulose and make it accessible for metabolism. Cellulases may be generally categorized as endo- or exoglucanases. Endoglucanases randomly cleave segments of the chain, and thereby produce new ends upon which exoglucanases can attach to further break down segments into cellobiose, the disaccharide subunit of cellulose (Lynd et al. 2002). The final enzyme that cleaves free

subunits is a glucosidase, also known as cellobiase. The specific mechanism by which these enzymes are implemented, however, may vary between organisms. For example, aerobic cellulolytic bacteria release cellulases in copious concentrations that begin deconstructing the cellulose structure in unison, while anaerobes use a cellulosome, an extracellular multi-enzyme system anchored to a single structural protein.

Due to the numerous enzymes required and structural soundness of cellulose, complete hydrolysis to CO_2 and H_2O is not easily achieved by natural microbial communities, much less single organisms (Schwarz 2001). Fortunately, many intermediate products of cellulose degradation are valuable resources. Sugars like glucose and starch produced from partial digestion of cellulose are useful as food products or chemical commodities, but fermentation of these sugars to produce bioethanol is currently of greater interest. With growing concern over the continued use of fossil fuels, cellulose represents a cheap and readily accessible source of renewable energy. However, there are still many challenges to overcome in order to make cellulose a feasible alternative fuel source. Mainly, researchers seek to accelerate the slow rate of enzymatic hydrolysis and to discover a single-step process for direct conversion of biomass to fuel (Schwarz 2001; Lynd et al. 2002). Further study of cellulolytic microorganisms from diverse habitats and discovery of new effective enzymes is required to overcome these challenges.

Cellulolytic bacteria are physiologically diverse and can be found in a variety of habitats including soils, aquatic environments, and intestinal tracts of animals and insects (Cross 1981; Martin et al. 1991; Kopecný et al. 2004; Russell et al. 2009). While many studies focus solely on thermophilic cellulose-degraders due to their function in high-temperature bioreactors, mesophilic cellulose-degrading bacteria offer the benefit of lower energy costs associated with bioreactor operation (Rastogi et al. 2009). Examples of prominent mesophilic cellulolytic bacteria include *Bacillus*, *Ruminococcus*, *Micromonospora*, and *Streptomyces* (Lynd et al. 2002).

The present study seeks to investigate mesophilic cellulolytic microbial communities in alkaline serpentinizing springs to elucidate which consortia are most abundant at high pH. Alkaline cellulose degradation has applications in many industries, including paper processing, detergent manufacturing, and biofuel production (Fujinami and Fujisawa 2010; Vilanova et al. 2012). Results from this study have strong implications in expanding strategies for culturing target organisms for alkaline cellulolysis.

4.2 Materials and Methods

4.2.1 Enrichment of cellulolytic bacteria in alkaline conditions

Environmental samples from sites ML2 and CC1 in Manleluag Springs National Park, Luzon, the Philippines were used for inoculation of enrichment cultures. For site descriptions and sampling information, refer to Chapter I, section 1.5.

Cellulolytic bacteria from environmental samples were cultured using a basal salts medium supplemented with cellulose as the sole carbon source. The recipe for the basal salts medium was adapted from Mohagheghi et al. (1986), and altered for the purposes of this study. In 1 L solution: 1.0 g NH_4Cl; 1.0 g KH_2PO_4; 0.1 g $Na_2HPO_4 \cdot 7H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; $CaCl_2 \cdot 2H_2O$; 0.5 g yeast extract; 1.25 g SigmaCell cellulose, microcrystalline 20 um particle size (Sigma-Aldrich); 1.25 g D-(+)-cellobiose (Sigma-Aldrich). For alkaline cultures, pH was adjusted with 10 M NaOH to values of 8, 9, 10, and 12. 10 mL of cellulose media was dispensed into Balsch tubes, and 100 uL of sediment-fluid slurry from environmental samples was used for inoculation. For anaerobic cultures, gas headspace was flushed with 95% N_2, 5% H_2. Cultures were incubated at 35°C. Gas headspace was refreshed at 7 days, and experiments were incubated for a total of 14 days.

4.2.2 Community analysis of environmental samples and enrichment cultures

DNA was extracted from environmental samples and enrichment cultures using PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). PCR amplification of gDNA was performed using 27F/1492R primers, and products were verified with agarose gel electrophoresis. Next Generation Sequencing (NGS) of the 16S rRNA gene was carried out by DNA Services at the University of Illinois at Chicago. Region-specific primers (515F/806R) were used to amplify a segment of the 16S rRNA gene. FASTQ files were merged using PEAR (Paired-End Read Merger), and were then imported to CLCBio for quality and length trimming. A Q20 quality trim was performed and sequences <225 bp were discarded. Sequences were run through a QIIME pipeline (Caporaso et al. 2010). Chimeras and 515F/806R primers were removed from the dataset, and sequences were clustered and taxonomically classified. For relative abundance charts, OTU's with less than 1000 sequence reads were discarded.

4.2.3 Isolation and identification of cellulolytic bacteria from environmental samples

Cellulolytic bacteria were isolated using minimal (Mm) agar medium supplemented with carboxymethylcellulose (CMC) powder. Mm media consists of the following (in g/L): 1.0 g $NaNO_3$; 1.0 g K_2HPO_4; 0.5 g $MgSO_4$; 1.0 g KCl; 0.25 g yeast extract; 7.5 g bacteriological agar (Sigma-Aldrich); 5.0 g carboxymethylcellulose (Sigma-Aldrich). The pH was adjusted to 7.2. Media was autoclaved at 250°C for 40 min. 100 µL aliquots of environmental samples were spread onto Mm-CMC agar with a sterile L-spreader, and incubated at 35°C for 7 days. Cellulase activity of colonies was determined using the Congo red staining technique as described by Meddeb-Mouelhi et al. (Meddeb-Mouelhi et al. 2014). CMC agar plates were flooded with 0.2% Congo red solution and allowed to stain for 15 min. Congo red solution was then decanted, and agar was de-stained by rinsing 3x with 1 M NaCl. Transparent clearings observed surrounding colonies demonstrates the inability of Congo red to bind with hydrolyzed CMC, and is indicative of endoglucanase activity by organisms. As Congo red is toxic to bacteria, colonies were

preselected and transferred to fresh Mm-CMC agar prior to staining. Colony locations were marked on Petri plates and given specific designations. After staining, selected isolates that were observed to produce a hydrolytic clearing zone were chosen for further analysis.

For procedures involving DNA extraction, 16S rRNA sequencing, and phylogenetic analyses of bacterial isolates, refer to Chapter II, section 2.2.4.

4.2.4 Screening of isolates for cellulose degradation in alkaline conditions

Isolated bacterial strains were examined for potential to utilize CMC in alkaline conditions. Mm-CMC agar media was adjusted to pH 8, 9, 10, and 11 using 10 M NaOH. Cell suspensions for each isolate were prepared from stock plate cultures, and 10 μL aliquots were spotted onto alkaline Mm-CMC plates. Plates were incubated at 35°C for 7 days. Cellulolytic activity was determined by presence of transparent clearing zones surrounding bacterial growth using the Congo red staining technique previously described.

4.3 Results

4.3.1 Community analysis of serpentinizing environments

16S rRNA gene sequences reveal diverse phyla in environmental samples, and considerable variation between sites ML2 and CC1 (Fig. 27). The most abundant phyla in ML2 include *Actinobacteria* (~30%) and *Bacteroidetes* (~28%), followed by *Proteobacteria* (~15%) and *Firmicutes* (~10%). In contrast, CC1 is dominated by two main phyla, *Thermi* (~40%) and *Proteobacteria* (~35%), while *Actinobacteria* and *Bacteroidetes* represent <10% of the community. Total number of assembled sequences are shown in Table XIX in section 4.3.2.

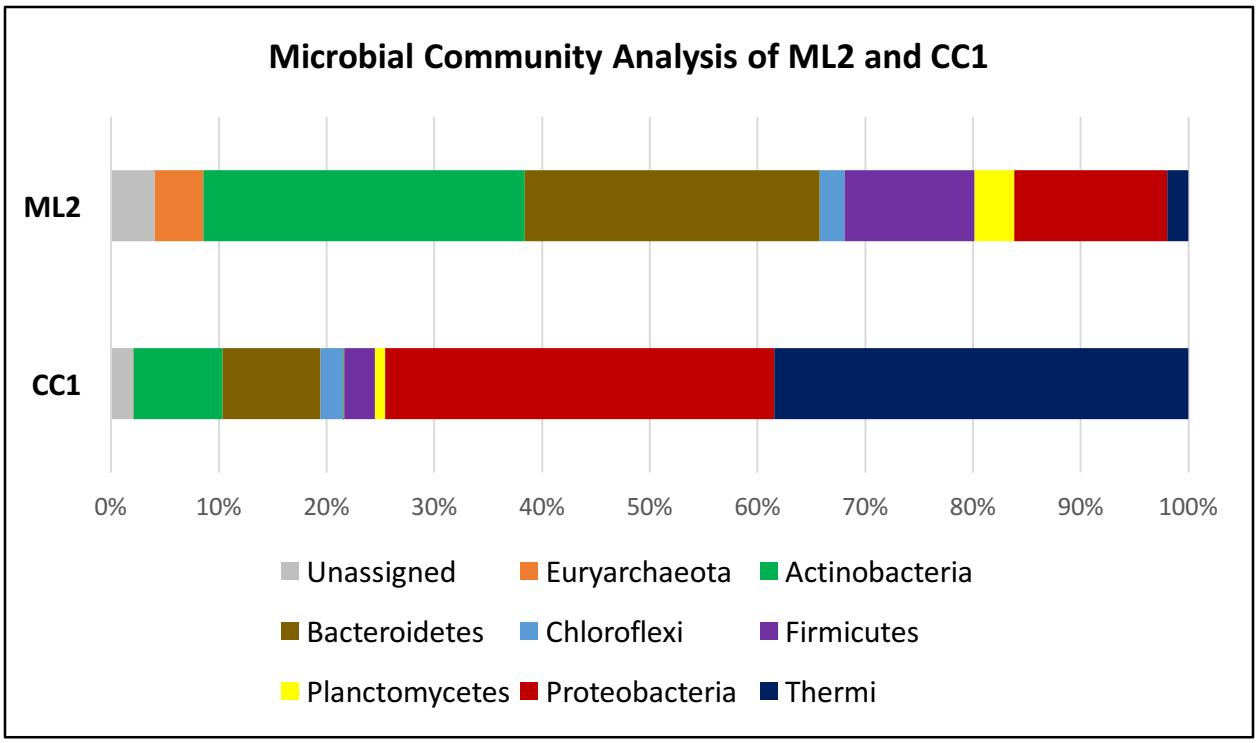

Figure 27. Relative abundances of various phyla in environmental samples. "ML2" refers to Manleluag serpentinizing spring (source). "CC1" refers to a site 10 m down the outflow channel of ML2.

4.3.2 Community analysis of alkaline cellulolytic enrichment cultures

Turbidity and other visual changes were observed in aerobic and anaerobic cellulolytic cultures growing in alkaline conditions. Anaerobic cultures at pH 12 inoculated with ML2 exhibit black to dark green particles (1-2 mm) in lieu of the white cellulose powder initially added to the medium; no other cultures show morphological alteration of cellulose powder. Gas production is also demonstrated in many aerobic culture tubes as evidenced by the presence of bubbles in liquid; approximately 2 – 4 mL of gas was collected via syringe from ML2 and CC1 cultures at pH 8 – 10, while there was no discernible gas production at pH 12. Gases were not analyzed for composition.

