Abstract

PHYSIOLOGICAL AND PHYLOGENETIC STUDIES OF THE BIOGEOGRAPHY OF ALKALIPHILIC HETEROTROPHIC BACTERIA FROM SERPENTINIZING HABITATS

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Serpentinization occurs when ultramafic rocks containing the mineral olivine react with water to produce highly reducing conditions, which are commonly coincident with high concentrations of hydrogen. Hydrogen provides an energy source for microbial metabolism and in combination with mineral catalysts can lead to the abiogenic synthesis of small organic compounds, such as methane. Serpentinite habitats contain abundant potential electron donors, but microorganisms presumably have limited accessibility to electron acceptors. A consequence of serpentinization is the production of highly alkaline (pH 10-12), highly reducing fluids. The stress exerted on microorganisms in such ecosystems can make fundamental cellular processes such as maintaining a proton motive force or stabilizing RNA a difficult task and limit microbial diversity in these environments. It is reputed that the microbial communities in these extreme habitats are able to adjust to the ultrabasic conditions through biochemical and metabolic adaptations. However, it remains to be discovered how microorganisms have physiologically adapted to the serpentinite environment.

Taxonomic characterization of novel isolates from different serpentinite habitats allows us to examine microbial biodiversity and assess whether certain microorganisms are found only at a particular site or whether their occurrence is widespread and correlated with specific environmental conditions. Comparisons of microbial diversity of the numerous serpentine sites our laboratory group is working on in the United States, Canada, and Italy to previously published studies provides insight into the biogeography of alkaliphiles in serpentinizing habitats at a global scale. Detailed physiological studies focused upon a high-resolution environmental data set from the Coast Range Ophiolite Microbial Observatory (CROMO) site in California have been used to relate physiological data from a subset of new isolates to environmental characteristics of the environments from which they were sampled.

Alkaliphilic microorganisms were isolated and grown aerobically from samples collected from three different serpentinite habits located in California, Italy, and Canada between 2009 and 2013. Total genomic DNA from the isolates was used to sequence their 16S rRNA gene to establish the taxa present in each serpentinite habitat as well as in "background" soils and water. Culture-dependent analyses on a subset of isolates investigated the physiology of the isolated alkaliphilic heterotrophs. This research contributes to our understanding of microbial life in ultrabasic habitats associated with serpentinization, explores the biodiversity of the subsurface microorganisms, and characterizes their physiological adaptations.

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CHAPTER 1: Review of Literature

Microorganisms are the most diverse living entities on Earth, and have adapted to a vast array of environments. Due to their flexibility to acclimate to varying surroundings and their extensive history microbial populations are often phenotypically and genotypically distinct from one another, even over very small scales. There are such a broad spectrum of microbial species and conditions that favor their growth that major portions of microbial biodiversity have not been thoroughly characterized. Despite these challenges, it is important to find ways to describe microbial communities in nature in order to understand their biogeography and relationships to their environment.

Since the early days of Microbiology, many studies have focused on microbial diversity at the surface of our planet, influenced by higher taxonomic groups, by photosynthetically derived organic carbon, the atmosphere, and the water cycle. Until relatively recently little investigation has gone into characterizing the subsurface microbiome, in particular in rocks originating from the deep subsurface. As a result, microbial activities and distributions in the subsurface are not altogether understood. However, with recent technological advances in gene sequencing techniques, it has been more accessible to characterize microorganisms that have yet to be cultured due to the extreme environments they live in. Typically "normal" environments that are moderate in terms of pH, nutrient availability, salinity, etc., support relatively high microbial abundances and community diversity. In contrast, an extreme environment may limit the diversity of microorganisms present, although it does not necessarily suggest a low abundance of microorganisms if they are sufficiently adapted to meet the environmental challenges. An example of an extreme environment would be one undergoing active

serpentinization, often characterized by its high pH's and low oxidation-reduction potential (Eh). Serpentinite sites can exert specific evolutionary selective pressures on the microorganisms present, and are often coincident with low cell abundances and microbial diversity. Due to their ubiquity and antiquity, and the low diversity present in the serpentinite sites, they are an ideal location to investigate microbial evolution and physiological adaptations.

What environmental factors are the driving forces for determining the microbial diversity of a particular environment (Fierer et al., 2006)? A famous statement by the Dutch Microbiologist Lourens Baas Becking states "everything is everywhere, but the environment selects", if there are no dispersal barriers and the habitats are physically and chemically similar; we should see the same groups of microorganisms at all sites (Wit et al., 2006). Recent work by Reno, et al. on the biogeography of the archaeon *Sulfolobus islandicus* pangenome suggests that there are some contradictions to Bass Becking's broad hypothesis (Reno et al., 2009). The authors examined the biogeographical distribution of multiple genes in several genomes of closely related *S. islandicus* strains from varying geographical locations. It was found that in *S. islandicus* the core genome between strains were highly similar, while the variable genome shows changes between lineages that were indicative of strains evolving independently from one another based on their geographical distribution (Reno et al., 2009). However, there remains little evidence of whether microorganisms are site-specific and the role of physiological traits in defining distinct species (Rutger et al., 2006). The research presented in this dissertation focuses on the microbial biogeography of highly alkaline habitats associated with serpentinization as another type of model system to study microbial diversity and biogeography.

Serpentinization

Serpentinization starts with ultramafic rocks, which are a major part of the Earth's lithosphere and upper mantle and are enormous reservoirs for carbon (Brazelton et al., 2012). Serpentinization occurs when ultramafic rock is exposed to water during tectonic uplift or from fluid flow of underground, producing different serpentinites (Brazelton et al., 2012). Exposure to water results in the hydration and oxidation of the minerals comprising the ultramafic rock (Sleep et al., 2004). Exposure frequently occurs in tectonically active areas near mountain belts or slow spreading mid-ocean ridges. As plate tectonics progresses, partially serpentinized oceanic rocks are accreted onto the continental margins to form structures known as ophiolites. Ultramafic rocks continue to form and are exposed by tectonic activities today on modern-day Earth (Sleep et al., 2004). The oxidation of minerals in ultramafic rocks results in the formation of molecular hydrogen and methane, and leads to high alkalinity (Muntener et al., 2010). Mineral composition, pressure, and temperature of the environment results in some variability between serpentinite sites, however the products of serpentinization are consistent between sites (Schulte et al., 2006). Serpentinization at low temperatures often results in a high pH environment, typically over 10 (Schrenk et al., 2013). This alkaline environment is a result of released Ca^{2+} and OH^- into the fluid systems of actively serpentinizing habitats can result in the precipitation of calcium carbonate upon reaction with atmospheric CO_2 (Tiago et al., 2004). Minerals, such as olivine at these serpentinite sites are able to buffer metamorphic fluids, which result in highly reducing conditions that produce an abundance of molecular hydrogen gas (Sleep et al., 2004). Additionally, magnetite and iron-nickel composites can catalyze the reaction to enhance the amount of hydrogen, hydrocarbons, and methane produced (Muntener et al., 2010). The

abiogenic hydrogen and organic compounds may be a critical energy source for microorganisms in such settings (Schrenk et al., 2013).

$$6(Mg, Fe)_2 SiO_4 + 7H_2O = 3(Mg, Fe)_3 Si_2O_5 (OH)_4 + Fe_3O_4 + H_2$$
$$\text{olivine + water = serpentine + magnetite + hydrogen}$$

Equation 1: The equation above refers to the serpentinization process of the mineral olivine. The mineral olivine constitutes 60-80% of the Earth's upper mantle. (https://www.ldeo.columbia.edu/gpg/projects/carbon-sequestration)

Additionally, depending upon the setting of the serpentinizing ophiolites and the characteristics of the circulating fluids, organic carbon and other nutrients can also be contributed by marine paleo-sedimentary organic matter, or by meteoric waters circulating into subsurface aquifer (Morrill et al., 2013; Schrenk et al., 2013). The details of the carbon sources and sinks in many cases remain to be well articulated, and are an area of active research.

Serpentinite	Coast Range	Coast Range	Bay of Islands	Cabeço de Vide	Liguria Gruppo di Voltri
Country	Cazadero, CA, USA	Del Puerto, CA, USA	Tablelands, Canada	Portugal	Italy
Temperature (°C)	20	17.8 to 24.2		17.1 to 19.8	10.5 to 23
pH	11.54	8.6	11.8 to 12.3	10.7 to 11.1	9.95 to 11.86
Eh (mV)			-609 to 121	-177 to -39	-525 to -388
CO_2 (mg/L)	0	466 to 639	1.1 to 27.25	1.14 to 2.82	0.53 to 160
Na^+ (mg/L)	19	5.4 to 9.6		37.0 to 55.9	3.9 to 84
K^+ (mg/L)	1.1	0.31 to 0.6		4.15 to 5.22	0.51 to 10.8
Ca^{2+} (mg/L)	40	3.5 to 8.1		5.1 to 22.5	0.6 to 61.9
Mg^{2+} (mg/L)	0.3	110 to 150	0.06 to 7.57	0.16 to 0.3	0.001 to 15.4
Cl^- (mg/L)	63	4.8 to 9.5	166 to 479		8.96 to 97.4
NO_3^- (mg/L)	0.1			5.57 to 7.60	<0.01 to 1.51

Table 1: Table describing the geochemical characteristics of several ophiolites from California, Canada, Portugal, and Italy (Schrenk et al., 2013)

Cell densities in fluids of many subsurface serpentinite habitats are less than 1,000 cells per milliliter (Schrenk et al., 2013). However, the interface between the energy rich subsurface fluids and surface waters in serpentinites can support higher cell densities of 10^5 cells ml^{-1} or greater (Brazelton et al., 2013). Microbial communities were found to correlate with the subsurface ultrabasic fluids that supported them and communities differed from one another based upon the depth of the groundwater it inhabited (Suzuki et al., 2013; Brazelton et al., 2013). Serpentinite sites create numerous challenges for the support of microbial life, by having an environment that is high in pH, with few electron acceptors, and low nutrient availability (Schrenk et al., 2013). These inhospitable conditions beg the question; "What kinds of microorganisms are found in these extreme habitats?" and "What mechanisms do they utilize to enable them to grow in such settings?"

An interesting point-of-comparison to habitats associated with serpentinization are the much better studied alkaline ecosystems associated with soda lakes. These lakes are, closed basins of water that results in a concentration of anions such as CO_3^{2-} and Cl^-.due to extensive evaporation (Jones et al., 1999). Soda lake ecosystems, such as those found in Mono Lake, CA and in northern Africa have been well studied and yielded numerous strains of alkaliphilic microorganisms that have been shown to depend upon the high concentration of Na^+ to aid in the transfer of various substrates across cell membranes (Horikoshi et al., 1999). In contrast, serpentinite environments are non-saline alkaline environments, and therefore the resident microorganisms cannot rely on the extensive availability of sodium ions to cope with alkalinity.

Microbial Biogeography and Serpentinizing Environments

Due to the range of methods available to microbiologists in the modern era, it is possible to investigate the biogeography of microorganisms from a number of different perspectives, using both cultivation-dependent and -independent approaches (Green et al., 2008). Studies since the early days of Microbiology have employed enrichment culture approaches and the isolation of distinct species to quantify both the number of different types of bacteria and to characterize their physiology and biochemistry. In the past several years, sequence based approaches have sought to obtain an unbiased picture of microbial diversity and distributions. However, the benefits of the wealth of sequence data are offset by the frustration that many gene sequences serve unknown functions. Nevertheless, substantial advances have been made in understanding microbial activities and biogeographic distributions. A study by Reno, et al. examined the genomic sequences of several closely related strains of the hyperthermophilic archaeon *Sulfolobus islandicus* from geographically distant sites in the USA and Russia (Reno, et al., 2009). The authors were able to identify both a "core genome" common to all strains, and adaptive genomes specific to each site. They also found that the extent of sequence divergence corresponded to geographic distance. In contrast, a study by Fierer, et al. (2006) using molecular methods found differences in the composition and diversity of microbial communities in soils worldwide, with the physical-chemical characteristics of the soils being a huge controlling factor. Similar approaches are being applied to the study of various habitats of the human body through the Human Microbiome Project (http://commonfund.nih.gov/hmp/index).