Total number of assembled sequence reads for each culture and environmental samples are shown in Table XIX.

Table XIX. Total number of sequences obtained from 16S rRNA gene sequencing before and after quality trimming and chimera removal. Information is shown for environmental samples (env) and cellulolytic enrichment cultures.

Sample	Initial	Quality Trimmed
Manleluag 2 (env)	30762	30643
Caustic Cascade 1 (env)	60217	60051
CC1 pH 8 (aerobic)	64597	61800
CC1 pH 9 (aerobic)	75771	75087
CC1 pH 10 (aerobic)	86403	82798
CC1 pH 12 (aerobic)	81358	73449
ML2 pH 8 (aerobic)	112777	111790
ML2 pH 9 (aerobic)	107722	105840
ML2 pH 10 (aerobic)	123836	121908
ML2 pH 12 (aerobic)	94036	83059
CC1 pH 8 (anaerobic)	75155	72857
CC1 pH 9 (anaerobic)	71462	66231
CC1 pH 10 (anaerobic)	71331	69237
CC1 pH 12 (anaerobic)	114989	104953
ML2 pH 8 (anaerobic)	84706	81908
ML2 pH 9 (anaerobic)	127882	125002
ML2 pH 10 (anaerobic)	87334	80878
ML2 pH 12 (anaerobic)	99926	89051

All cellulolytic enrichments from both sites are almost exclusively dominated by bacteria belonging to the orders *Clostridiales* and *Bacillales* (Fig. 28), both of which are in the phylum *Firmicutes*. Some important distinctions can be made in community structure at the order level with varying culture conditions. In aerobic conditions, there are roughly equal abundances of *Clostridiales* and *Bacillales*, while there is a considerable community shift towards *Clostridiales* in anaerobic conditions. Furthermore, in all pH 12 cultures, members of the order *Clostridiales* account for more than 80% of the community, while there is a dramatic reduction in abundance of *Bacillales*. Another interesting point to consider is the appearance of *Enterobacteriales* only above pH 10 from CC1 samples; these organisms appear to be more abundant in aerobic conditions, and show a marked increase in in aerobic pH 12 culture.

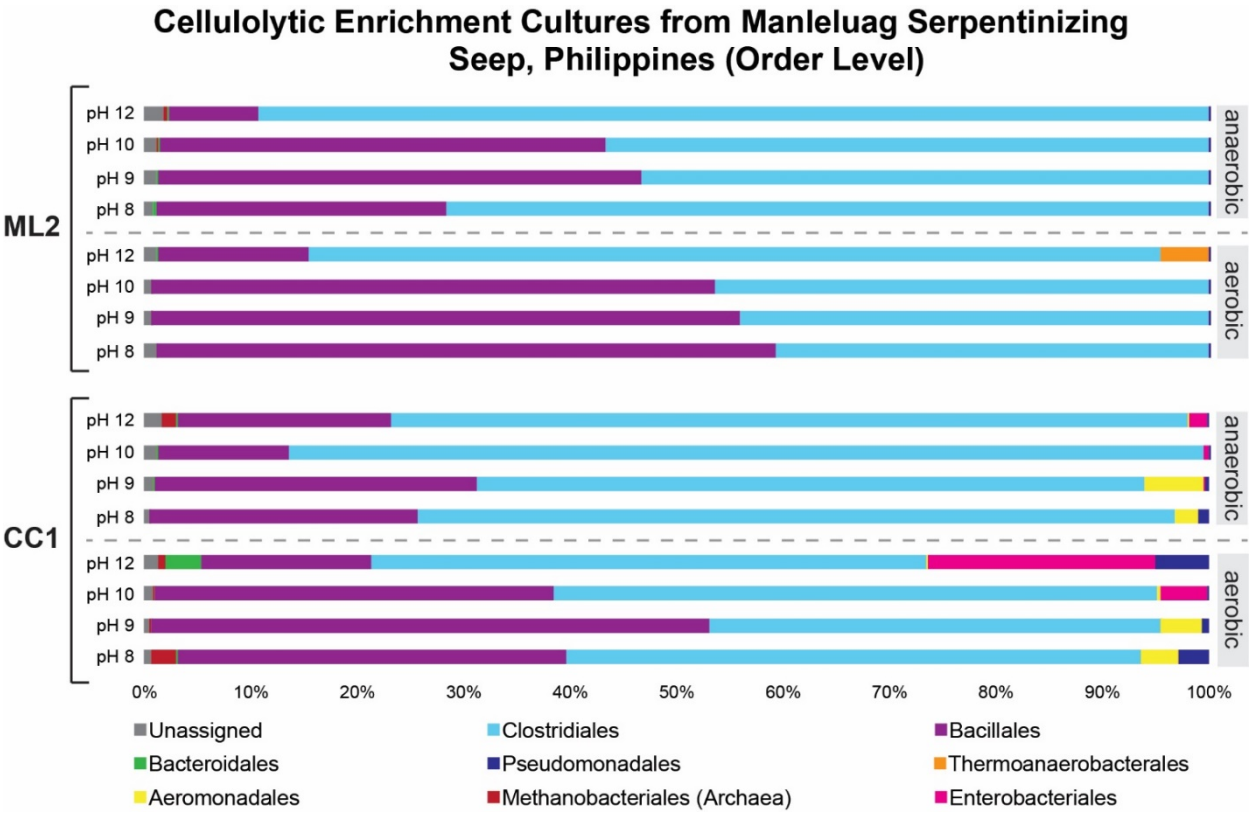

Figure 28. Order level comparison of microbial communities in cellulolytic enrichment cultures from ML2 and CC1. Colored bars represent relative abundances (%) of various taxa. Samples are grouped by site and then by aerobic or anaerobic conditions. Site names and pH are denoted to the left of the bars. Aerobic vs. anaerobic conditions are denoted to the right of the bars.

At the family level, most cellulolytic cultures are dominated by *Clostridiaceae* and *Bacillaceae* (Fig. 29). The exceptions are presented by the most alkaline culture conditions. At pH 12, roughly 20 – 30% of the community belong to the family *Veillonellaceae*; interestingly, these organisms constitute >20% of the community above pH 10 and exhibit relatively consistent abundance in both aerobic and anaerobic conditions. Other families that comprise a significant fraction of pH 12 cellulolytic enrichments include *Lachnospiraceae*, *Christensenellaceae*, and *Ruminococcaceae*. In pH 12 enrichments, there generally does not appear to be a significant difference in composition between aerobic and anaerobic cultures, though there are small variations in relative abundances of taxa; one exception is that *Enterobacteriaceae* from CC1 are approximately 90% more abundant in aerobic conditions at pH 12.

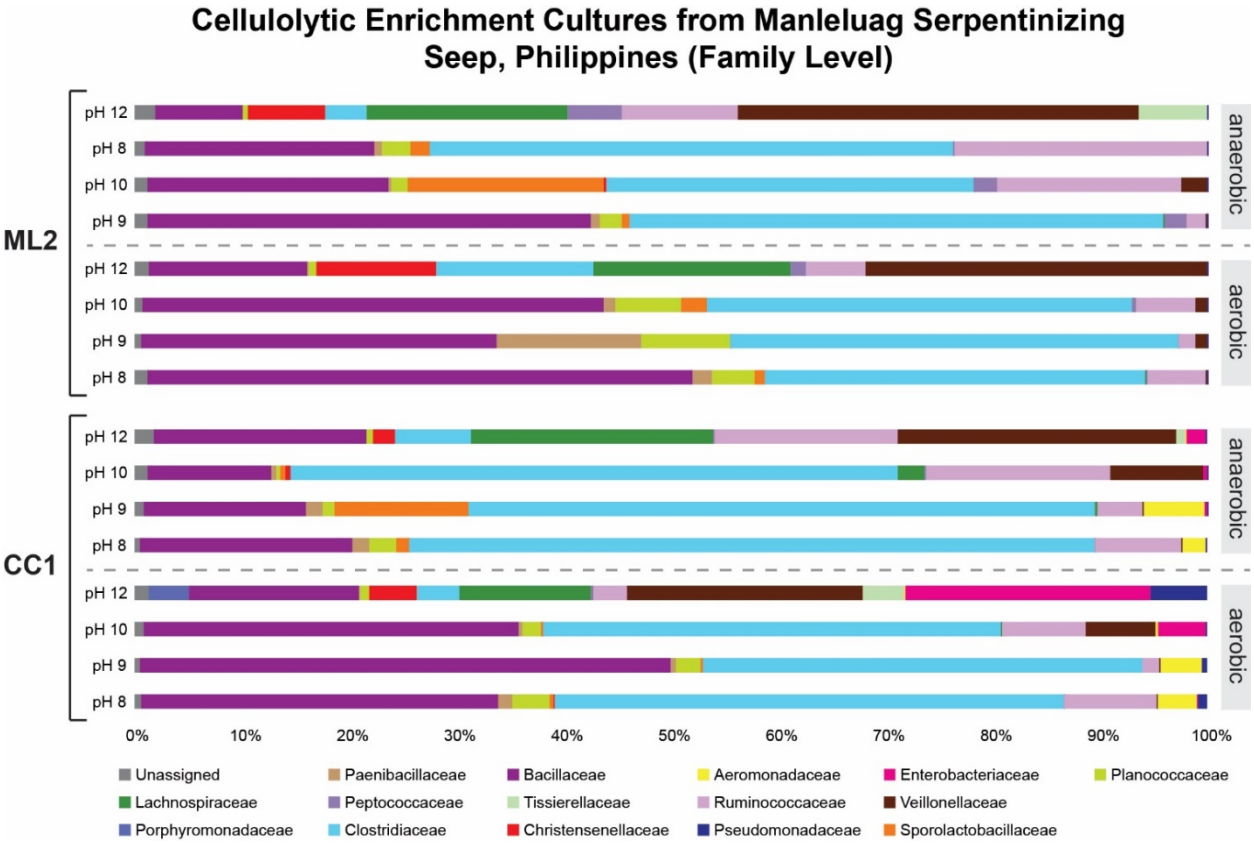

Figure 29. Family level comparison of microbial communities in cellulolytic enrichment cultures from ML2 and CC1. Colored bars represent relative abundances (%) of various taxa. Samples are grouped by site and then by aerobic or anaerobic conditions. The sampling site and pH are denoted to the left of the bars. Aerobic vs anaerobic conditions are denoted to the right of the bars.

Cellulolytic enrichment cultures exhibit similar relationships at the genus level. Generally, most cultures are dominated by *Clostridium* and *Bacillus*, but more generic variation can be observed with respect to oxygen availability and pH (Fig. 30). For example, *Ethanoligenens* comprises about a quarter of the community in pH 8 anaerobic cultures from ML2, but are significantly reduced in the presence of oxygen at the same pH. One of the most striking differences is the drastic reduction of *Clostridium* coupled to the increase of *Coprococcus* organisms at pH 12 in both aerobic and anaerobic cultures from both ML2 and CC1. *Coprococcus* accounts for approximately 20 – 30% of the community at pH 12, yet appear to be completely absent at lower alkalinity. Similarly, *Sporomusa* species also appear exclusively at pH 12, and are more predominant in ML2 than CC1; it is also worth noting that their abundance remains constant in both aerobic and anaerobic conditions.

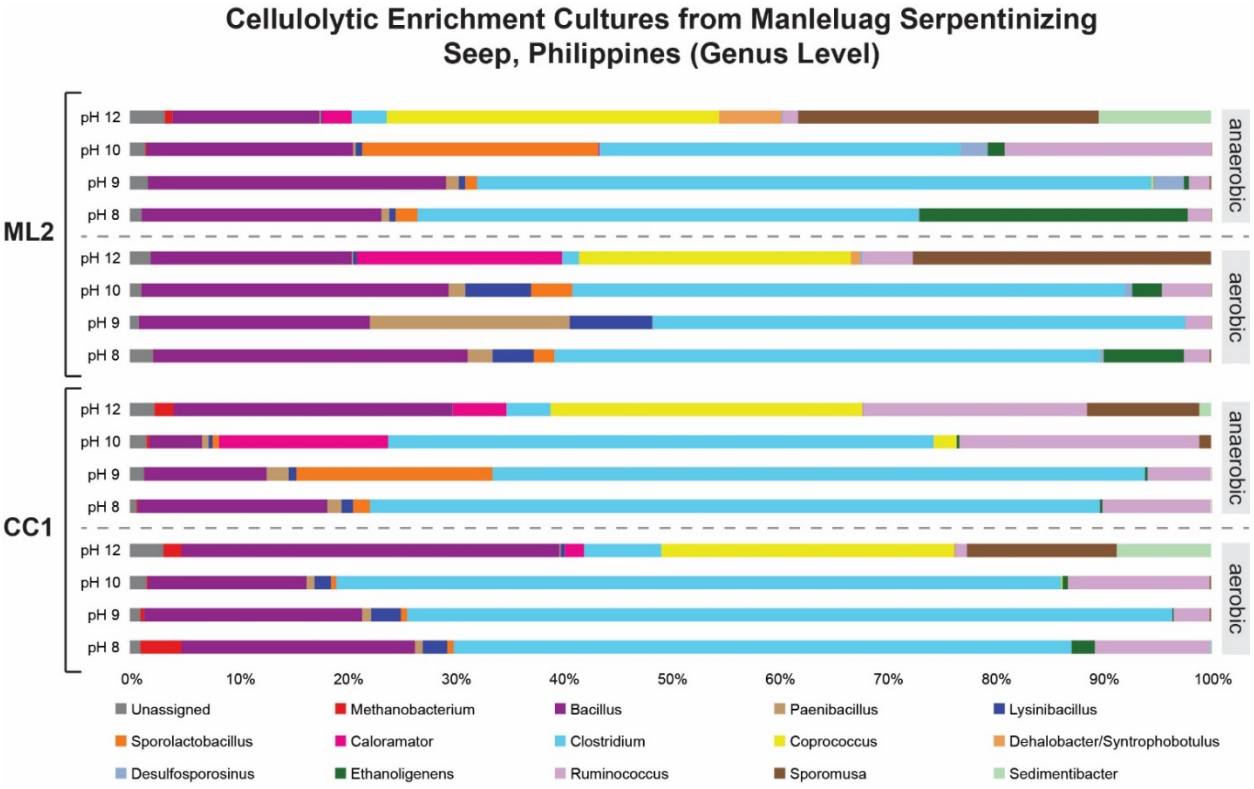

Figure 30. Genus level comparison of microbial communities in cellulolytic enrichment cultures from ML2 and CC1. Colored bars represent relative abundances (%) of various taxa. Samples are grouped by site and then by aerobic or anaerobic conditions. The sampling site and pH are denoted to the left of the bars. Aerobic vs anaerobic conditions are denoted to the right of the bars.

4.3.3 Isolation and identification of cellulolytic bacteria from environmental samples

Subsequent to staining of CMC-agar plates with Congo red solution, zones of clearing were observed surrounding several of the selected colonies (Fig. 31). In total, five of the selected isolates were determined to exhibit cellulolytic potential and were purified via two to three transfers to fresh media. Partial 16S rRNA sequences determined that all five isolates are closely related. Four strains belong to the genus *Micromonospora*, while one is 98.6% related to *Catellatospora*, also within the radiation of *Micromonosporaceae* (Table XIX). Phylogenetic relationships of the isolates and their closest taxonomic relatives are demonstrated in Fig. 32.

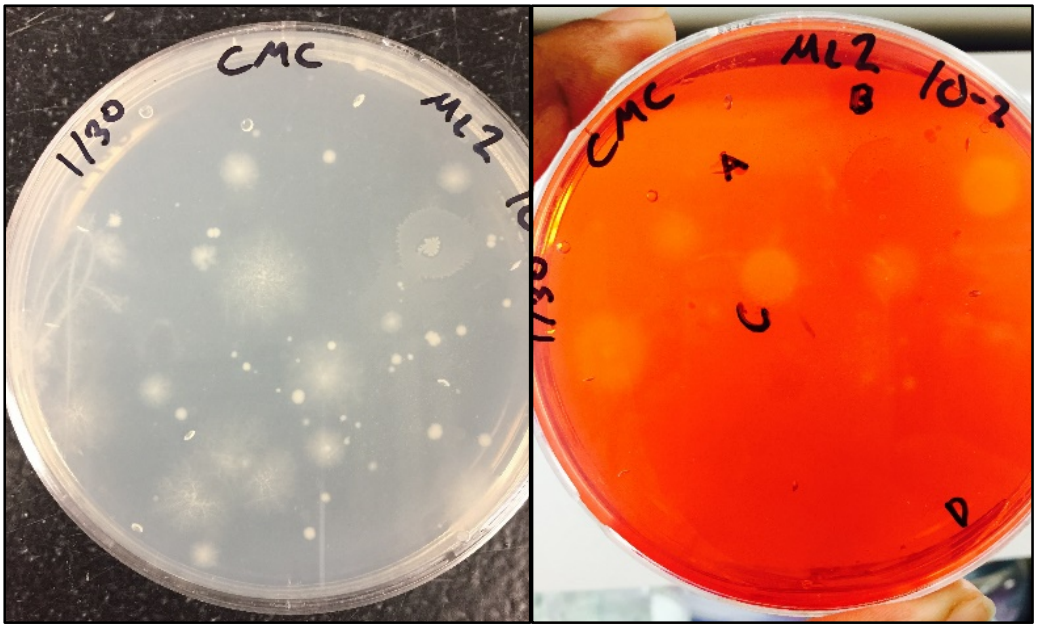

Figure 31. CMC-agar plates inoculated with ML2 before (left) and after (right) staining with 0.2% Congo red solution. Lighter zones of clearing on the stained plates indicate zones of hydrolysis surrounding cellulolytic colonies.

Table XX. Cellulolytic bacteria isolated from serpentinizing environments and their closest taxonomic relative as determined by 16S rRNA sequences.

Strain	Source	Closest Taxon	Similarity (%)
ML2-E	ML2 seep	*Micromonospora sediminis*	99.41
ML2-G	ML2 seep	*Micromonospora peucetia*	99.60
ML2-I	ML2 seep	*Micromonospora soli*	98.74
CC1-C	CC1 outflow	*Catellatospora chokoriensis*	98.64
CC1-D	CC1 outflow	*Micromonospora terminaliae*	99.52

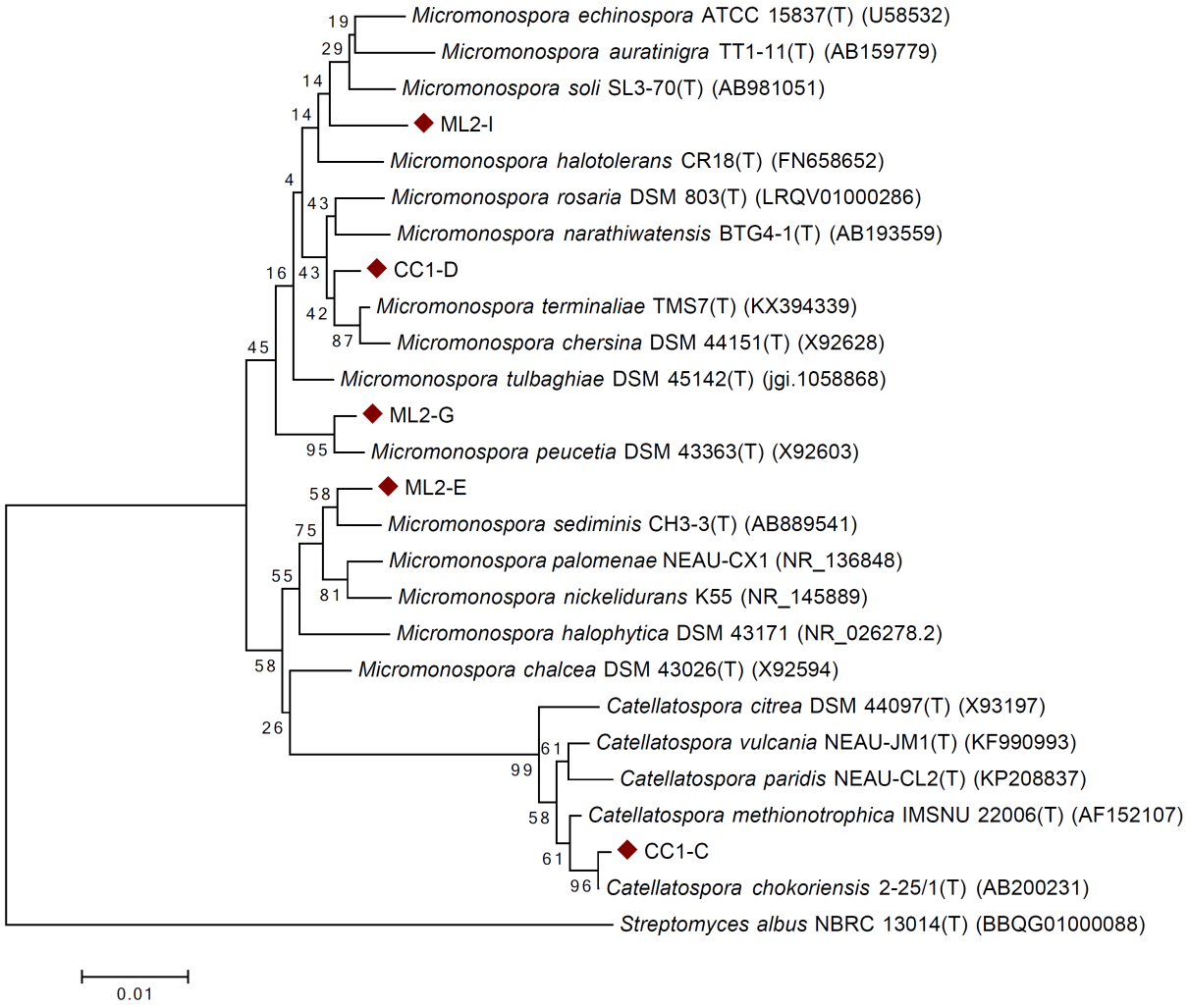

Figure 32. Phylogenetic dendrogram of isolates obtained in this study (denoted with red diamonds) and their position within the radiation of members of the genus *Micromonospora* and closely related taxa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bar, 0.01 nt substitutions per site.