The subsurface habitats associated with serpentinization provide an interesting comparison to the previously mentioned studies. Although serpentinizing habitats are found throughout the world, their characteristic harsh conditions make them distinct from intervening

habitats. The fact that many of these organisms exist in subsurface aquifers, often in discontinuous fracture networks, may further limit the dispersal and proliferation of microorganisms within such habitats. In near surface environments microbial populations are presumed to disperse via aerosols in the atmosphere or via the water cycle. It is unknown whether subsurface populations, particularly those that are adapted to extreme environmental conditions follow such a pattern. Microbial adaptation to highly alkaline environments can be a result of extensive evolution of specific lineages of bacteria found just in these serpentinite sites or the occurrence of horizontal gene transfer between microorganisms containing adaptive genes.

Overall, there have been very few studies on microbial diversity and abundances at serpentinite sites. Previous work by Oline et al., sought to determine if serpentinite –hosted microbial populations differed from non-serpentinite microorganisms (Oline et al., 2006). Microbial communities were observed using culture-independent techniques and compared to adjacent non-serpentinite microbial communities in serpentine soils of northern California and southern Oregon. It was concluded in this study that serpentinite soil microbial communities are more compositionally similar to each other than they are to their non-serpentinite counterparts (Oline et al., 2006). It was suggested that perhaps the unique chemistry of the serpentinite soils resulted in constraints upon the microbial community (Oline el al., 2006). The previously mentioned paper by Fierer and colleagues acknowledged that environmental pH as a major predictor of microbial diversity at a continental scale (Fierer et al., 2006). Environmental pH was found to be more influential on microbial diversity than geographic distance or temperature. Microbial diversity was found to be higher in more neutral habitats than in the acidic soils studied.

Tiago and colleagues conducted a study of ultrabasic groundwater wells in Portugal using a cultivation-dependent approach where heterotrophic alkaliphiles in a non-saline serpentine environment were isolated and characterized (Tiago et al., 2004). It was found that a majority of the microorganisms found in the non-saline serpentinite environment were unrelated to other alkaliphilic species found in high pH habitats (Tiago et al., 2004). Tiago also found that under microorganisms that were isolated from high pH serpentinite samples were unable to proliferate at the same pH under lab conditions. The number of cultivatable bacteria from these sites in a lab setting was also relatively low and were predominantly best suited to a medium that was slightly alkaline or neutral in pH. It seemed that when replicated in the lab, the alkaliphiles preferred more moderate pH levels (Tiago et al., 2004). This may be the result of picking up microorganisms from the serpentinizing environment that have the capability to tolerate growth at a high pH, but may not be growing optimally in this type of environment.

Unlike their microbial counterparts there has been extensive investigation of macroorganisms in serpentinite environments, such as the plant communities that inhabit such areas. Through previous research serpentinite soils are known to reduce the abundance and diversity of the plants that inhabit these sites (DeGrood et al., 2004). Plants present in serpentinite habitats exhibit metal hyper accumulation and high levels on endemicity, related to the abundance of toxic heavy metals and low nutrient availability. These unusual plant characteristics are thought to be a result of the evolutionary pressures that the serpentine habitat exhibits on the plants. Perhaps similar evolutionary forces allow for the microbial communities in the serpentinite sites to also have unique characteristics found just in these habitats (Oline et al., 2006).

Previous research also suggests that there are different microbial communities within the serpentinite ecosystem, depending on whether you look at serpentine soil or fluid habitats. With regard to the microbial communities established in serpentinite soils there was dominance in the phylum Actinobacteria at geographically distinct sites (DeGrood et al., 2004; Tiago et al., 2004; Daae et al., 2013). In the alkaline fluid systems of the Del Puerto Ophiolite, California clone libraries of 150 16S rRNA gene sequences were evaluated from microbial mats located by nearby springs that found microbial communities were dominated respectively by phyla Proteobacteria, Bacteroidetes, and Firmicutes (Blank et al., 2009). Similar results were found in Tiago's 2013 culture independent study of the Cabeço de Vide Aquifer in Portugal, where Proteobacteria and Bacteroidetes were found to be prominent. Microbial populations found at Cabeço de Vide were surprisingly diverse and strongly indicated the utilization of metabolisms associated with H_2, sulfur, and methane (Tiago et al., 2013).

Particularly in the mixing zones of the fluid systems at serpentinite sites there is a predominance of Betaproteobacteria within the Genus *Hydrogenophaga* (Brazelton et al., 2013; Tiago et al., 2013; Ishii et al., 2013; Daee et al. 2013). These mixing zones consist of the hydrogen rich serpentinite fluid mixing with the oxygenated surface water (Brazelton et al., 2013). *Hydrogenophaga* is a type of facultative autotrophic hydrogen-oxidizing bacterium, characterized as chemoorganotrophic or chemolithoautotrophic (Willems et al., 1989). *Hydrogenophaga* can use hydrogen as an electron donor and oxygen as its electron acceptor-facilitated by hydrogenase, an enzyme that results in the oxidation of hydrogen. This enzyme occurs in three different forms in *Hydrogenophaga* species, membrane-bound, cytoplasmic soluble, and regulatory hydrogenase, although it is unknown which variety is preferred at high pH (Yoon et al., 2008). *Hydrogenophaga* species are known to be rod-shaped Gram-negative

bacteria, that are catalase variable and oxidase positive. This genus can be found in a variety of habitats such as soil, geothermal, freshwater/marine, and rhizosphere ecosystems (Yoon et al., 2008). It can be hypothesized that the reason that *Hydrogenophaga* exists in many of the fluid systems of these serpentinite sites are that they are known hydrogen oxidizers who take advantage of the release of hydrogen from active serpentinization. In fact metagenomic research on serpentinite hosted microbial communities found hydrogen oxidation genes belonging to *Hydrogenophaga* (Brazelton 2012). Past research suggests that evidence of hydrogen oxidizing microbial communities indicate that hydrogen production at active serpentinite sites sustains the microbial life present and that the presence of *Hydrogenophaga* can be an indicator of active serpentinization (Daae et al., 2013; Brazelton et al., 2013). As more studies accumulate, it will be essential to understand if these microorganisms are just found where serpentinization appears or can they be discovered everywhere and have just adapted to the alkaline conditions.

Microbial Adaptations to High pH

One of the major challenges for microorganisms to survive in a serpentinite environment is the extreme pH. This alkaline environment of serpentinizing habitats is caused by the release of Ca^{2+} and OH^- during serpentinization and the associated precipitation of calcium carbonate (Tiago et al., 2004). The calcium carbonate not only contributes to an extremely alkaline environment, but also sequesters inorganic carbon away from the microbial communities. A recent study on several strains of *Serpentinomas,* a new genus being proposed that is highly related to *Hydrogenophaga*, has been found to employ the novel ability to utilize solid calcium carbonate as its sole carbon source. These *Serpentinomas* strains were found to be highly adapted to the serpentinizing environment in the sense that they use the serpentinite product hydrogen as an electron donor and calcium carbonate as carbon source in an environment

otherwise deficient in carbon (Suzuki et al., 2014). In addition to the challenges of limited carbon availability extremely alkaline conditions external to the cytoplasm interfere with the pH homeostasis of microbial cells, which in turn affects protein stability, enzymatic process, metabolism, and other biochemical processes. The internal pH of most microorganisms is nearly neutral and cannot deviate far from the optimum cytoplasmic pH. For example, *Bacillus subtilis* can proliferate and grow only if the cytoplasmic pH range stays between 7.4-7.8, while the external pH can range anywhere from 5.0 all the way to 9.0. Mechanisms in which bacteria maintain their cytoplasmic pH within that narrow range can vary between different species of bacteria, which could require alkaliphiles to consume more energy than neutrophiles to maintain homeostasis due to the extreme difference of the internal and external pH (Slonczewski et al., 2009; Sorokin et al., 1999).

There are several mechanisms microorganisms are known to employ in order to adapt to an alkaline environment, an increase in acid production through use of urease activity, morphological changes in the cell wall to retain cytoplasmic protons or to increase production of anionic phospholipids, protection from sudden shifts in pH, and an increase in the number of cation/proton antiporters (Padan et al., 2005; Slonczewski et al., 2009). Most of these microbial adaptations are to acclimate to the effects of alkalinity on ATP production. The production of ATP is dependent upon the electrochemical potential of the pH gradient across the microbial cell membrane. Typically in neutrophiles the transmembrane pH gradient is more acidic outside the membrane, due to the constant pumping of hydrogen ions in the electron transport chain, while inside the membrane remains more basic. This relationship is reversed in alkaliphiles where outside the cell membrane there is an extremely alkaline environment, when compared to inside the cell membrane. This reversed pH gradient interferes with the pumping of hydrogen ions into

the cell membrane through ATP synthase to produce the ATP (Yumoto et al., 1997). Unlike microorganisms that inhabit alkaline soda lakes, serpentinite-hosted microorganisms have a more limited ability to used alternative cations such as Na^+ and K+ to help pump protons across the cell membrane since high concentrations of cations are not readily available (Slonczewski et al., 2009; Schrenk et al., 2013).

It is also found that in many alkaliphiles there is an increase in cytochrome expression, when compared to neutrophiles. Cytochromes assist in the transfer of electrons from the electron donor to the electron acceptor, thus playing a central role in creating an electrochemical potential across the cellular membrane during ATP production (Yumoto et al., 1997). Once protons are pumped outside into the alkaline environment, there is evidence that there may be mechanisms to locally sequester them, in which the protons stay rooted near the membrane to be pushed through ATP synthase (Slonczewski et al., 2009). Many of the mechanisms mentioned previously such as utilizing Na^+ to pump more hydrogen ions, antiporters, and increased cytochrome expression, help compensate for the deficiency of the electrochemical potential across the cell membrane in highly alkaline environments other than serpentinite sites. It is unknown whether microbial adaptations in serpentinizing environments are similar to other alkaline habitats or whether these microorganisms have entirely different methods of adaptation.

In addition to the high pH, one of the trademarks of serpentinite soil systems are the high concentration of heavy metals (chromium, nickel, etc.) and the deficiencies in nutrients such as nitrogen, phosphorus, and potassium (DeGrood et al., 2004). Organic carbon sources may also be present, either as the byproduct of chemosynthesis by autotrophic microbial communities, such as the *Hydrogenophaga* species mentioned earlier, as the alteration products of ancient organic materials, or as the product of abiogenic synthesis processes influenced by

serpentinization. The microbial populations in serpentinizing ecosystems must expend additional energy to compensate for these challenges, An important source of this energy are the iron containing minerals of the ultramafic rock, which undergo oxidation when exposed to water which results in the release of hydrogen (Okland et al., 2012). As hydrogen is generated and mixed with more oxidized fluids, it can act as a potential electron donor for chemoautotrophic organisms. Hydrogen can also be combined with the surrounding carbon dioxide by methanogenic bacteria, known to inhabit certain serpentinite sites, to produce methane (Schulte et al., 2006). Hydrogen is one of the primary energy sources for the microbial life that live at these serpentinite sites and may select for microbial species that are able to utilize this electron donor (Daae et al., 2013). When the hydrogen rich fluids mix with the atmosphere or with oxidized surface fluids they create an energy-rich interface that microbial communities can harness and use to cope with a variety of environmental stressors. Due to the paucity of studies on serpentinites, it is unknown whether the microorganisms in these ecosystems have evolved similar mechanisms those from soda lakes or if they utilizing other, unknown adaptations.

CHAPTER 2: Research Objectives

The emphasis of my research is to understand the identity and distribution of alkaliphilic heterotrophic bacteria in serpentinite habitats and to characterize the physiology of microorganisms isolated from these environments. The research plan can be summarized by three complementary objectives, described below.

Document the Biodiversity of Alkaliphilic Heterotrophs in Serpentinites

This research characterized and isolated microorganisms from several distinct serpentinite locations. Tables 3A-3C describe the number of isolates obtained from each of the sampling sites at serpentinizing habitats in Italy, Canada, and California. Isolated microbial cultures had their DNA extracted, and their 16S rRNA gene sequenced to identify the isolate. The 16S rRNA gene is a convenient taxonomic identifier used for most prokaryotes, because it is highly conserved within microbial genomes and of a sufficient length for comparative studies. This objective provides baseline data to relate the microbial diversity of cultivable alkaliphiles from serpentinites to other published work.

Investigate Patterns in Microbial Distribution

The sequenced bacterial isolates provide an opportunity to investigate, which species are living in a particular serpentinite habitat, and compare them in a phylogenetic sense both locally and globally. Essentially this project aims to distinguish whether the same organisms are seen across all serpentinite sites sampled.