4.3.4 Screening of isolates for cellulose degradation in alkaline conditions

All isolates were positive for growth on control CMC-agar media which was adjusted to pH 7. Additionally, all strains were positive for growth at pH 8. Only ML2-E and ML2-G exhibit growth at pH 9, and only ML2-G can grow at pH 10. After staining, a zone of hydrolysis could be seen surrounding growth of isolates on control media, pH 8, and pH 9. At pH 9, ML2 -E produces a larger clearing zone than ML2-G (Fig. 33), indicating more cellulase production by this organism. Although one isolate displays growth at pH 10, a lighter clearing zone could not be distinguished due to darker tinting of the media at extreme alkalinity. Therefore, it is indeterminable whether ML2-G is capable of degrading cellulose at pH 10.

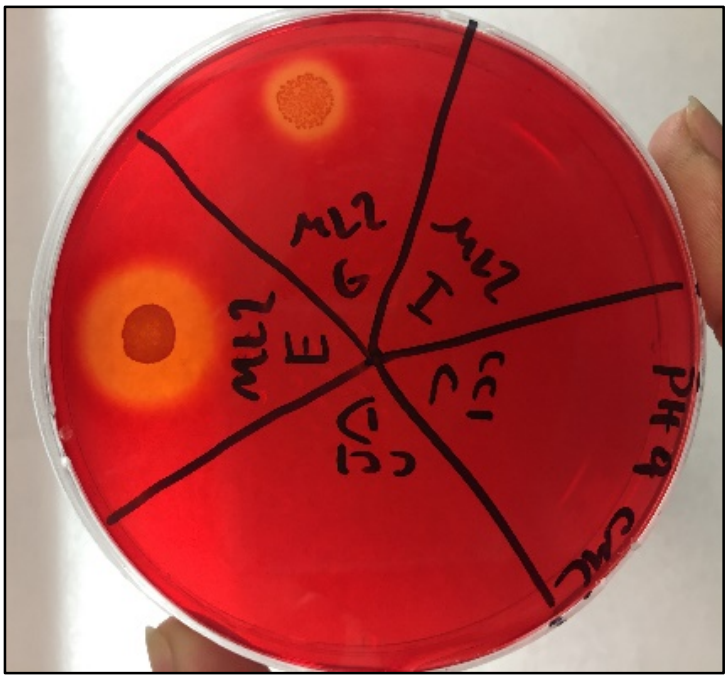

Figure 33. Zones of hydrolysis surrounding bacterial growth on pH 9 CMC-agar stained with Congo red; clearings indicate cellulase activity only by strains ML2-E and ML2-G.

4.4 Discussion

4.4.1 Microbial taxa in alkaline cellulolytic enrichment cultures

Whereas there is diversity seen among bacterial phyla in the environmental samples, all cellulolytic enrichment cultures consist exclusively of organisms in the *Firmicutes*. These results agree with those from another study investigating cellulolytic ability of microorganisms from the deep subsurface (Rastogi et al. 2009), reinforcing the importance of this particular phylum not only in global carbon cycling, but potentially in biofuel research as well. While the following discussion assumes cellulolytic activity by all abundant taxa indicated by 16S rRNA gene analysis, it must be mentioned that there is a likelihood that some organisms in enrichments may not be active cellulose degraders, but are utilizing the byproducts of cellulose degradation for metabolism. These enrichments represent communities, and the community structure may very well involve those organisms benefiting from the metabolic action and waste products of other species. For example, sugars formed from cellulose hydrolysis may facilitate abundant growth of heterotrophic bacteria living alongside cellulose-degrading organisms. However, all major taxa that will be discussed have been previously cited in studies as having cellulolytic potential, and thus they will be treated as such for discussion purposes.

As expected, *Bacillus* and *Clostridium* dominate all cultures, as these genera are known to harbor many cellulolytic organisms (Lynd et al. 2002; Rastogi et al. 2009). However, interesting variations are exhibited with changing pH and oxygen availability. Most notably, it is demonstrated that extreme alkalinity (pH 12) significantly reduces the abundance of *Clostridium*, suggesting that enzymes of most of these organisms may be limited in high pH environments and applications. This is important when considering that organisms like *C. thermocellum, C. cellulolyticum,* and *C. cellulovorans* are commonly studied and utilized in industrial cellulose degradation (Lynd et al. 2002). In contrast, *Bacillaceae* appear to maintain relatively constant abundances across varying pH and oxygen availability, implying that their cellulases may have a more widespread utility in biotechnology. Many cellulolytically active organisms at

this end of the pH spectrum appear to be those commonly found in animal intestinal tracts, such as those in the families *Ruminococcaceae* and *Enterobacteriaceae*. This is intriguing as digestive fluids in animals are typically acidic, but there are examples of cellulolytic insects with extremely alkaline midguts that harbor appropriately alkalitolerant symbiotic microbes (Vilanova et al. 2012); however, the extremely alkalitolerant taxa identified in this study largely do not correlate with those identified in alkaline insect digestive tracts, intimating that high pH cellulose degraders in serpentinizing systems are unique. The genera *Coprococcus* and *Ruminococcus* are especially prevalent in pH 12 cellulolytic enrichments, and are predominant organisms of the human colon (Kopecný et al. 2004) and in the rumen of livestock animals (Russell et al. 2009), respectively. This provides strong motivation for potentially expanding the search for alkaline-stable cellulases to encompass human and animal digestive tracts, as well as serpentinizing ecosystems.

The abundance of the genus *Ethanoligenens* in pH 8 anaerobic cultures of ML2 provides compelling support for potential applications of microbiota from serpentinizing environments in biotechnology. These strictly anaerobic organisms demonstrate the ability to produce many valuable fermentation products such as ethanol, acetic acid, and hydrogen (Xing et al. 2006). Interestingly, this genus only contains one validly described species, *Ethanoligenens harbinense*, that was initially isolated from activated sludge of molasses wastewater (Xing et al. 2006). Thus, this study marks the first cultivation of organisms in this genus from serpentinizing fluid-associated sediments, reinforcing the claim that these environments harbor many biotechnologically relevant microorganisms that are yet to be discovered. Considering the prevalence of *Ethanoligenens* only in pH 8 cultures, it may be speculated that these organisms are sensitive to more alkaline conditions, providing valuable data that may be used in optimization of cellulose fermentation strategies using enzymes from members of this genus.

4.4.2 Cellulolytic bacterial isolates and alkaline cellulolysis

Surprisingly, the five isolates obtained in this study are not representative of any of the predominant taxa identified in enrichment cultures. *Micromonosporaceae* belong to the phylum *Actinobacteria*, ubiquitous soil-dwelling organisms that also consist of numerous cellulolytic bacteria (Schwarz 2001; Lynd et al. 2002). Interestingly, these bacteria have been previously detected in hyperalkaline serpentinizing springs in the Voltri Massif in Italy (Quemeneur et al. 2015), implying that they may easily adapt to tolerate the extreme conditions of this particular environment. Prior studies involving cellulolytic ability by *Micromonospora*, however, are limited and primarily constrained to soils and freshwater habitats (Cross 1981; De Menezes et al. 2008). The isolates from this study demonstrate the production of cellulases in alkaline media up to pH 9 and possibly at pH 10, although this was not able to be confirmed using the methods described. This marks the first evidence for cellulose degradation by organisms in this genus in the alkaline pH range. It is important to consider that, while the CMC-Congo red staining technique has been shown to be reliable in detecting endoglucanase production, it has been found that many organisms capable of degrading CMC are not always capable of hydrolyzing crystalline cellulose such as that used in enrichment cultures (Schwarz 2001; Lynd et al. 2002). Thus, further investigation is needed to determine the true cellulolytic potential of these isolates. It is also worth noting that one of these isolates, designated CC1-C, potentially represents a novel species of *Catellatospora* based on 16S rRNA gene sequence, and warrants further physiological and biochemical characterization.

4.5 Conclusion

Microbial biotechnology has been implemented by humans for thousands of years, but the current rate of growth and expansion in the field is unprecedented. A rapidly growing global population necessitates the discovery of novel approaches to fulfilling increasing energy demands, and microbial conversion of lignocellulose to biofuel has the potential to be the most cost-efficient and environmentally

friendly solution. This study provides a comprehensive analysis of cellulose-degrading communities from serpentinizing environments that are active at a range of extremely alkaline conditions. Data from this study may be used in targeted culturing of cellulolytic bacteria that demonstrate functionality in alkaline conditions that are often characteristic of various industrial processes. Ultimately, it has been shown that while some prominent cellulolytic taxa are ubiquitous, other less-studied organisms may display increased efficiency in certain physicochemical extremes, and increased research is necessary to properly evaluate the potential of these microorganisms.

V. Cellulolytic Bacterial Communities from Acidic Hydrothermal Springs, Yellowstone National Park, WY

5.1 Introduction

Biotechnologically-relevant, cellulolytic organisms are commonly thermophiles that can function at extreme temperatures (Mohagheghi et al. 1986; Rainey et al. 1993; Schwarz 2001; Hamilton-Brehm et al. 2010). This is because bioreactors operating at high temperatures can more quickly and thoroughly degrade cellulose than low-temperature reactors, with the caveat of being more costly to operate (Shiratori et al. 2006). Therefore, hot spring environments are logical study sites for the discovery of cellulases produced by natural microbial communities that are stable at high temperatures as well as low pH. Many hot springs are characterized by inherently low organic carbon content (see Table II in Chapter I, section 1.6); therefore, indigenous microbial communities may benefit from the addition of exogenous carbon from meteoric runoff, which may include cellulose from vegetation surrounding hot springs. While many studies have described thermophilic, cellulolytic bacteria from hot springs, investigations into microbial cellulolysis at pH values lower than 5 are extremely limited. The following investigation seeks to elucidate thermophilic, cellulolytic communities from hydrothermal environments that function at a range of acidic conditions for potential applications in biotechnology.

5.2 Materials and Methods

5.2.1 Enrichment of cellulolytic bacteria in acidic conditions

Environmental samples from several hot springs in Yellowstone National Park were used for inoculation of enrichment cultures. Sites include “Wood Chip Beach”, “Log Jam”, and “Obsidian Pool”. For site descriptions and sampling information, refer to Chapter I, section 1.6.

For enrichment media recipe, refer to Chapter IV, section 4.2.1. A basal salts medium was used with pH adjusted to 2, 3, 4, and 5 using 10 M HCl. For anaerobic cultures, gas headspace was flushed with

95% N_2, 5% H_2. Gas headspace was refreshed at 7 days. Cultures were incubated at 65°C for a total of 14 days. Uninoculated controls containing growth media were incubated alongside enrichment cultures.

5.2.2 Community analysis of environmental samples and enrichment cultures

Refer to Chapter IV, section 4.2.2 for procedures involving DNA extraction, PCR, and 16S rRNA gene sequencing for environmental samples and enrichment cultures.

5.2.3 Quantification of cellulolytic potential in hydrothermal samples

The three environmental samples from Yellowstone National Park were examined for cellulolytic potential using a filter paper degradation assay. The assay involves adding a known mass of filter paper to culture media, inoculating with environmental sample, and determining change in weight of the filters post-incubation. Whatman 0.2 μm cellulose filters were cut into 0.5 x 0.3 cm strips and weighed prior to addition into culture media; the media recipe is identical to the basal salts medium described in Chapter 4, section 4.1.1 but without cellobiose or cellulose powder. Instead, approximately 0.03-0.04 g of paper strips (2-3 strips) were added to each culture as the sole source of carbon. Additionally, media was adjusted to pH values of 3, 4, and 5 with 10 M HCl to determine degradation potential in varying acidity. 100 μL of hydrothermal fluid-sediment sample was used to inoculate serum vials containing 10 mL of basal salts medium and pre-weighed paper strips at each pH. Only aerobic conditions were used for filter paper degradation experiments. Serum vials were incubated at 65°C for 14 days. After incubation was complete, paper residue was collected via centrifugation of cultures at 3500 rpm for 30 minutes and oven-dried overnight at 80°C. Dry paper residue was weighed to determine mass loss attributed to degradation of cellulose filters.

5.2.4 Obsidian Pool cellulolytic culture analysis

A paper filter-degrading culture from Obsidian Pool (pH 4) was analyzed for microbial community composition. DNA was extracted from the filter paper culture and community analysis was performed as described in Chapter IV, section 4.2.2.

5.3 Results

5.3.1 Community analysis of hydrothermal field sites

Total number of sequences obtained from 16S rRNA gene sequencing for environmental samples and cellulolytic enrichment cultures is shown in Table XXI. Community analysis reveals remarkable similarity of phyla and their relative abundances between each of the three hydrothermal sites. In general, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are the major constituents of each spring (Fig. 34). Some notable differences include the higher abundance of *Aquificae* in Obsidian Pool relative to the other two sites, and the presence of *Thermi* exclusively in "Log Jam."

Table XXI. Total number of sequences obtained from 16S rRNA gene sequencing before and after quality trimming. Information is shown for environmental samples and cellulolytic enrichment cultures.

Sample	Initial	Quality Trimmed
Log Jam (150729LJ)	43489	39045
Wood Chip Beach (150728)	37632	33477
Obsidian Pool (150727)	46921	41288
LJ pH 3 culture	36138	33631
LJ pH 4 culture	21947	20170
OP pH 3 culture	25429	23001
OP pH 4 culture	24025	20634
OP pH 5 culture	41395	34842
WCB pH 4 culture	134434	126721
WCB pH 5 culture	30561	26647

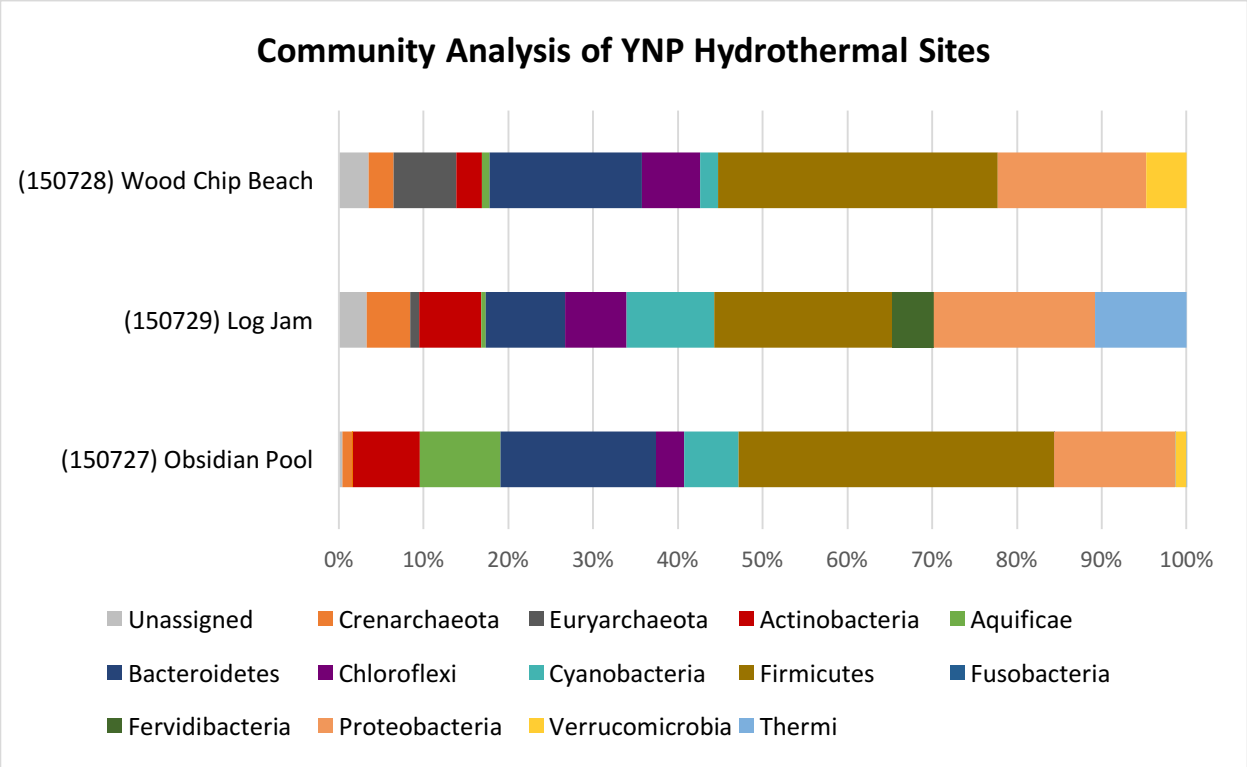

Figure 34. Relative abundances of various phyla in three hydrothermal springs in Yellowstone National Park (YNP). Site names are listed on the left; sample inventory number is shown in parentheses.

5.3.2 Community analysis of acidic cellulolytic enrichment cultures

Several cellulolytic enrichment cultures from each site appeared turbid after 1 week of incubation. Generally, aerobic cultures appeared more turbid than anaerobic cultures. In fact, most anaerobic cultures visually resembled uninoculated controls. Additionally, at the lowest pH, there was no evidence for microbial growth in any of the cultures when compared visually with the uninoculated controls. Turbidity of culture media appeared to increase with increasing pH. Thus, only the enrichments that exhibited observable growth (turbidity) relative to controls were analyzed for microbial community composition. All anaerobic and pH 2 enrichments were therefore excluded from community analysis.

At the phylum level, cellulolytic enrichments from all three hydrothermal sites are comprised of similar bacterial taxa in relatively equal abundances. In all cultures, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are the most abundant, and each represent roughly 25-30% of the total community (Fig. 35). The only notable difference is in the Wood Chip Beach pH 4 enrichment, that is dominated by approximately 80% *Firmicutes*.

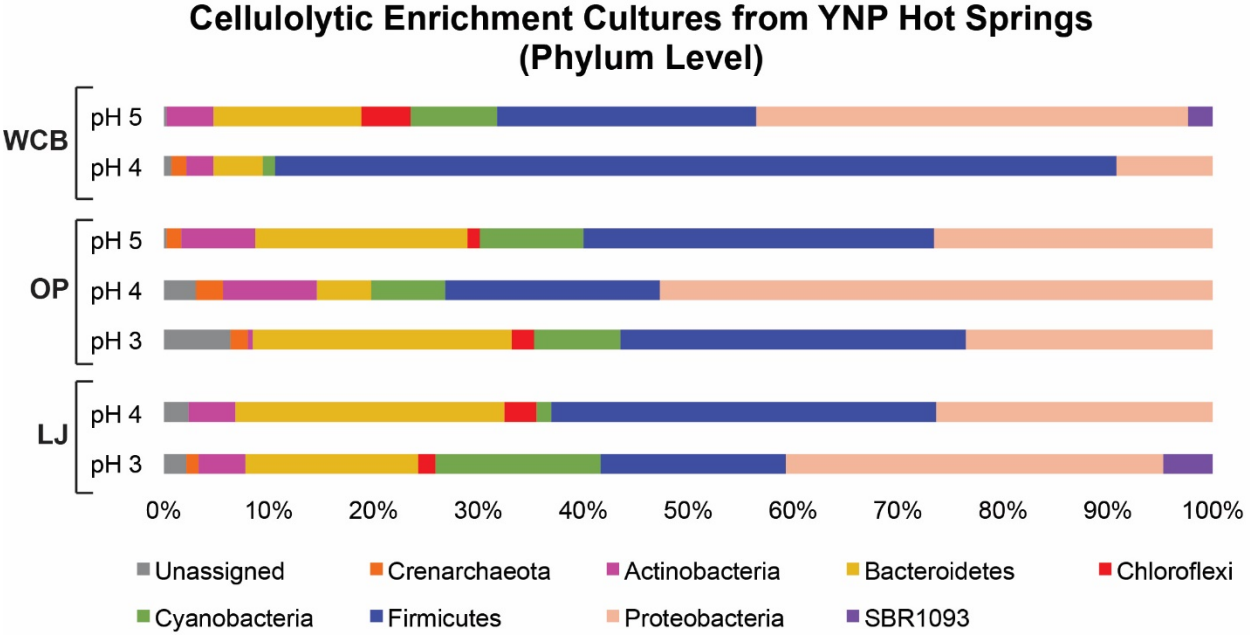

Figure 35. Relative abundances of various phyla in cellulolytic enrichments of environmental samples from three hot springs in Yellowstone National Park (YNP). Site designations and pH are denoted on the left: WCB = "Wood Chip Beach"; OP = "Obsidian Pool"; LJ = "Log Jam"

Comparison of enrichments at the family level reveals a similar diversity. Generally, the most abundant groups common to all cultures are S24-7 and *Streptococcaceae* (Fig. 36). Otherwise, there are limited differences in community composition between sites and varying pH. One interesting observation is the presence of *Enterobacteriaceae* in Obsidian Pool and Log Jam enrichments, but not in Wood Chip Beach enrichments. The Wood Chip Beach pH 4 culture that was previously noted as being comprised almost entirely of *Firmicutes*, primarily consists of bacteria in the family *Alicyclobacillaceae*.