The samples used in this study include a variety of soils, rocks, and fluids. Identification of isolates from these various sites has provided an improved understanding of the microbial biodiversity of serpentinizing habitats. The identity and phylogenetic diversity of the isolates has allowed us to visualize how particular subsets of bacteria vary over a global scale in similar

environments and whether there are geographic or environmental patterns. Phylogenetic analysis of the 16S rRNA gene allows better resolution to describe any relationships between the isolates from the same serpentinizing habitat or in geographically distinct separate serpentinizing habitats. It is important to understand the geographical distribution of microorganisms from these serpentinizing habitats, since there have been very few studies on the topic although serpentinization is a major ongoing process on Earth.

Understanding Relationships Between Microbial Physiology and the Environment

Culturing novel isolates provides working cultures that can be manipulated for physiological and biochemical characterization. Several phenotypic traits of the isolated organisms have been characterized, to note if there are any trends in physiology between sites. This was used as a way to distinguish if microbial community composition is heavily based upon the attributes of the environment it lives in or if there are geographic factors influencing species occurrence.

With water samples from various serpentinite wells on the California field site, the Coast Range Ophiolite Microbial Observatory (CROMO), I have characterized the phylogeny of new isolates and conducted tests of their physiology to establish whether there are trends between species occurrence and environmental characteristics of these habitats. Test of the microbial physiology in terms of pH, temperature, and oxygen usage have been used to determine if the isolates possess adaptations to live in the serpentinizing environment. The physiological data collected from the isolates has been compared to the environmental data such as pH, temperature, Eh, dissolved oxygen, and conductivity of each of the well sites. A detailed table displaying characteristics of the isolates are found in Appendix A.

CHAPTER 3: Hypotheses

Microbial Diversity will be Low in Serpentinizing Habitats

Due to the selectivity of the alkaline serpentinite environment, lineages will be constrained in number, which will result in low microbial diversity. The serpentinite habitat is a harsh environment with low nutrient availability, limited electron acceptors, and high alkalinity. The combination of these three components will present physiological challenges which likely impact the types of microorganisms found in serpentinite habitats and the overall diversity of microbial communities.

Serpentinite Environments will have Similar Microbial Communities

Serpentinite environments of geographically distinct locations are expected to share the same microbial communities. If indeed there are no barriers to microbial dispersal, as Baas Becking predicted, environments with similar environmental characteristics should be expected to have similar microbial community diversity. Serpentinite environments are so harsh that there are only select microorganisms that can survive here. There should be the same groups of microorganisms found throughout all serpentinizing habitats, with very few differences in the microbial communities associated with each particular habitat.

Microbial Physiology will Reflect Environmental Characteristics of Serpentinites

Physiological characteristics of the alkaliphilic heterotrophs will show a strong relationship with the characteristics of the serpentinite habitat. For example, it is hypothesized that a sample site that is more neutral in pH should in turn have more microbial diversity and that the isolates would grow best in more neutral pH environment. If this hypothesis is valid, the experimental physiological data of the isolates should closely relate to the environmental data of the CROMO well it is isolated from. Physiological testing can also be used to determine if the

microbial populations are flexible enough to adapt to other environmental stresses (*i.e.* they are "tolerant") or if they have evolved to be so adapted to their serpentinite habitat (they are "-philic"). Describing the physiology of different isolates and comparing it to the environmental data has been used to investigate relationships between microbial adaptations and habitat characteristics.

Essentially this project aims to document the diversity of cultivable microorganisms in serpentinite habitats and whether the same organisms are seen across all sampled sites in Canada, Italy, and California. Subsets of the isolated organisms have been physiologically characterized, to note if there are any trends in physiology between sites. This was used as a way to distinguish if microbial community composition is heavily based upon the attributes of the environment it lives in or if there are local and global geographic factors influencing species occurrence.

CHAPTER 4: Methods

Isolation of Novel Microorganisms

Rock cores, soil, and water samples were collected from serpentinite sites in Liguria, Italy, Tablelands, Canada, and the Coast Range Ophiolite Microbial Observatory, California between 2009 and 2013. Soil samples from College Hill and the Howell Science Building at East Carolina University Greenville, NC were taken in November 2012 and used as background samples for comparative purposes. Samples were sent to our lab at East Carolina University and stored at 4°C until use.

Figure 1: Map showing displaying several of the well sites located at the Coast Range Ophiolite Microbial Observatory, California USA

To promote growth from water samples approximately 45 ml of sample was centrifuged at 8,000-10,000 × *g* for 20 minutes at 4°C and the resulting supernatant was removed. The pelleted biomass was re-suspended in 4.5 ml of pH 11 yeast-peptone liquid media comprised of 0.5% wt of yeast extract, 0.5% wt of peptone in 0.1M sodium carbonate buffer at pH 11. Rock and soil samples were homogenized and re-suspended in 4.5 ml of pH 11 yeast-peptone media. Approximately 500 µl of sub-samples were suspended in 1000 µl of pH 11 yeast-peptone media. Following suspension anywhere from 100-500 µl, dependent upon cell density of the sample, of the mixture was aseptically spread-plated onto pH 11 yeast-peptone agar plates liquid media comprised of 0.5%% wt of yeast extract, 0.5%% wt of peptone, 1.5% bacto agar and 0.1M sodium carbonate buffer at pH 11. Plates were incubated at room temperature (~25°C), in the dark, for two weeks aerobically and anaerobically. High pH was used as a selective pressure to enrich for any alkaliphile or alkalitolerant organisms that were present in the sample. Colonies that grew were isolated and sub-sampled based upon differences in colony shape and color onto a separate pH 11 enriched media plate for further analysis (Figure 2). A chosen colony was picked with a sterilized loop and aseptically transferred to a separate pH 11 media plate using the 4-quadrant streaking method to streak for isolation and ensure a pure culture (Appendix A.)

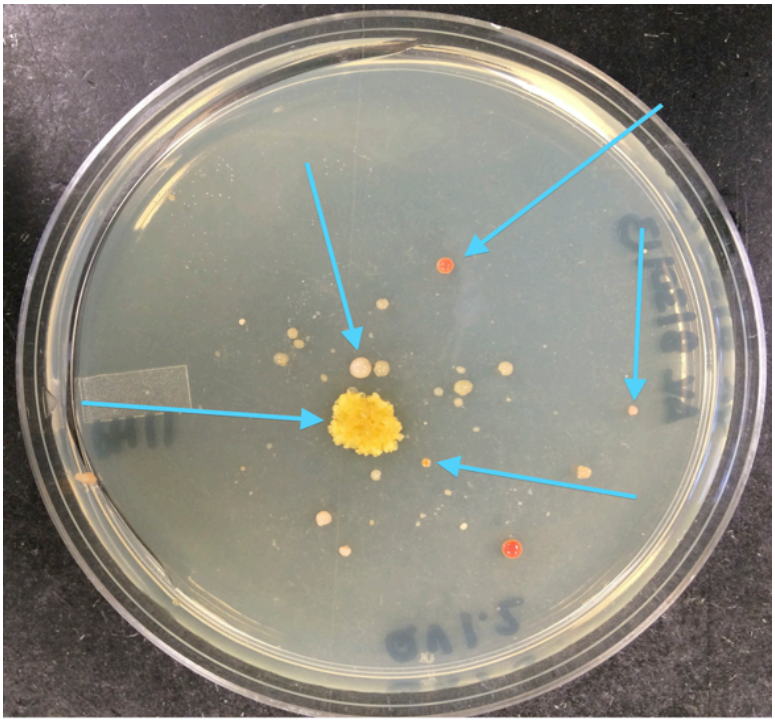


Figure 2: Mixed microbial culture from a water sample at the serpentinizing environment CROMO California, USA. A colony was chose to be isolated based upon different morphologies and colors. Arrows indicate colonies that would be considered for isolation.

Determining Colony Forming Units of CROMO Wells

Samples were sent to our lab at East Carolina University and stored at 4°C. To promote growth from water samples approximately 45 ml of sample was centrifuged at 8,000-10,000 × *g* for 20 minutes at 4°C and the resulting supernatant was removed. The pelleted biomass was re-suspended in 4.5 ml of pH 11 yeast-peptone liquid media comprised of 0.5 % wt of yeast, 0.5 % wt of peptone, and 0.1M sodium carbonate buffer at pH 11. Approximately 150 µl of the suspension, a 10-fold dilution, was aseptically spread plated once onto the pH 11 yeast-peptone plates and aerobically/anaerobically cultured in the dark at room temperature for 2 weeks. Colonies were counted to calculate the Colony Forming Units per ml (CFU/ml) of each well to determine microbial abundance and the numbers of cells per milliliter obtained by direct microscopic counts are also included for each well for comparison (Table 6).

Figure 3: An image of alkaline groundwater wells at the CROMO site in California.

Extraction of Total Genomic DNA

Following isolation of the cultures, DNA was extracted from each individual isolate using the MoBio Ultra Clean Microbial DNA isolation kit (MoBio Labs: Carlsbad, CA) following the manufacturer's protocol. Microbial cell biomass used for the DNA isolation kit was harvested from the isolated sub-cultured bacteria from the pH 11 yeast-peptone agar plates using a sterilized loop; several "loopfuls" of biomass was used for sufficient extraction. Extracted DNA was quantified using a NanoDrop 2000 UV-Vis spectrophotometer. The nucleic acid content and the absorbance at 260 and 280 nm, which determine sample purity, were recorded for each isolate's extracted DNA. Approximately 30 ng/µl DNA was needed per PCR reaction for each sample. However, due to the prolific growth of these cultures on organic-rich media, the nucleic acid content of the extracts often exceeded 100 ng/µl.

PCR-Based Assays to Amplify the 16S rRNA gene

The Polymerase Chain Reaction (PCR) was used to amplify the 16S rRNA gene from genomic DNA of the isolates. PCR reagents for 1 sample (20 µl reaction): 4.0 µl 5× go*Taq* Buffer, 2.0 µl 2 mM DNTPs, 1.0 ul 5 µM 27F Primer 1.0 µl 5 µM 1492R Primer, 10.8 µl molecular grade Water, 0.2 µl GoTaq polymerase enzyme (Promega). The thermal cycling profile was [94.0°C for 5 min. 32 cycles of 94°C for 30 sec., 54°C for 45 sec., 72.0°C for 2 min.] followed by a 10 min. extension step at 72.0°C for 10 minutes. The reactions were checked by gel electrophoresis and were purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's protocol. Purified product was sent to ECU's Core Genomics Facility to have the product sequenced with the following primers: B27F, B519R, B907R, B926R, B1492R, B515F (Table 2).

BACTERIAL 16S rRNA PRIMERS

Primer Name	Primer Sequence
27F	5'-AGAGTTTGATCCTGGCTCAG-3'
1492R	5'-GGTTACCTTGTTACGACTT-3'
515F	5'-GTGGCASCMGCCGCGGTAA-3'
519R	5'-GWATTACCGCGGCKGCTG-3'
926F	5'-GGTTAAAACTYAAAKGAATTGACGG-3'
907R	5'-CCGTCAATTCCTTTRAGTTT-3'

Table 2: Oligonucleotide primers (Supplied by Invitrogen) used to amplify the 16S rRNA gene during PCR assays

Sequences were cleaned and assembled into contigs using the program Sequencher (Gene Codes Corp.) to generate approximately 1,500 b.p. of bidirectional sequence. Contigs were compared to the sequence database on GenBank (www.ncbi.com) to determine the identity of the isolate.

Construction of Phylogenetic Trees

Phylogenetic trees were made to compare the 16S rRNA gene of the alkaliphilic isolates to each other and to determine their relatedness. Following the assembly of contigs and sequence editing, FASTA files from isolates were exported out of the program Sequencher (Gene Codes Corp.), and grouped into three different phyla, Actinobacteria, Bacilli, and Proteobacteria. Each phylum was then aligned with reference sequences on Silva, a comprehensive ribosomal RNA database, http://www.arb-silva.de/aligner. After isolated sequences were aligned with three "reference" sequences for each isolate, they were exported out of Silva and imported into the program MEGA, molecular evolutionary genetic analysis (Tamura et al., 2013). Imported aligned sequences were then used to construct a maximum likelihood tree through a MEGA utility. Pairwise distances of the 16S rRNA sequences were also constructed through the computation of the MEGA utility (data not shown).

Determining Optimum Temperature and pH

Nutrient rich plates were made as previously described at a range of different pH conditions. Different buffer solutions were made for each pH range. These included *pH 7 Buffer*: 1M monobasic sodium phosphate and 1M dibasic sodium phosphate, made into a 50.0 ml buffer solution and measured with a pH meter. *pH 8 Buffer*: 1M TRIS and 1M HCL, made into a 50.0 ml buffer solution and measured with a pH meter. *pH 9 Buffer*: 1M sodium carbonate and 1M sodium bicarbonate, made into a 50.0 ml buffer solution and measured with a pH meter. *pH 10 Buffer*: 1M sodium carbonate and 1M sodium bicarbonate, made into a 50.0 ml buffer solution and measured with a pH meter.

Plates were sectored individually into 6 different segments to hold 6 different isolates (Figure 4). Isolates were aseptically streaked onto the sector plates in their designated segment.

Each isolate was streaked on a pH 7, 8, 9, and 10 plates for three sets of each pH. Therefore for each isolate there were a total of 12 plates. For the three plates per pH, each plate was stored at a different temperatures of 20°C, 30°C, and 37°C. Subsequently, this was done for each range of pH the isolate was streaked on. Growth was monitored for a week and optimum growth conditions were determined based upon how fast the microbe grew and how dense the growth was.

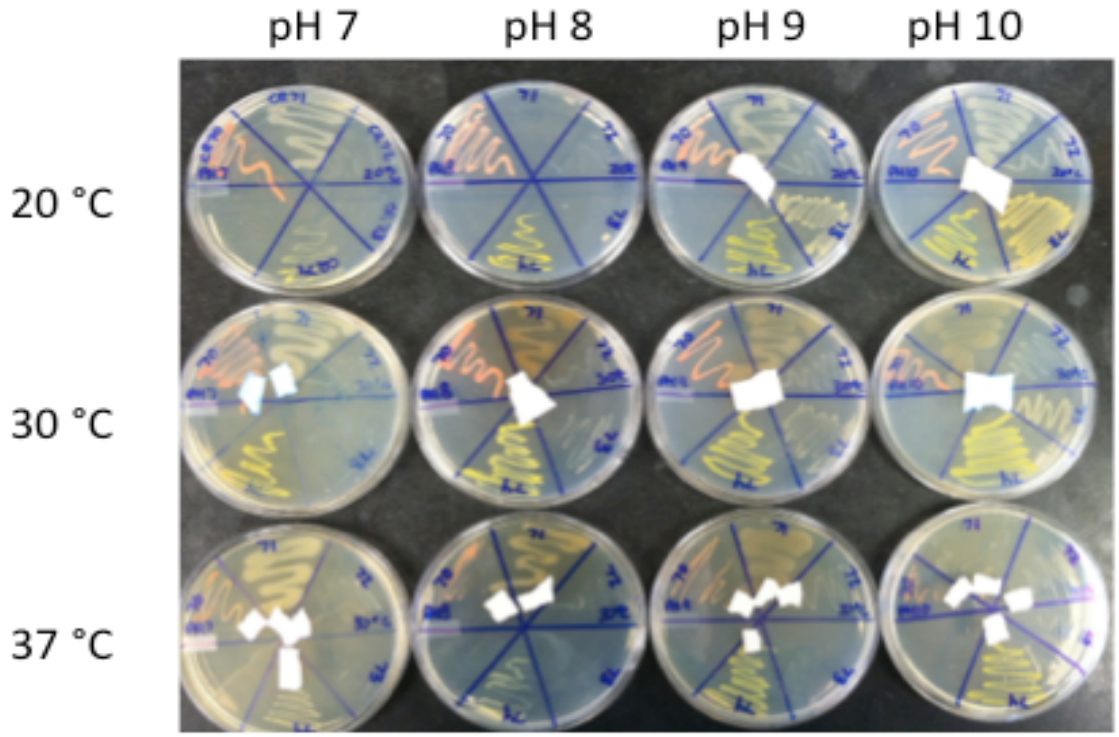


Figure 4: An example of the sector plates used to determine optimum pH and temperature. Each wedge represents a different isolate culture.

Oxidase Test

Microbial cultures grown for a maximum of two weeks on yeast-peptone agar plates were sampled with a sterile swab. A drop of BD Oxidase reagent was applied to the inoculated swab. If the reagent turned a dark purple color within a 30 second time period, the isolate was denoted

as oxidase positive. The oxidase test is an indicator if the organism has cytochrome c oxidase, which helps facilitate the transfer of electrons to the electron acceptor oxygen.

Catalase Test

A drop of 3 % hydrogen peroxide solution was released onto an isolate cultured plate and if bubbles were produced the microbe was considered catalase positive. An isolate was determined as catalase positive if there was the presence of bubbles after the hydrogen peroxide was added. The bubbling effect is indicative of the release of oxygen as a result of the catalase reaction (Figure 5).

Figure 5: An example of a positive catalase test result on isolates.

CHAPTER 5: Microbial Biodiversity and Biogeography of Serpentinite Habitats

Total Microbial Biodiversity

Alkaliphilic microorganisms have been isolated and identified from samples collected from three different serpentinite habitats located in California, Italy, and Canada (Table 3). Ultrabasic waters and soil samples were collected from Canada's Tablelands Ophiolite, which has been estimated to have been accreted to the North American continent nearly 500 Ma. The site has extensive travertine deposits that indicate past serpentinization, alkaline springs at pH ranging from 11-12 and highly reducing conditions. The 200 Ma Liguria Springs in Italy are generally similar to the Tablelands site in the sense that it too has a pH ranging from 11-12, is highly reducing, and rich in H_2 and CH_4. The youngest serpentinite habitat is the Coast Range Ophiolite in northern California at 65 Ma. The Coast Range Ophiolite site is unique in that fluid and sediment samples have been obtained directly from the serpentinizing subsurface and a long-term microbial observatory has been established. Samples collected in California include soil and water samples from the Quarry Valley (QV) and Core Shed Well (CSW) sites.

To examine the microbial diversity of these serpentinite habitats, it was imperative to examine if all habitats share common microbial communities. From the three geographically distinct serpentinite sites there was an overall dominance of the phyla Firmicutes, Actinobacteria, and Proteobacteria from the isolated cultures (Figure 6). It can also be noted that several isolates from the phylum Bacteroidetes were found at the Canadian serpentinite environment. Similar results were found through previous research on microbial diversity at serpentinite sites through culture dependent analysis (Tiago et al., 2004) although similar results have been observed in culture independent sequencing studies as well (DeGrood et al., 2004; Daae et al., 2013; Blank et al., 2009; Tiago et al., 2013). When comparing total distribution of phyla from all the isolates at

the three serpentinite sites, Firmicutes were found to make up 54.0%, Proteobacteria 22.8%, Actinobacteria 21.0% and 0.02% Bacteroidetes. Individual serpentinite sites also showed a strong dominance in Firmicutes, with varying distributions of Proteobacteria and Actinobacteria (Figure 6). This indicates that there are overall similarities in the phyla of microorganisms that can be found at these serpentinizing habitats, even though they are geographically distinct from one another. That perhaps these dominant phyla are physiologically more suited to the serpentinizing environment than other microbial phyla. Previous research done on the microbial diversity of soils show there to be at least 32 phyla groups, with as many 52 phyla can be present. In typical soils Proteobacteria, followed by Acidobacteria, dominate a majority of the soil composition, while Firmicutes contribute very little to the typical soil microbial community (Janssen 2006). This is interesting in that Firmicutes make up a large majority of these serpentinite systems, which compels the idea that perhaps these Firmicutes have adaptations that allow them to survive in these harsh serpentinite habitats.

A. Liguria Springs, Italy

Site	Type	Number of Isolates
Gor 2.1	water	1
Gor 2.2	water	1
Gor 2.4	water	1
Gor 3.A	water	1
Gor 3.B	water	1
ClIO	water	1
L43	water	1
LA1	water	1
BR2	water	1
BA2	water	1
LER20	water	4

B. Tablelands, Canada

Site	Type	Number of Isolates
TLED	water	2
WHC2A	water	9
WHC2	water	8
B30-2	water	1
DLB-1	water	1
A4A-2	water	1
A4D-2	water	1
A4A-1	water	1

C. CROMO, California

Site	Type	Number of Isolates
CSW 1.1	water	7
CSW 1.2	water	2
CSW 1.3	water	4
CSW 1.5	water	5
CSW 2	core	1
CSW 20	core	2
CSW 22	core	1
CSW 23	core	2
CSW 5	core	2
CSW 9	core	10
CSW OLD	water	17
NO8A	water	3
NO8B	water	8
NO8C	water	1
QV 1.1	water	8
QV 1.2	water	7
QV 1.3	water	5
QV 10	core	2
QV 18	core	2
QV 33	core	1
QV 41	core	1
QV 8	core	2

Table 3: 3A-C describes the number of isolates obtained from sampling sites in the serpentinizing environments of Italy, Canada, and California.

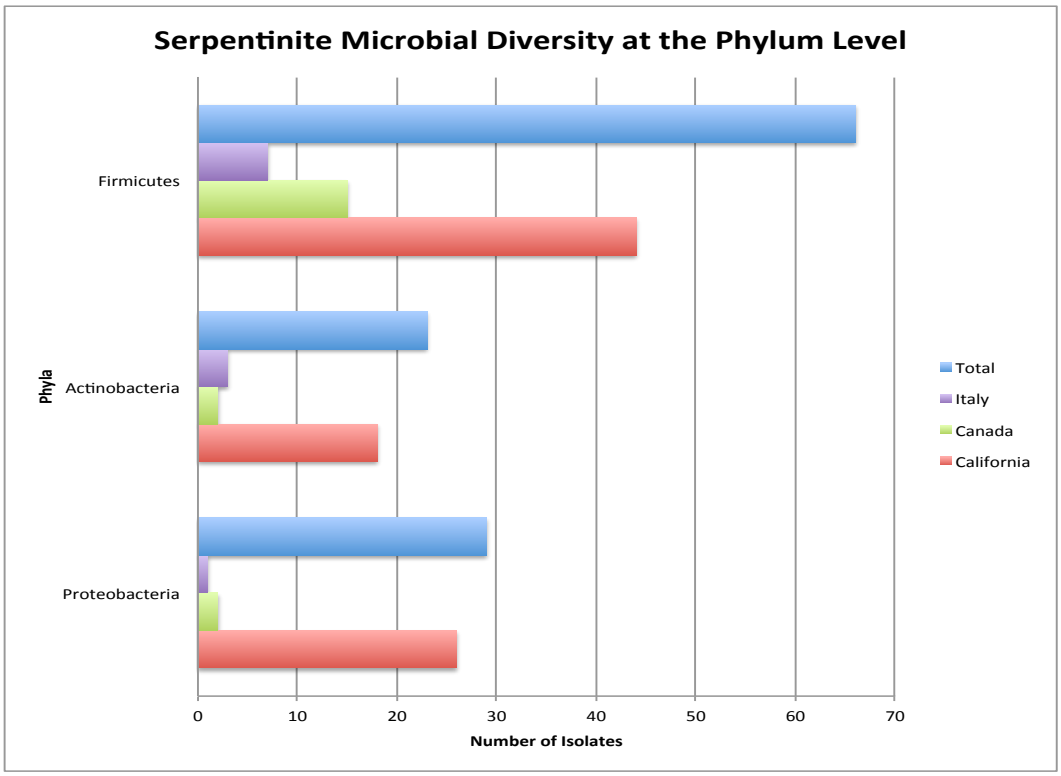


Figure 6: Displays the three dominant phyla from isolated cultures from Italy, Canada, and California

Microbial Biogeography

Magnified at the genus level, we can compare similarities of the serpentinite-hosted microbial communities more thoroughly between the different sites. For comparison, background soil samples from Greenville, NC were included and were isolated by the same procedure used for serpentinite soils. The isolates were categorized into the appropriate groups at a Genus level by looking at the 16S rRNA gene sequence similarity through NCBI BLAST (Table 4). *Bacillus* was the only genus found throughout all three serpentinizing habitats, the background sample, and data from the Portuguese hyperalkaline wells from Tiago 2004. It is interesting that *Bacillus* is found throughout all of the serpentinite sites studied and that it was even present in the background sampling. *Bacilli* are spore-forming microorganisms, which allow them to be better suited for dispersal, and perhaps more adaptive which may be why they are common in all of the sites.