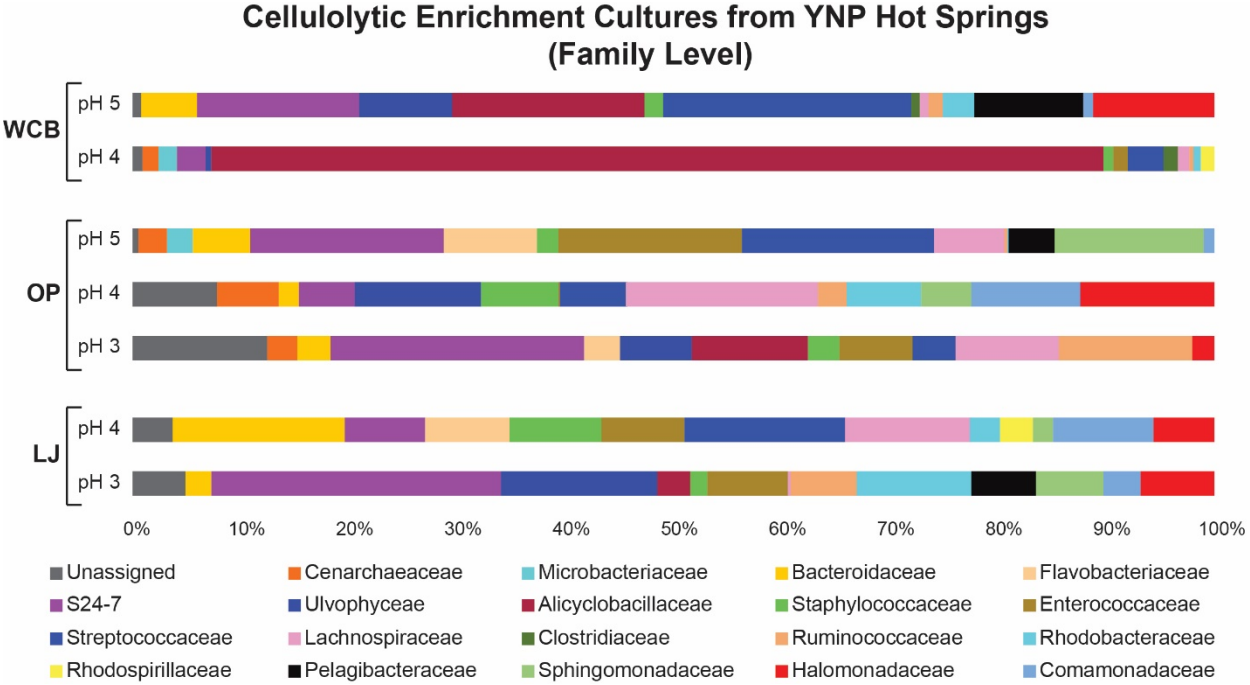

Figure 36. Relative abundances of various taxa at the family level in cellulolytic enrichment cultures from three hot springs in Yellowstone National Park (YNP). Site designations and pH are denoted on the left. [WCB = "Wood Chip Beach"; OP = "Obsidian Pool"; LJ = "Log Jam"]

5.3.3 Quantification of cellulolytic potential in hydrothermal samples

After one week of incubation, only one culture exhibited obvious degradation of filter paper strips. Paper strips from all other cultures were dried and weighed, but no change was observed in the mass of filter paper from any other culture at any pH. Comparatively, the paper strips in the Obsidian Pool pH 4 culture were completely deconstructed, leaving a white amorphous residue in the serum vial. In lieu of weighing the sample, the culture was analyzed for microbial community composition to elucidate the identity of the highly cellulolytic consortium.

5.3.4 Obsidian Pool paper filter-degrading culture analysis

Community analysis reveals that the filter paper-degrading culture is predominantly composed of *Clostridiaceae* (Fig. 37). The second most abundant taxon is *Caldicellulosiruptoraceae*. Other families

comprise <3% of the total community. The corresponding dominant genera in the culture are *Thermoanaerobacterium* and *Caldicellulosiruptor.*

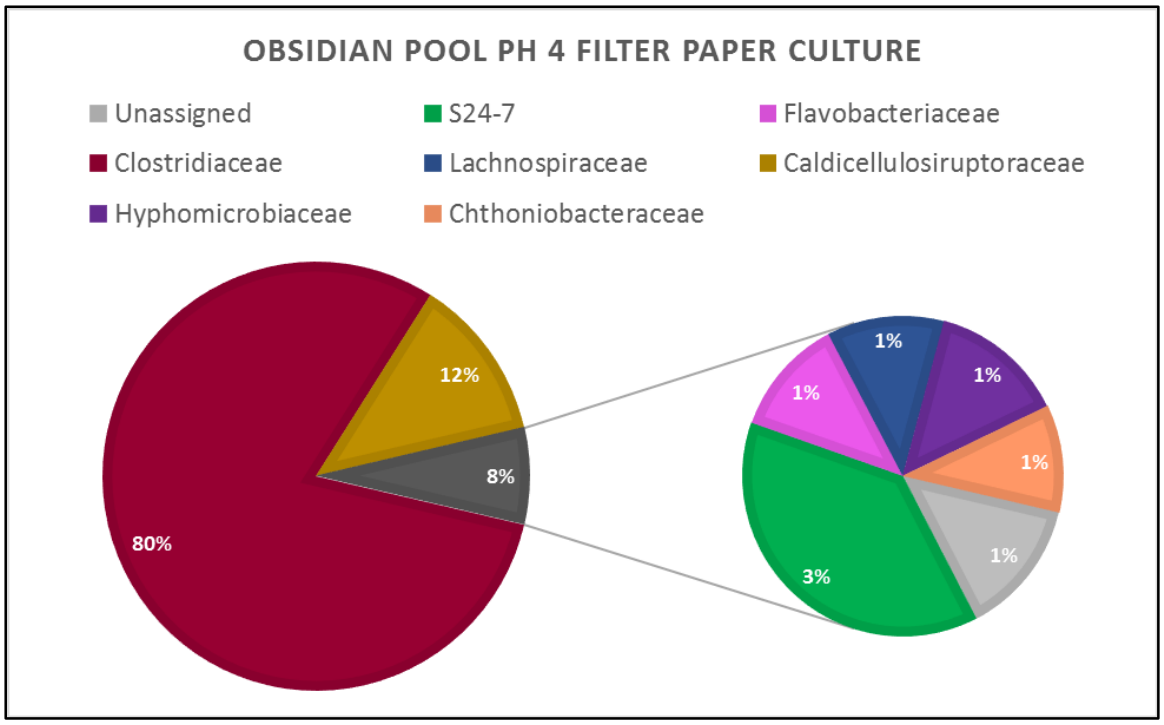

Figure 37. Relative abundances of dominant taxa at the family level in Obsidian Pool pH 4 filter paper culture. The smaller pie chart reveals detailed composition of the 8% wedge in the larger pie chart.

5.4 Discussion

5.4.1 Microbial taxa in acidic cellulolytic enrichment cultures

Community analysis reveals diverse groups of bacteria present in all cellulolytic enrichments. Interestingly, there are no significant variations between enrichments from different springs or in relation to changing pH. This potentially suggests that utilization of cellulose in these acidic hydrothermal environments may be common to many organisms across the same general phyla. This is supported by the overall lack of a single dominant phylum or family in any given culture. However, an alternate

explanation presents when comparing enrichment culture communities to those of the environmental samples. The high degree of similarity between environmental communities and enrichment cultures predicates the likelihood that most enrichments may not have been successful.

One exception is represented by "Wood Chip Beach" pH 4 enrichments, that were found to consist almost exclusively of *Alicyclobacillus*. The type species of this genus, *A. acidocaldarius*, was originally isolated from acidic fluids in Norris Geyser Basin, and incidentally exhibits optimal growth in the conditions used for enrichments in this study (Darland and Brock 1971). Furthermore, this species has also been shown to demonstrate optimal cellulolytic activity at pH 4 and 80°C (Eckert and Schneider 2003), the same pH at which this organism is most abundant. As "Wood Chip Beach" exhibits abundant cellulosic substrate and is located in Norris Geyser Basin, the findings from this study support previous work regarding *Alicyclobacillus* and cellulose degradation in its natural acidic habitat. Strong evidence is therefore presented for the successful enumeration of cellulolytic bacteria in this enrichment.

Interestingly, one taxon, S24-7, is common to every culture and is also one of the most abundant. S24-7, a family in the *Bacteroidales*, represents roughly a quarter of the community in most enrichments, and is especially prevalent at pH 3 in "Log Jam" and "Obsidian Pool." This family, also known as *Muribaculum*, is well cited as being the most dominant bacterial group in mouse intestines (Seedorf et al. 2014; Lagkouvardos et al. 2016). Although this may seem unusual, mice are likely abundant in the environment, and mouse fecal matter may be washed into hot springs after rainfall. Although there appears to be no previous evidence for cellulose degradation by these organisms, it is feasible that bacteria living in digestive tracts of mice would be adapted to degrade a wide variety of substrates, including cellulose. Alternatively, the previously mentioned possibility that cellulolytic communities were not enriched in these experiments must also be considered. When referring to the community analysis of field sites at the family level (data not shown), S24-7 is observed to comprise ~10-20% of the total

community from each spring. Therefore, this may be further evidence that some cellulolytic enrichments were not successful, and simply reflect relative abundances of natural environmental communities.

5.4.2 Obsidian Pool paper filter-degrading community

The prevalence of *Caldicellulosiruptor* and *Thermoanaerobacterium* in the paper filter-degrading culture is anomalous, because both genera are characterized as being strictly anaerobic (Lee et al. 1993; Rainey et al. 1994). As cultures were designed to be aerobic, it must be speculated that oxygen availability dramatically decreased with growth of other organisms. It may be possible that the initial growth phase of other aerobic organisms consumed the supply of oxygen, and optimized conditions for the growth of these obligate anaerobes. Both organisms are well-known cellulose degraders that can produce useful fermentation byproducts such as ethanol and acetate (Rainey et al. 1994; Lynd et al. 2002). However, this may be the first reported case of these organisms actively degrading cellulose filter paper below pH 5. This finding has enormous implications in developing strategies for the direct conversion of cellulosic biomass to useful end products, a critical goal of current biofuel research.

5.5 Conclusion

Results from this study support continued investigations in hot springs environments for thermophilic, acidophilic cellulose-degrading organisms. While there were generally no dominant organisms that were functional in acidic conditions, considerable diversity of taxa is observed in enrichments. However, due to significant similarities between enrichment communities and environmental samples, it may be likely that enrichment cultures were largely unsuccessful. In contrast, the presence of several well-known cellulolytic bacteria such as *Alicyclobacillus*, *Caldicellulosiruptor*, and *Thermoanaerobacterium* in "Wood Chip Beach" pH 4 cultures and paper filter-degrading cultures are undeniable. These results strengthen support for the continued use of these organisms for industrial applications. Specifically, the latter two genera were shown to almost completely deconstruct whole

paper filters within a short time, and may even represent the first reported instance of cellulose degradation by these organisms at pH 4. Physical deconstruction of cellulose fibers is the slowest step in cellulose fermentation, as the produced sugar byproducts can be metabolized much more quickly. Thus, the results may provide useful data in developing strategies for pretreatment of cellulosic biomass with enzymes from these organisms to increase degradation rate.

VI. Conclusion

6.1 Microbes and metal tolerance in serpentinizing and hydrothermal springs

To first order, trace metal analysis from both serpentinizing and hydrothermal fluids determined that environmental metal concentrations are very low. In the Philippines, it is possible that extreme alkalinity facilitated the precipitation of metal cations from the aqueous phase, forming solid minerals that may have accumulated in spring sediments. In Yellowstone, acidic spring fluids and high temperatures allow for metals to remain in solution, but concentrations were typically observed in the ppb range in the sites sampled for culturing. In either case, it was demonstrated that highly metal tolerant organisms are present and can be cultivated from both environments. Furthermore, these organisms display a range of metal tolerance mechanisms. It is likely that these bacteria adapted to tolerate the presence of toxic metal cations in low concentrations, but possess mechanisms that can function at higher concentrations of metals as well. It is worth mentioning that isolates from serpentinizing environments are generally more metal-tolerant than those from hydrothermal environments, as several organisms from serpentinizing environments exhibit growth above 600 mg/L of at least one meta. Conversely, the MIC for all isolates from Yellowstone was determined to be below 600 mg/L.