Phylum	Genus	Canada	California	Italy	Portugal data from Tiago 2004	Greenville, NC
Actinobacteria	Arthrobacterium			1	X	
Actinobacteria	Brachybacterium		1			
Actinobacteria	Cellulosimicrobium			1		
Actinobacteria	Dietzia	2	7		X	
Actinobacteria	Kocuria			1		
Actinobacteria	Microbacterium		1	2	X	
Actinobacteria	Microcella	2	5			
Actinobacteria	Micrococcus		3		X	
Bacteroidetes	Aquiflexum	2				
Firmicutes	Bacillus	13	25	6	X	6
Firmicutes	Erysipelothrix		2			
Firmicutes	Exiguobacterium		4			
Firmicutes	Geomicrobium	3	5			
Firmicutes	Paenibacillus	1	5			
Firmicutes	Planococcus			1		
Firmicutes	Planomicrobium		1			
Firmicutes	Solibacillus		1			
Firmicutes	Sporosarcina		2			
Proteobacteria	Alishewanella		8			
Proteobacteria	Agrobacterium			1		
Proteobacteria	Hydrogenophaga	1	10			
Proteobacteria	Malika		1			
Proteobacteria	Pseaudomonas		7			
Proteobacteria	Thauera		1			
Total:		**24**	**88**	**13**		**6**

Table 4: Comparison of isolates at the genus level between the three serpentinite sites, background samples, and previous data from Tiago 2004.

When comparing the overlap of different genera between the three serpentinite sites studied, there were more similarities between the isolates found in Canada and California than when either was compared to Italian isolates. *Bacillus, Dietzia, Geomicrobium, Hydrogenophaga, Microcella,* and *Paenibacillus* were found both in the serpentinizing environments in Canada and California. Isolates from Italy were most similar to the data from Tiago 2004 with comparable results in the genus *Arthrobacterium, Bacillus, and Microbacterium*. Broad similarities between the populations found in Canada/California compared with Italy/Portugal suggest that serpentinizing sites being on the same continental

plate may harbor more similar organisms. In can be possible that perhaps geographical distance and dispersal barriers do play a role in the microbial distribution of certain microorganisms between similar environmental habitats. However, this result can also be a result of under sampling and further analysis will rectify when the same trend is found with more isolates.

In contrast, the genera *Bacillus* and *Microbacterium* can be found both in the serpentinite sites of Italy and California, consistent with the earlier discussion about alkalitolerant organisms enriched from background soils and the possibility that some species are better suited for dispersal, due to the ability to form spores. Perhaps with more exhaustive sampling and isolation efforts there will be more similarities among the genera found between the serpentinite sites. There is a strong bias towards the California serpentinites in the current data set, and under sampling of the Canadian and Italian serpentinite sites. Further sampling will remedy that bias and provide better resolution for comparisons between microbial communities. The current study identified what microorganisms resided at these various serpentinite sites through conventional culturing and sequencing approaches. For future analyses, sequencing the genomes of the closely related isolates will lead to greater resolution of both local and global effect of the evolution of microbial populations.

Phylogenetic Relatedness of Isolates

To examine whether isolates were genetically more related to other isolates from the same geographic region, a phylogenetic tree was constructed to understand the relationships among all of the isolates (Figure 7). There was a relatively even distribution between isolates of the same genus, but from geographically distinct separate serpentinite habitats. There are few cases where distinct genera represented entirely by one serpentinite environment; in cases where we see this instance are probably results of under sampling. In general we see the same genera

present at all serpentinite habitats. Thus, reinforces Bass Becking's hypothesis that dispersal barriers do not account for microorganism. Perhaps the serpentinite environment is selecting for particular microbes based upon genetic adaptions that allow for survival at these serpentinite habitats, which is why you see the same types of microbes in separate environments in California, Canada, and Italy. However variations and geographic separation between the serpentinite sites may have allowed for genetic evolution to be recorded in the organism's whole genomes, not just in the 16S rRNA gene. It would be interesting to examine the presence of genes in isolates that may be related to survival in the serpentinite environment, to see if these organisms are truly adapted to the habitat or merely just tolerant. Evaluation of the isolates genomes would allow for better resolution to see if the same relatedness is found between isolates, when looking at genes beyond 16S rRNA. This would be similar to the previous work discussed earlier Reno 2009. The resolution of similarities between the isolates would be better visualized by looking at whole genomic sequences, as was done in the Reno 2009 study. By comparing the genomic content of isolates the biogeographic patterns of the microbial isolates would be more apparent, and this is the intent of follow on work with these isolates.

Serpentine Soils vs. Fluids

Previous studies through culture-independent research have determined that microbial communities in serpentinite systems varied from the serpentinizing deep subsurface environments. It was found that H_2 oxidizing Betaproteobacteria dominated the fluid systems and Clostridia, containing [FeFe]-hydrogenases used to help catalyze hydrogen production, are prevalent in the deep subsurface (Brazelton et al., 2012; Brazelton et al., 2013). Phylogenetic relationships of isolates were compared to one another based upon if they originated from serpentinite rock/soils or waters (Figure 7). While there are higher instances of soil/rock isolates

from the phylum Firmicutes, there were no instances where there were specific phyla found only on a particular sample type. It can be speculated that the microorganisms in the fluid component of the serpentinite habitat are washed off from the soil and rock of that particular environment. However, it can be noted that isolates that originated from the same sample type such as soil, were clustered closer together on the phylogenetic tree than if they were from a different sample type such as soil vs. water. Different geochemical characteristics occur in the fluid systems of the serpentinite environment than its soil habitats, which could perhaps be the reason why isolates from the same sample type are more genetically similar to one another.

Divergence from Known Alkaliphiles

To evaluate whether the new isolates were closely related to other known alkaliphiles, a phylogenetic tree was constructed to determine the relatedness of reference bacteria from the Silva database. Phylogenetic trees were broken down into three groups consisting of phylum Actinobacteria and the genera *Bacilli and Betaproteobacteria.* As previously discussed, there are some close relationships between isolates from geographically different serpentinizing habitats seen in the phylum Actinobacteria (Figure 8). What is even more interesting is that many of the isolated cultures from the studied serpentinizing habitats are closely related to microorganisms found in the non-saline serpentinizing environment in Portugal. This suggests that perhaps there are some genes that are specifically required to survive in a serpentinite habitat. However, a majority of the reference sequences were microorganisms that were identified in environments unrelated to serpentinization, aside from the sequences from Portugal, meaning they are not strict alkaliphiles.

Figure 7: Radial Phylogenetic tree displaying relationships among isolates from geographically distinct serpentinite sites. The tree was constructed using 16s rRNA gene of the isolates to create a maximum likelihood tree using a muscle alignment on the program MEGA. Colors indicate the habitat from which samples were isolated. CROMO, California USA: Pink. Tablelands, Canada: Green. Liguria, Italy: Blue. Background samples from Greenville, NC: Orange. Isolates with a green arrow indicate microorganisms that originated from serpentinite soil/rock; isolates without the arrow indicate microorganisms originating from serpentinite water

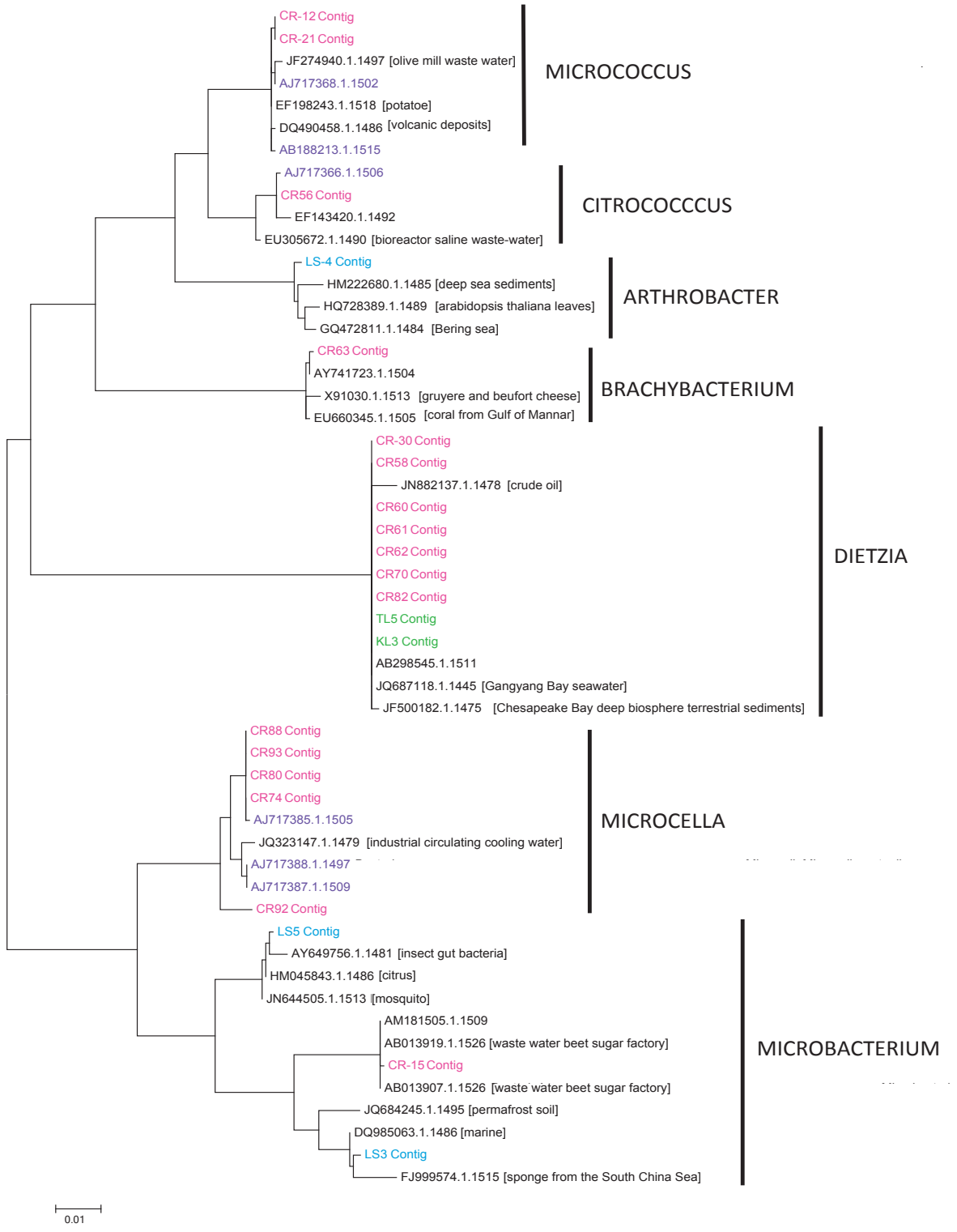


Figure 8: Phylogenetic tree of isolates from the phylum Actinobacteria. Colors indicate the serpentinite site from which the isolated originated. California, USA: Pink. Liguria, Italy: Blue. Tablelands, Canada: Green. Sequences that are purple correlate to microorganisms originating from the serpentinizing habitat in Portugal.

Isolated cultures may be able to tolerate the high alkalinity of the serpentinite sites, but may not be able to grow optimally in these habitats. There is a prominence in the number of isolates from the genera *Dietzia* and *Microcella,* which could imply that these microorganisms may contain physiological traits that enable adaptation to the serpentinite environment as compared with isolates from other taxa. The data from the phylogenetic tree of the phylum Actinobacteria suggests that you do in fact see some of the same microbes at all the serpentinite sites. These serpentinite isolates are also more closely related to other serpentinite microbes of geographically distinct habitats than they are to the references sequences that did not come from a serpentinizing habitat. This same relationship is found when looking at the phylogenetic relationships at the genus level through separate phylogenetic analysis (Fig 9, 10).