The data suggest that for most bacterial isolates, an efflux pump is the likely mechanism of metal tolerance as evidenced by ICP data showing constant metal concentrations in cultures. Bacterial membrane proteins responsible for shuttling ions across the cell membrane are not specific to trace elements required for metabolic function, thus allowing for toxic elements in the environment to also enter the cell. Therefore, it is logical that in environments with low trace metal concentrations, an efflux pump would be the most efficient mechanism of action to remove the relatively small amounts of toxic ions from the cytosol. Data from this study demonstrate that the efflux transporters utilized by bacteria

in serpentinizing and hydrothermal systems may be highly competent and functional even when metals are elevated well beyond the low concentrations in their natural habitat.

Exclusively in the presence of Cu, several bacteria from the serpentinizing environment showed the ability to considerably reduce Cu concentration in culture media. Considering the decrease in Cu concentration and presence of brown precipitates in cultures, it can be reasonably assumed that these organisms are immobilizing Cu through biomineralization. However, the precise mechanism is currently unclear. It is often difficult to distinguish between extracellular precipitation via the secretion of chelating compounds and intracellular sequestration and precipitation in the cell envelope, as the two processes sometimes coincide (Haferburg and Kothe 2007). Therefore, further analysis, preferentially electron microscopy, is required to elucidate the precise mechanism of Cu removal exhibited by these bacteria. Additionally, *Bacillus wiedmanii* organisms from Iron Fist outflow in Yellowstone potentially indicate intracellular accumulation and biomineralization of Ni as evidenced by green pigmentation of cellular biomass on media supplemented with 200 mg/L Ni. This coloration can be compared with the green crystals produced by isolate ML-15 from Manleluag, which were determined to be likely a Ni-chloride hydrate. Additional electron microscopy and EDS analysis may confirm the character of the green-pigmented cells.

Lastly, isolate CC-8, *Streptomyces misionensis*, demonstrated the use of extracellularly active melanin to chelate metals and thereby detoxify its surroundings. Secondary metabolite production is a common phenomenon in *Streptomyces* organisms. Furthermore, it has been shown that metals can promote the production of other secondary metabolites, including antibiotics, and may therefore offer a novel strategy to synthesize valuable chemicals from metal resistant *Streptomyces* organisms.

6.2. Cellulolytic communities in alkaline serpentinizing springs and acidic hydrothermal springs

Although cellulose as a substrate for metabolism may not be ubiquitous across serpentinizing environments, it is especially prevalent in Manleluag Spring National Park. Given the elevated pH that is characteristic of serpentinization and the abundance of cellulose, cellulolytic microbial communities adapted to such an environment have significant implications in biotechnology. This study identifies active cellulolytic microbial communities across a range of alkaline pH values, and more specifically, elucidates which bacterial taxa are dominant in different conditions. The most important finding is that in extreme alkalinity (pH ~12), classic cellulolytic bacteria such as *Clostridium* and members of the *Actinobacteria* may not be as active as other taxa. It is demonstrated that in cases where cellulases may be required to operate at high pH, targeted efforts to find stable cellulases and effective cellulolytic consortia should focus on bacteria in the *Ruminococcaceae* and *Lachnospiraceae.* Additionally, it was observed that *Bacillus* organisms may be stable across a range of conditions, evidenced by their relatively consistent abundance over increasing pH and oxygen availability. Lastly, this study marks the first detection and cultivation of *Ethanoligenens* from a serpentinizing environment, although these organisms did not appear to tolerate conditions exceeding pH 8. However, this data may be useful in determining optimal conditions for cellulose degradation using enzymes from these highly biotechnologically relevant organisms.

Similar to serpentinizing environments, hot springs are not typically known for having abundant amounts of cellulose. However, limited nutrient availability necessitates that hydrothermal microbial communities be capable of metabolizing any carbon source that is offered. The cellulolytic communities that were enriched from hot springs in Yellowstone National Park were in stark contrast with those from serpentinizing environments. The main difference in community composition is evidenced by the significant variation observed at the phylum level. While alkaline enrichments from serpentinizing fluids are composed almost entirely of *Firmicutes*, those from acidic hydrothermal springs exhibit greater diversity. Whereas alkaline cultures were largely dominated but two main groups, acidic cultures are

composed of many taxa, each comprising roughly 10% of the community. For example, acidic enrichments show the presence of *Proteobacteria* and *Bacteroidetes* in almost equal proportions with *Firmicutes*. At high pH, *Bacillaceae* and *Clostridiaceae* dominated enrichments, but these groups were generally absent in acidic conditions, with the exception of a single enrichment in which *Alicyclobacillus* was the dominant genus. Perhaps the most intriguing result is the complete deconstruction of paper filter strips in pH 4 culture by a consortium consisting of *Thermoanaerobacterium* and *Caldicellulosiruptor*. The efficiency of this cellulolytic duo may prove useful in development of new cellulolytic technologies.

6.3 Geomicrobiology and potential applications in biotechnology

Ultimately, results from all experiments strongly support continued investigations of microbiology in extreme environments for use in biotechnology. Over billions of years of evolution, microorganisms have developed the most efficient ways to obtain energy and survive harsh environments. It is logical in that sense to learn from these creatures the best way to produce sustainable fuel for our own needs, and how to remediate toxic elements that threaten the environment as well as human health. It is well-known that only the smallest fraction of microorganisms is cultivable from any given environmental sample, insinuating the vast amount of knowledge yet to be gained from Earth's oldest living inhabitants. It is only through increased research and culturing efforts that we may discover novel microorganisms and processes that may prove practical and beneficial for human interests.

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APPENDIX A. Phase Contrast Images of Bacterial Isolates in the Presence of Metals

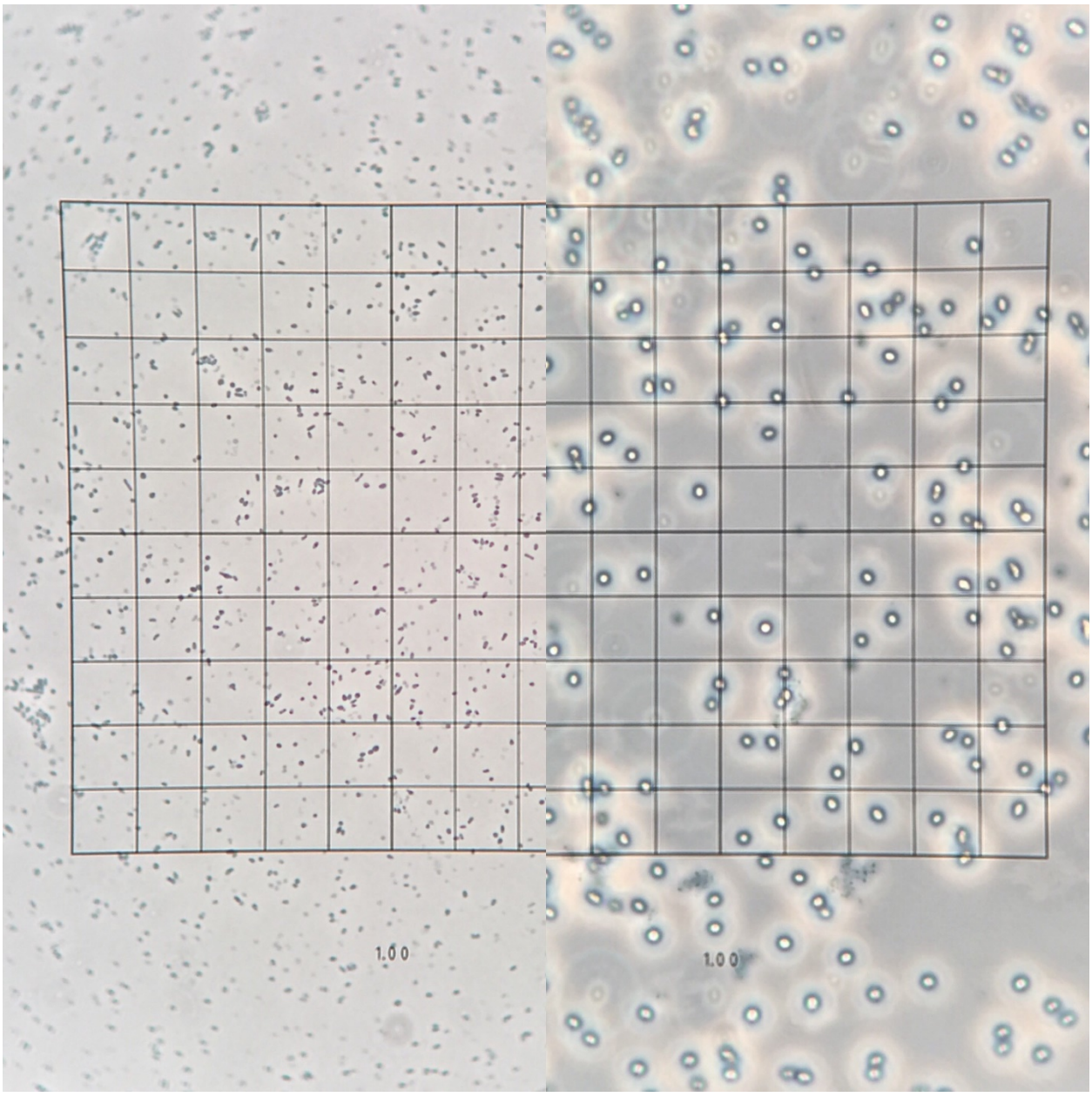

Figure A1. Comparison of cells of isolate ML-15 grown on LBA (right) and LBA + 600 mg/L Ni (left). Squares in grid are 10x10 µm.

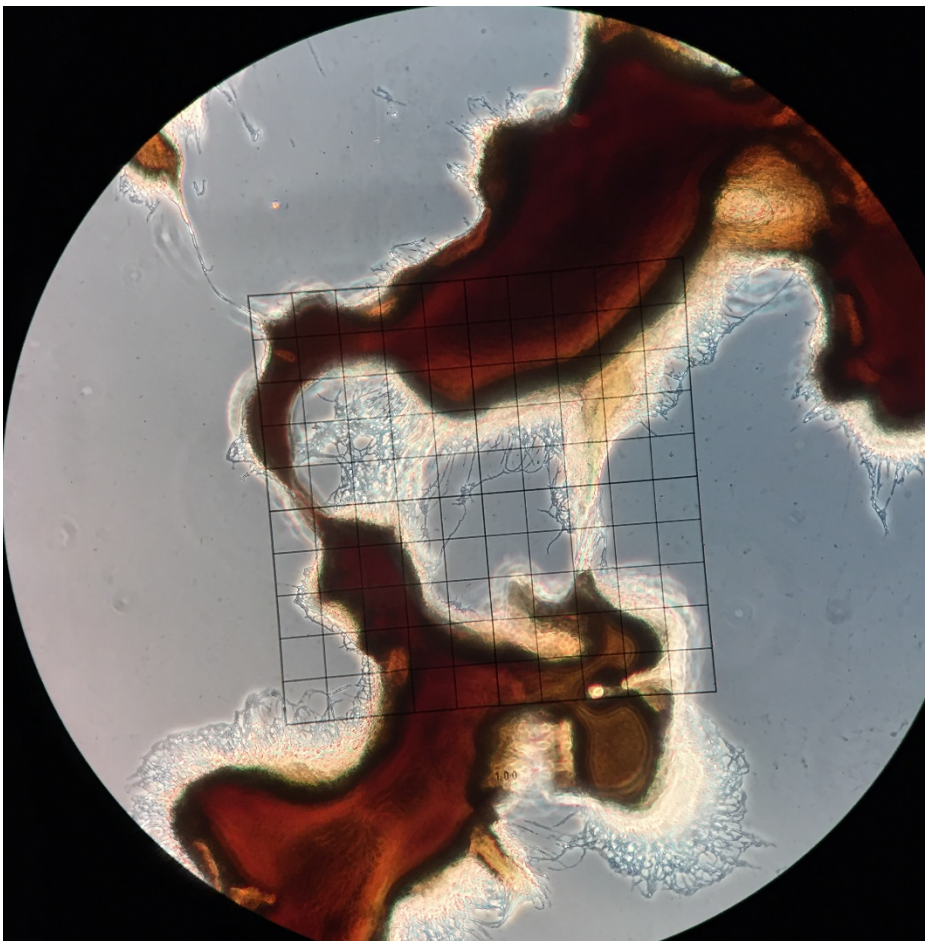

Figure A2. Image of isolate CC-8 grown in the presence of 200 mg/L Cu. Squares are 10x10 um. Filamentous cells can be seen protruding from solidified melanin.