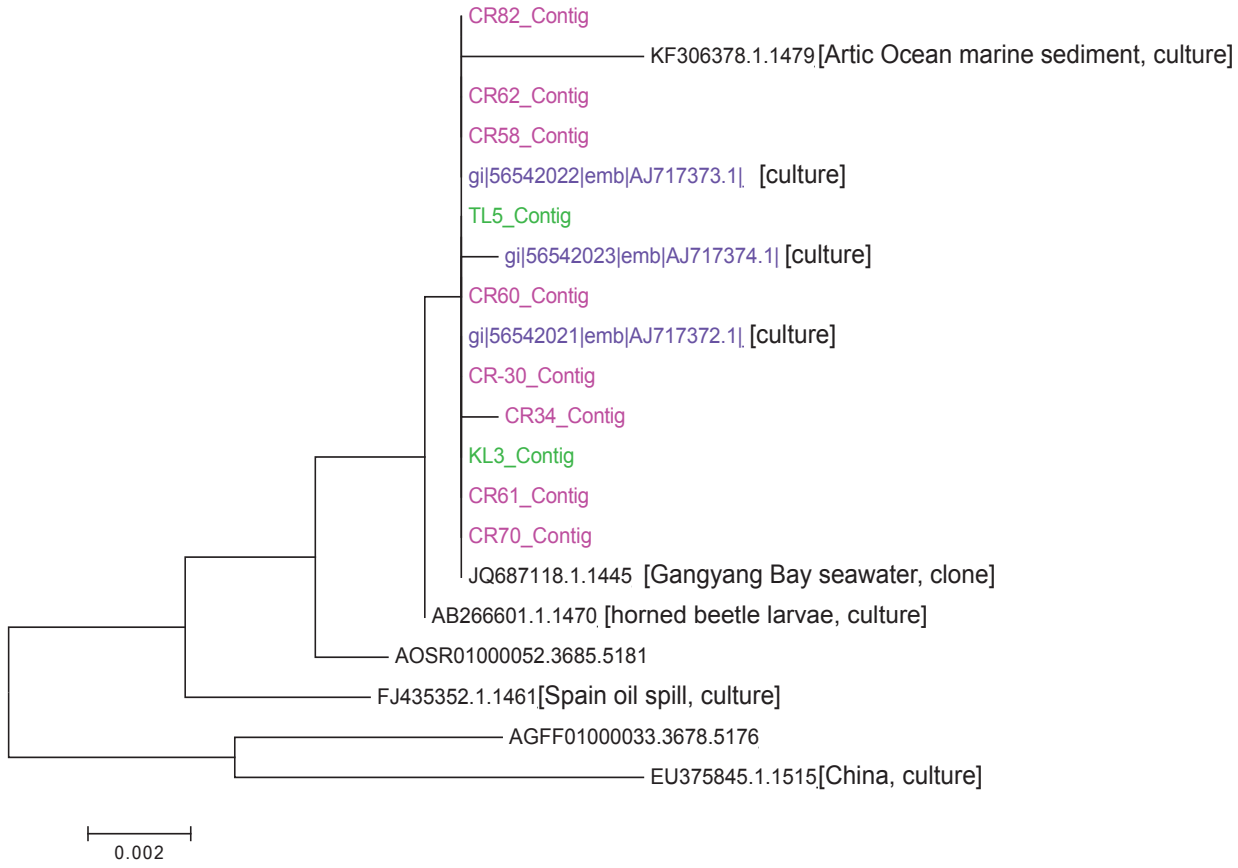


Figure 9: Phylogenetic tree of the genus *Dietzia*. Pink: California isolates, Green: Canada isolates, Purple: Portugal isolates

These relationships suggest that the serpentinite isolates may have genetic differences from their non-serpentinite counterparts that may or may not allow them to adapt to the serpentinite environment. That because of the way some of the same microorganisms are dispersed, you start to see evolutionary changes that occur due to geographical distance at the same types of habitats. It is also possible that genetic drift may play a role; where you would see some divergence from the reference sequences. Overall, the data suggest that you do see some of the same microbes at geographically distinct serpentinite habitat, but they are perhaps genetically attuned to the particular environment they are isolated from.

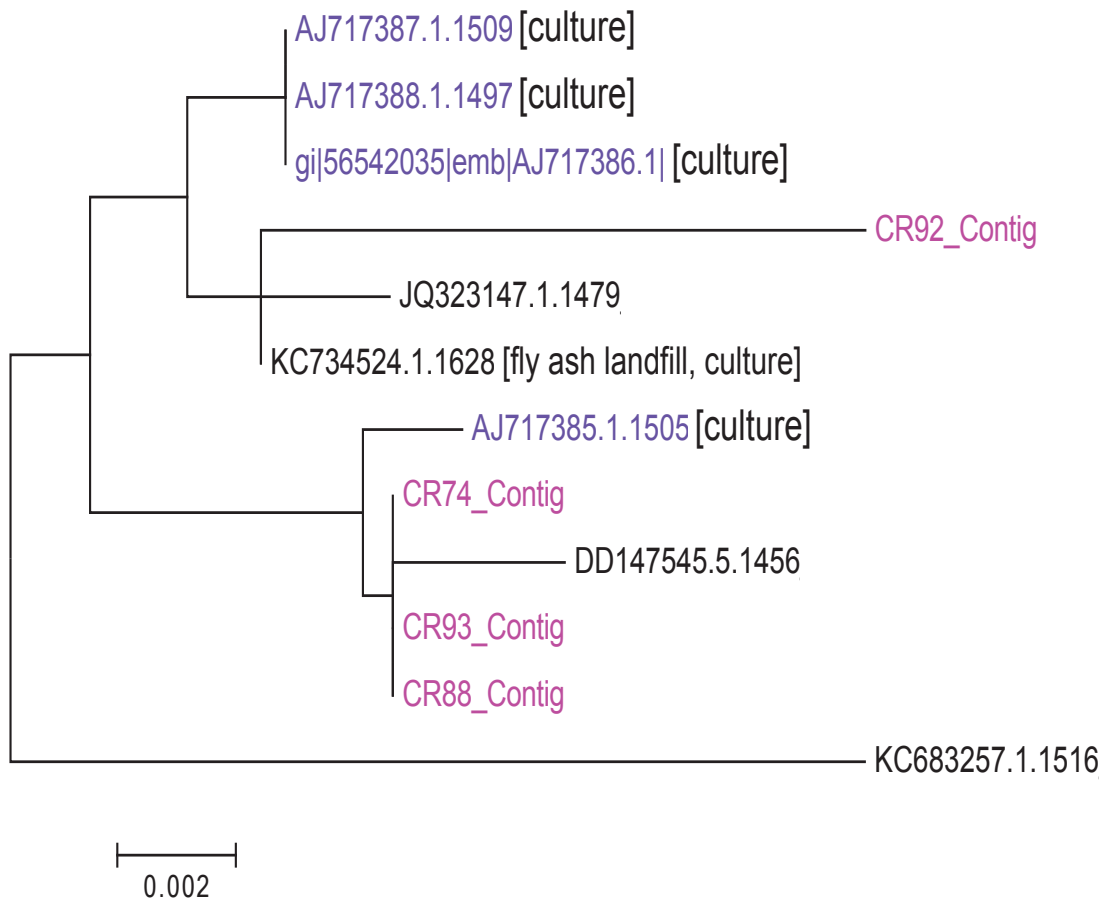
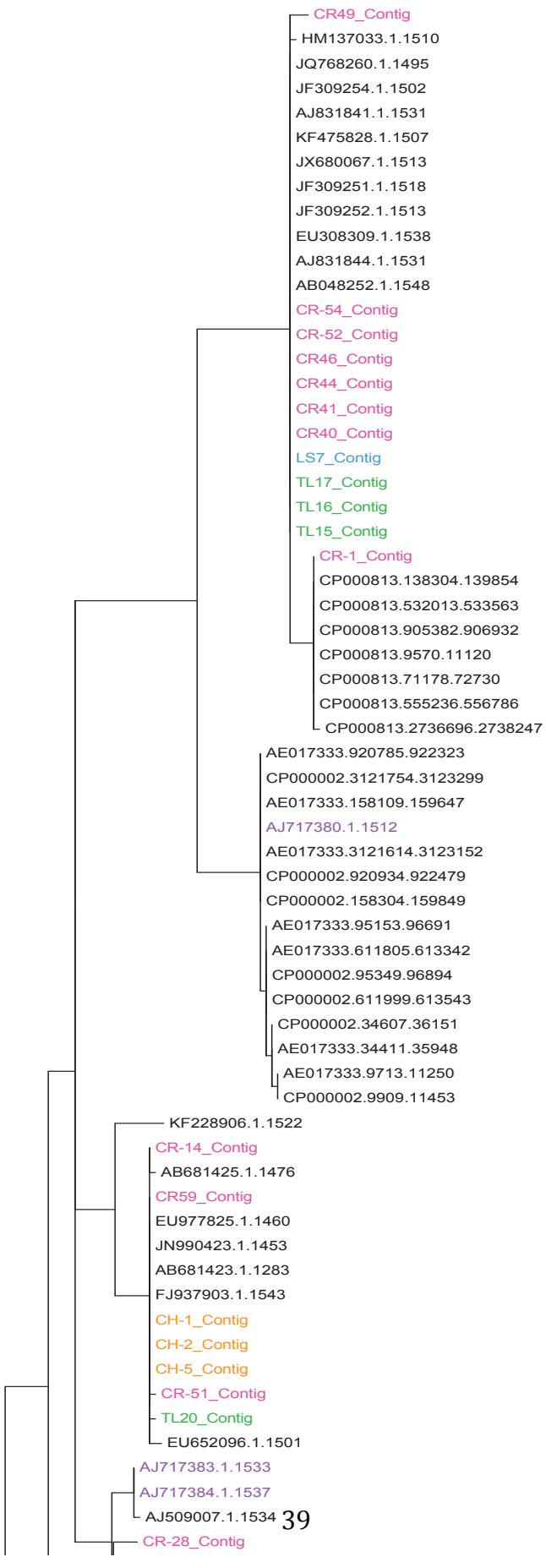


Figure 10: A phylogenetic tree of the genus *Microcella.* Pink: California isolates and Purple: Portugal isolates

As previously discussed the phylum Firmicutes were dominant in the serpentinite sites in California, Canada, and Italy. In particular the genus *Bacillus* was present in all three of the serpentinite sites and even in the background samples taken from neutral pH soil in Greenville, NC. To examine the relationship of the isolated *Bacillus*, sequences from the isolates and reference sequences from the Silva database were used to construct a maximum likelihood tree. In particular the genus *Bacillus* was investigated due to the fact that it was present in all of the serpentinite sites (Figure 11). It was curious to find that not only are the *Bacillus* which are able to live in the alkaline environment of serpentinizing habitats, distributed across all the sites, they were also genetically very similar to one another. The *Bacillus* isolates are very similar in the 16S rRNA gene, which can be shown by the short branch lengths on the phylogenetic tree (Figure 11). Comparing the 16S rRNA gene may not be enough to make comparisons between the *Bacillus* isolates and employing other genes may provide better insight. A previous study done by Kopac et al. 2014, looks at the biogeography of a group of *Bacillus* of the same species by examining different ecotype formation events that occur between species. The study found that when only conducting a three-gene analysis between the *Bacillus* species there were far fewer ecotype formations than when comparing entire genomes. There were more ecotype formation events when comparing *Bacillus* genomes by a factor of 192, which is far more effective than just looking at three genes (Kopac et al., 2014). This may be the same case with the serpentinite *Bacillus* isolates, that we are just not getting enough resolution in one gene to make adequate biogeographical comparisons.

Bacilli are known spore formers, which may allow them to be better adapted for microbial dispersal (Errington, 1993). Microbial isolates were more phylogenetically similar to one another than the background isolates and the reference sequences, shown by the distinct

branching on the phylogenetic tree (Figure 11). Perhaps microbial isolates share similar adaptive genes for the serpentinite environments, despite living in geographically distinct locations. However, since some of the isolates from California, Italy, and Canada are genetically closely tied to one another, it can be said that dispersal barriers do not apply to microorganisms. Since, we see the background samples related to other serpentinites, but with more genetic distance, it can be said that the serpentinite environment requires some genetic differences for a microorganism to live in that habitat. That there are microorganisms that contain the genetic capability to maybe not thrive in serpentinite habitats, but have the capability to survive in those harsh conditions.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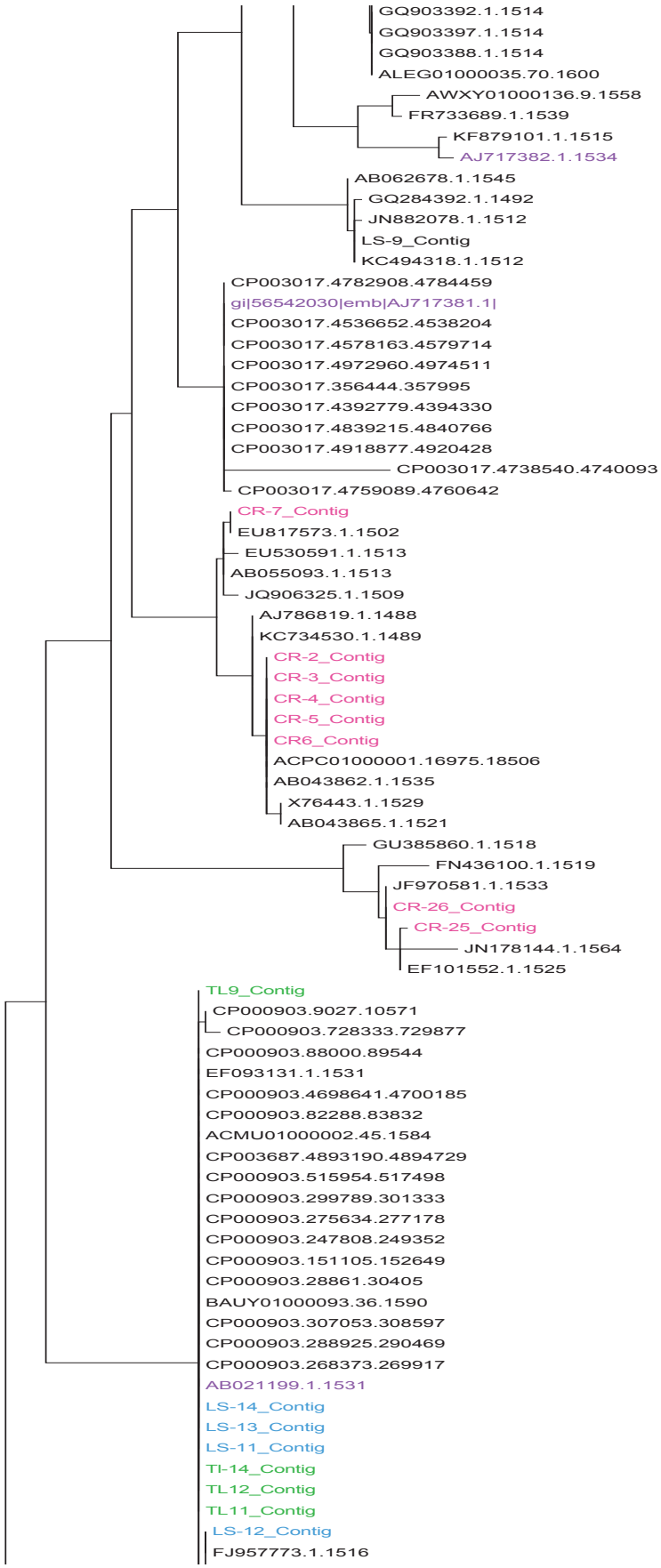
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GQ284392.1.1492
JN882078.1.1512
LS-9_Contig
KC494318.1.1512
CP003017.4782908.4784459
gi|56542030|emb|AJ717381.1|
CP003017.4536652.4538204
CP003017.4578163.4579714
CP003017.4972960.4974511
CP003017.356444.357995
CP003017.4392779.4394330
CP003017.4839215.4840766
CP003017.4918877.4920428
CP003017.4738540.4740093
CP003017.4759089.4760642
CR-7_Contig
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EU530591.1.1513
AB055093.1.1513
JQ906325.1.1509
AJ786819.1.1488
KC734530.1.1489
CR-2_Contig
CR-3_Contig
CR-4_Contig
CR-5_Contig
CR6_Contig
ACPC01000001.16975.18506
AB043862.1.1535
X76443.1.1529
AB043865.1.1521
GU385860.1.1518
FN436100.1.1519
JF970581.1.1533
CR-26_Contig
CR-25_Contig
JN178144.1.1564
EF101552.1.1525
TL9_Contig
CP000903.9027.10571
CP000903.728333.729877
CP000903.88000.89544
EF093131.1.1531
CP000903.4698641.4700185
CP000903.82288.83832
ACMU01000002.45.1584
CP003687.4893190.4894729
CP000903.515954.517498
CP000903.299789.301333
CP000903.275634.277178
CP000903.247808.249352
CP000903.151105.152649
CP000903.28861.30405
BAUY01000093.36.1590
CP000903.307053.308597
CP000903.288925.290469
CP000903.268373.269917
AB021199.1.1531
LS-14_Contig
LS-13_Contig
LS-11_Contig
TI-14_Contig
TL12_Contig
TL11_Contig
LS-12_Contig
FJ957773.1.1516

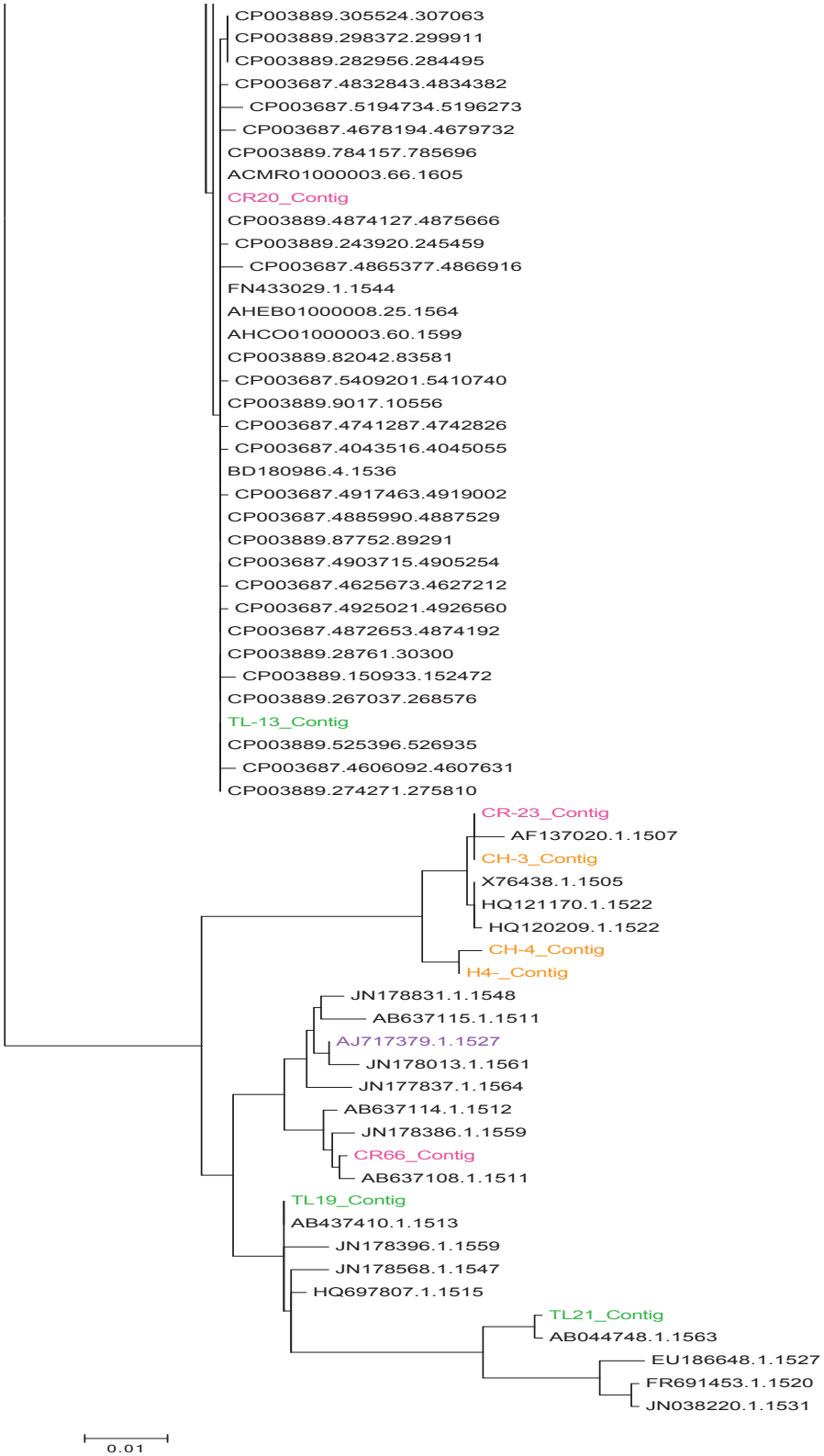

Figure 11: The tree displays isolates and reference sequences from the genus *Bacillus.* Isolates are color coordinated according to the serpentinite site they originated from. California, USA: Pink. Liguria, Italy: Blue. Tablelands, Canada: Green, Portugal: Purple. Background samples from Greenville, NC: Orange.

Previous studies have found that the class Betaproteobacteria, in particular the genus *Hydrogenophaga*, is very prominent in aquatic habitats associated with serpentinization (Brazelton et al., 2012 and 2013, Schrenk et al., 2013; Suzuki et al., 2014). It could be possible that this particular genus of bacteria are specifically adapted to the serpentinizing habitat and thrive off of the hydrogen that is produced from it. To examine if the *Hydrogenophaga* isolates were closely related to other alkaliphiles in the genus, a phylogenetic tree of *Hydrogenophaga* was constructed with references sequences from the Silva database (Figure 12). Phylogenetically there are two distinct groups of *Hydrogenophaga*. One group is closely related to *Hydrogenophaga* species found in the serpentinizing site in Portugal while the other isolates cluster into a separate distinct group of *Hydrogenophaga*.

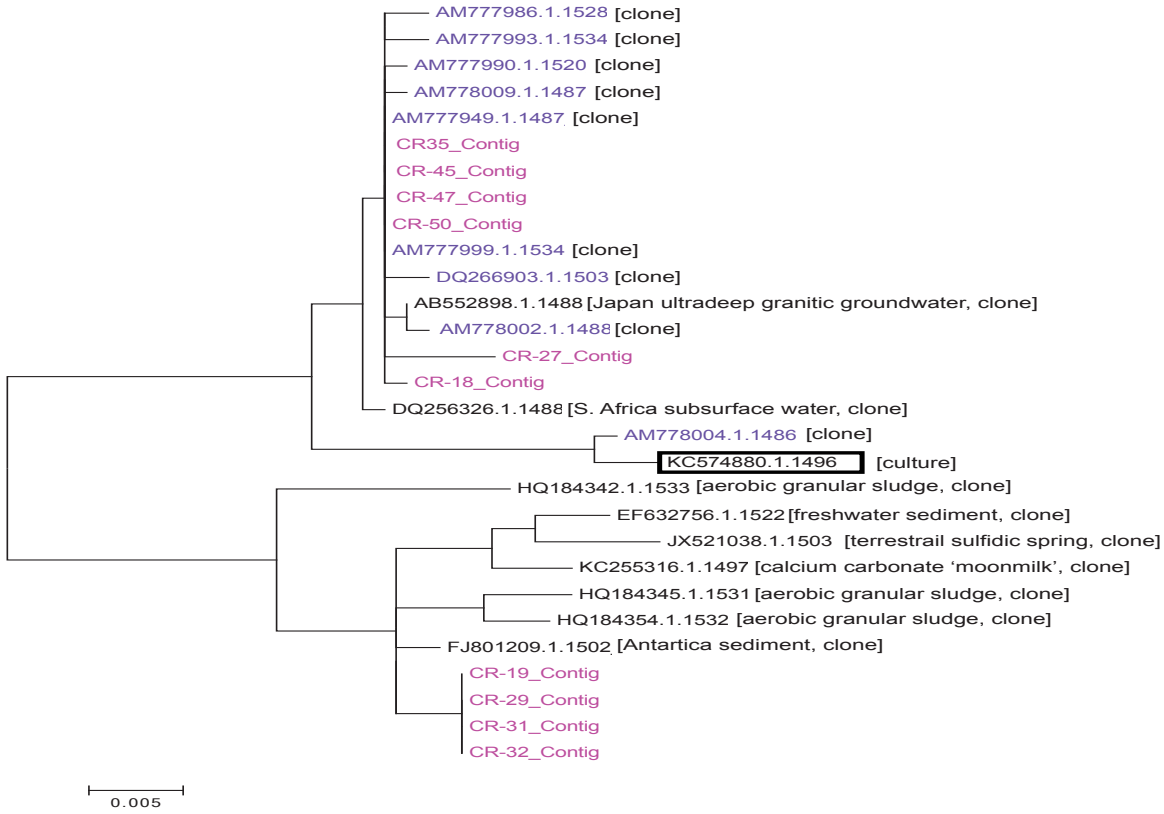

Figure 12: The tree displays isolates and reference sequences from the genus *Hydrogenophaga*. Isolates are color coordinated according to the serpentinite site they originated from. California, USA: Pink. Sequences that are purple indicate microorganisms originating from the serpentinizing habitat in Portugal. Boxed sequences are *Serpentinomas*.