APPENDIX B. Final pH of Metal-amended Cultures

Table A-I. Alteration of culture pH facilitated by isolates growing in the presence of 200 mg/L of Cu, Cr, and Ni.

Isolate	Metal conc. (mg/L)	Starting pH	Final pH
ML-15	200 Cu	7.07	8.83
BL-3	200 Cu	7.07	8.99
ML-8	200 Cu	7.07	8.56
ML-15	200 Cu	7.07	8.57
CC-7	200 Cr	7.55	8.90
ML-7	200 Cr	7.55	8.91
ML-9	200 Cr	7.55	8.81
CC-8	200 Ni	7.10	8.80
IF-3	200 Ni	7.10	8.77
BL-6	200 Ni	7.10	8.85

Appendix C. Screening of Metal-tolerant Isolates for Cellulase Activity

Table A-II. Ability of isolates to produce a hydrolytic clearing zone indicative of cellulase activity when grown on Mm-CMC agar. “+” indicates the presence of a clearing; “-“ indicates no clearing.

Isolate	Clearing Zone
BL-4	+
ML-9	+
CC-1	+
ML-4	-
ML-7	+
ML-8	+
CC-6	-
BL-6	-
IF-1	+
IF-5	+
FE-3	+

Appendix D. Community Analysis of Environmental Samples

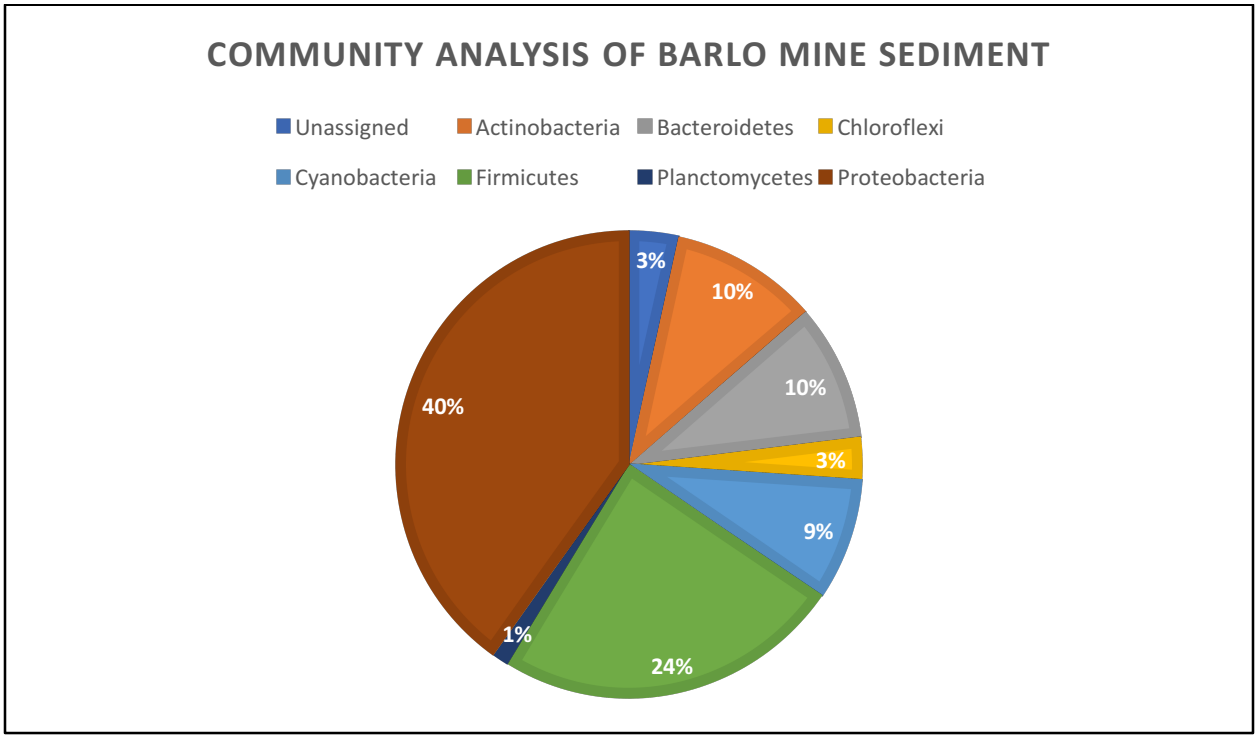

Figure A3. Relative abundances of various phyla in sediment collected from acid mine drainage in Barlo mine.

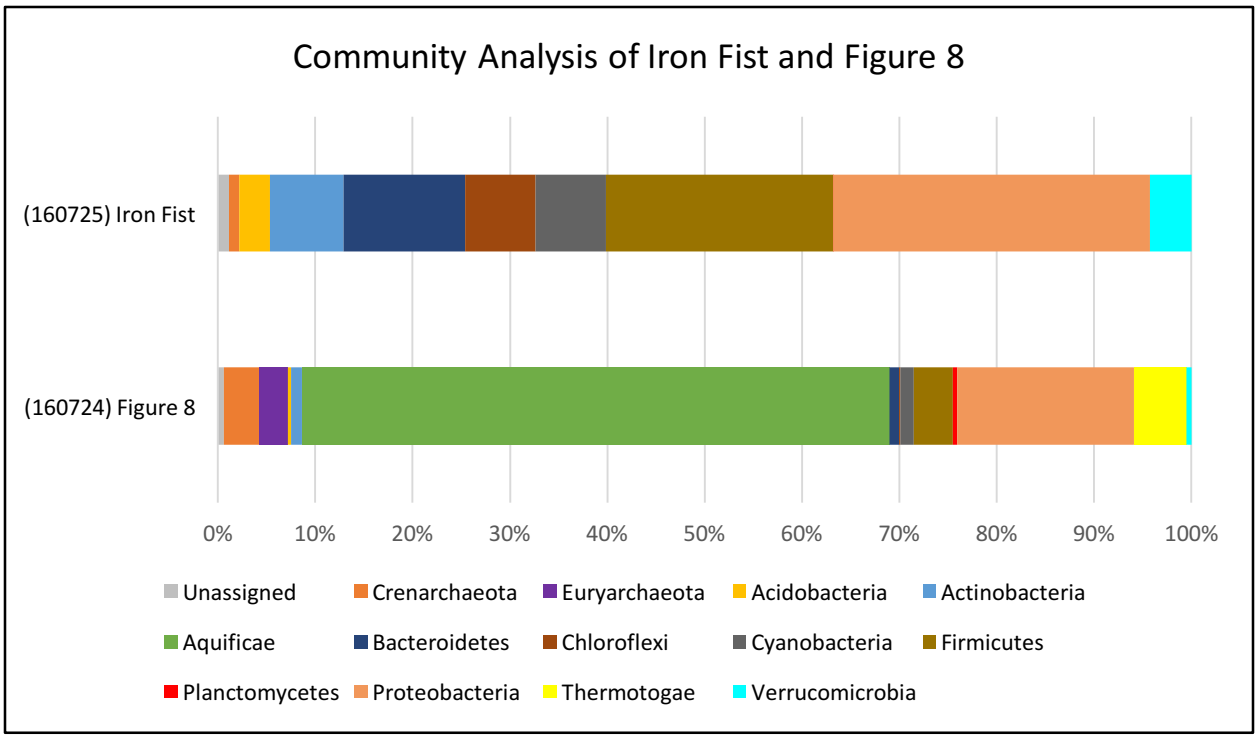

Figure A4. Relative abundances of various bacterial phyla in environmental samples from two hydrothermal springs, Iron Fist and Figure 8, in Yellowstone National Park.

Appendix E. Reference Diameter of Isolates Grown on LBA

Table A-III. Measurements correlate to diameter of bacterial growth on LBA (without metal). The values indicate the standard to which organisms grown on metal-amended media were compared.

Isolate	Colony Diameter
ML-1	1.0 cm
ML-4	1.0 cm
ML-6	1.5 cm
ML-7	1.3 cm
ML-8	2.8 cm
ML-9	1.5 cm
ML-10	1.2 cm
ML-14	1.0 cm
ML-15	1.0 cm
ML-16	1.0 cm
CC-1	1.5 cm
CC-2	1.7 cm
CC-3	1.3 cm
CC-4	3.2 cm
CC-6	1.3 cm
CC-7	1.4 cm
CC-8	1.0 cm
BL-3	2.5 cm
BL-4	1.1 cm
BL-6	2.2 cm
FE-1	3.0 cm
FE-2	3.0 cm
FE-3	3.0 cm
IF-1	3.0 cm
IF-3	3.0 cm
IF-5	3.0 cm
IF-6	3.0 cm
IF-8	3.0 cm

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PUBLISHED ABSTRACTS:

Vallalar, B., Meyer-Dombard, D., Cardace, D., Arcilla, C., "Heavy Metal Resistant, Alkalitolerant Bacteria Isolated from Serpentinizing Springs in the Zambales Ophiolite, Philippines" *AGU Fall Meeting*, San Francisco, CA. **2016**

Vallalar, B., and Meyer-Dombard, D., "Isolation of Cellulolytic Bacteria from High pH Serpentinizing Springs in the Philippines" *Astrobiology Science Conference*, Chicago, IL. **2015**

Meyer-Dombard, D.R., Cardace, D., Woycheese, K., Casar, C., **Vallalar, B**. Arcilla, C., "Geochemistry of microbial environments in serpentinizing springs of the Philippines" *Second Annual Midwest Geobiology Symposium*, St. Louis, MO. **2013**

Vallalar, B., Meyer-Dombard, DA., "Culturing Cellulolytic Bacteria from High pH Serpentinizing Springs" *Third Annual Midwest Geobiology Symposium*, Chicago, IL. **2013**

PUBLICATIONS IN PREP.

Vallalar, B., Meyer-Dombard, D., Cardace, D., Arcilla, C., "Multi-metal Resistant Bacteria Isolated from Serpentinizing Springs in the Zambales Ophiolites, the Philippines" *Applied and Environmental Microbiology*.

Vallalar, B., Meyer-Dombard, D.A., "Cultivation and Characterization of Cellulose-degrading Bacteria from Alkaline Serpentinizing Springs in the Zambales Ophiolite, the Philippines" *Applied and Environmental Microbiology.*