Both groups of *Hydrogenophaga* are hydrogen-oxidizing bacteria, as referenced by their genus name, which may be why they are so prevalent in the hydrogen producing serpentinite environment (Figure 12). The ubiquity of Hydrogenophaga at serpentinite sites is also reflected in culture independent data produced using next generation sequencing approaches (Sukuzi et al., 2013; Brazelton et al., 2013, Daee et al., 2013). Through culture independent surveys of bacterial populations the abundance of various *Hydrogenophaga* species at serpentinite sites in geographically different regions was evaluated. The heat map shown in Figure 13 includes some of the cultured isolates of *Hydrogenophaga*, in which the group of isolates that were closely related to Tiago's isolates from the serpentinite site in Portugal and to *Serpentimonas*, were in high abundance at many of the serpentinizing habits. While the other group of *Hydrogenophaga,* which were not closely related to the Portugal isolates, were not as abundant in many of these serpentinizing habitats.

This data suggest the isolated *Hydrogenophaga* can be categorized into two different groups, a group that has adapted to live successfully in a serpentinizing environment and a group that can be found more commonly in other habitats besides the serpentines. It would seem that one group of *Hydrogenophaga* has genetically evolved to become better optimized for the surviving the harsh serpentinite environment. This group of *Hydrogenophaga* may be part of a new proposed genus called *Serpentinomas. Serpentinomas,* as previously discussed, are close relatives to *Hydrogenophaga. Serpentinomas* were isolated from the serpentinizing habitat in The Cedars, California (Suzuki et al., 2014). The *Serpentinomas* isolates were able to grow heterotrophically in lab conditions, consistent with the current work. However, the *Serpentinomas* were found to couple hydrogen oxidation, typical for *Hydrogenophaga* species,

and utilize solid calcium carbonate from the serpentinizing habitat as a carbon source (Suzuki et al., 2014).

These *Serpentinomas* can be found through a wide range of alkaline habitats, whether they are artificial or naturally occurring. (Suzuki et al., 2014) With this proposed new genus and the presence of two distinctive groups of *Hydrogenophaga* from my isolates it is likely that one of the groups of *Hydrogenophaga* are actually part of the *Serpentinomas* genus which are highly adapted to serpentinizing habitats.

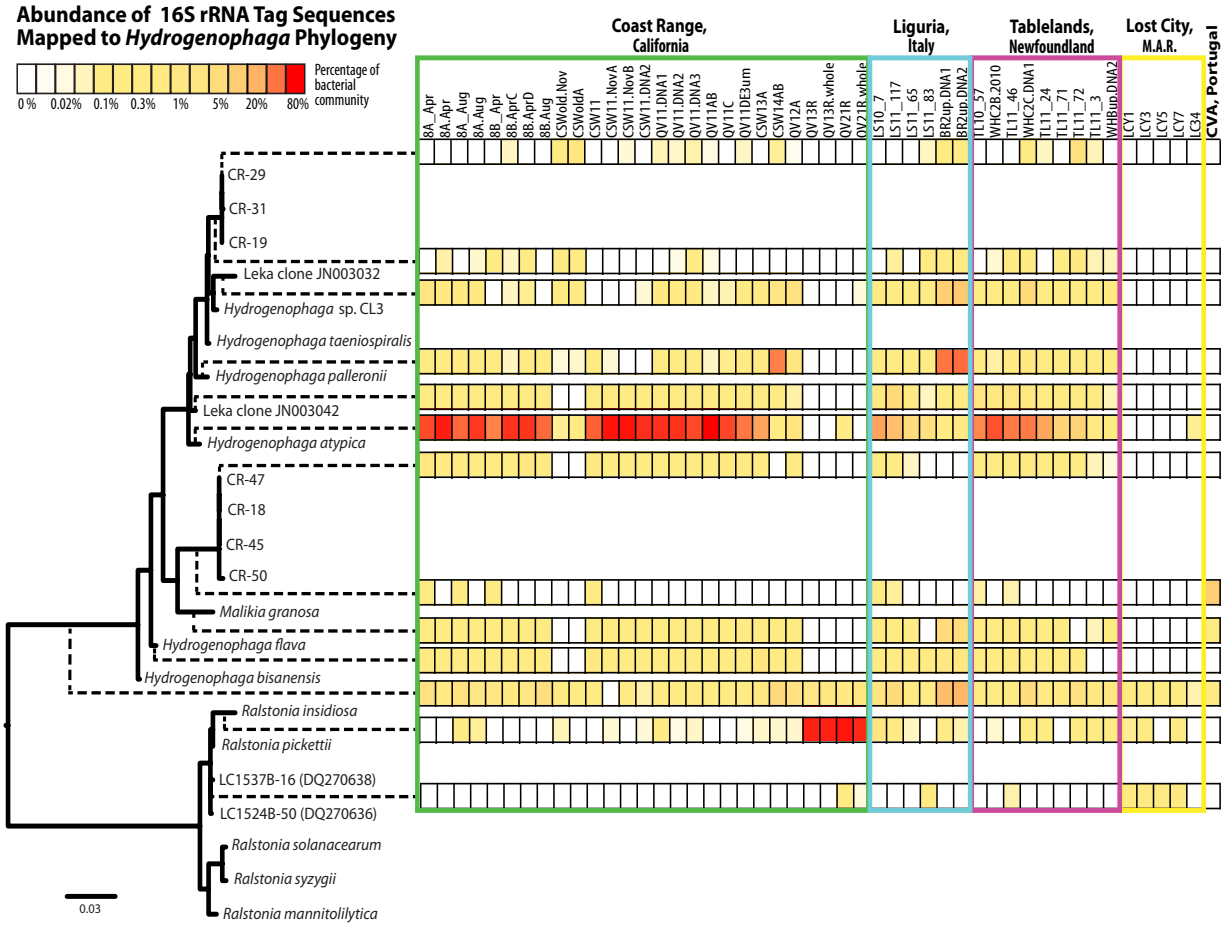

Figure 13: Figure made by Dr. W. Brazelton (University of Utah). The figure depicts the relative abundance of 16S rRNA tag sequences in environmental samples from different clades of *Hydrogenophaga* and their closest relatives. The figure above uses next generation tag sequencing of the 16S rRNA gene that shows the abundance of difference species of Hydrogenophaga at different serpentinite sites. Cultures that I have isolated and identified in the lab are also displayed in the figure as follows CR29, CR 31, CR 19, CR 47, CR 18, CR 45, and CR 50. Based upon where my isolates fall on the phylogenetic tree, there appears to be two different groups of Hydrogenophaga isolated.

CHAPTER 6: Microbial Physiology in the Context of the Environment

Coast Range Ophiolite Microbial Observatory, California

Experimental physiological data of isolates were compared to the environmental characteristics of different wells at the Coast Range Ophiolite Microbial Observatory located in California, USA. A series of fluid samples were collected for this study in August 2013. Table 5 displays the environmental pH, temperature, Eh (mV), dissolved oxygen (mg/L), conductivity (mS/cm^3), salinity, DOC, ammonia, nitrogen dioxide, total oxidized dissolved nitrogen species, and phosphate of the different CROMO wells; data was collected by colleagues in the Schrenk lab. The Eh measures the oxidation and reduction potential of the well, the more negative the potential of the solution the more likely it will lose electrons. The DO refers to the amount of dissolved oxygen in the water and conductivity is the measure of water purity. DOC is a measure of the amount of dissolved organic carbon present in each of the CROMO wells.

CROMO WELL	pH	Temperature °C	Eh (mV)	DO (mg/L)	Conductivity (mS/cm3)	Salinity (‰)	DOC (uM)	NH_4 (uM)	NO_2 (uM)	NO_x (uM)	PO_4 (uM)
NO8A	10.42	16	-139	0.19	5.88	4	195	398	0.07	5.71	0.56
NO8B	10.98	16	-65	0.31	3.07	2	104	222	0.09	0.20	0.12
NO8C	7.55	14	217.3	0.17	1.14	1	211	7.08	1.28	41.98	1.94
QV 1.1	11.64	16	-91.7	0.15	2.61	2	308	297	0.03	0.12	0.07
QV 1.2	9.07	16	-9.8	0.79	2.78	2	43	307	0.03	bdl	0.07
QV 1.3	9.63	16	-183.4	0.03	6.2	4	97	551	0.03	bdl	2.38
CSW 1.1	12.39	16	-255	0.2	4.49	2	989	1093	bdl	0.61	0.47
CSW 1.2	9.15	15	130.3	3.55	4.17	3	110	942	0.38	0.28	1.30
CSW 1.3	10.2	16	-135	0.14	4.69	3	84	1277	0.05	0.03	1.25
CSW 1.5	9.95	15	-223.5	0.27	4.74	3	139	1177	bdl	0.00	0.76

Table 5: August 2013 environmental data from several wells sites at the Coast Range Ophiolite Microbial Observatory, California.

Cell counts as determined by fluorescent microscopy of each well were relatively similar to one another, suggesting that the microbial abundance between the different serpentinizing wells were consistent. However, the number of colonies that grew in the alkaliphilic heterotrophic media varied between the different CROMO wells. This suggests that although the microbial abundance is similar between well sites, they share distinct microbial communities (Table 6). The identities of the isolates were found by evaluating the 16S rRNA gene, as previously described. It was found that the at the genus level, through comparisons by NCBI BLAST, that the microbial diversity did vary between the wells (Table 7). This variation of microbial communities may effect on how abundantly they grew on the alkaline, heterotrophic medium. This implies that the predominant microorganisms in some these wells with high CFUs were alkaliphiles and were able to grow in some cases abundantly on the heterotrophic alkaline medium.

CROMO WELL	CFU/ml	Cell Counts (cells/mL)
NO8A	66.7	8.97E+05
NO8A AN	66.7	8.97E+05
NO8B	66.7	6.15E+05
NO8B AN	200	6.15E+05
NO8 C	466.7	5.57E+05
CSW 1.1	133.3	2.95E+05
CSW 1.2	TNTC	3.65E+06
CSW 1.2 AN	TNTC	3.65E+06
CSW 1.3	266.7	2.14E+06
CSW 1.3 AN	4933.3	2.14E+06
CSW 1.5	TNTC	4.18E+06
QV 1.1 AN	133.3	2.66E+06
QV 1.2	TNTC	1.57E+06
QV 1.2 AN	66.7	1.57E+06
QV 1.3	7200	6.45E+06
QV 1.3 AN	7533.3	6.45E+06

Table 6: Colony Forming Units (CFU) per ml of each well at the CROMO, California site. TNTC= Too numerous to count

Genus	NO8 A	NO8 B	NO8 C	QV 1.1	QV 1.2	QV 1.3	CSW 1.1	CSW 1.2	CSW 1.3	CSW 1.2
Alishewanella						2		1	2	3
Bacillus							1			
Dietzia					1					1
Erysipelothrix					2					
Geomicrobium	1	1	1				1			
Microcella	1	1				1		1	2	
Pseudomonas						2				1
Thauera										
Total:	2	2	1	1	7	5	2	2	4	5

Table 7: Table displaying the number of isolates obtained from each serpentinizing CROMO well and the microbial diversity of each well. Identity of QV 1.1 isolate was unable to be identified due to issues with DNA extraction.

Experimental Optimum pH and Temperature of Isolates

Microbial isolates were then obtained on a separate pH 11 nutrient agar plate. Approximately 1-5 isolates were obtained from each water sample. Each isolate was then plated onto 3 sets of nutrient agar plates at a pH of 7, 8, 9, or 10. Microbial isolate sets plated at a specific pH were then individually incubated at three different temperatures 20°C, 30°C, and 37°C. Plates were monitored and observed daily for a week (Figure 4). Based upon how fast the isolated culture grew and how dense the growth determines the qualitative optimum pH and temperature of the alkaliphilic microbe. Growth was measured on a + scale, meaning + barely any growth, ++ for medium growth, +++ for high growth; the faster and the more dense the culture the more optimized the microbe is for that temperature or pH (Table 8)

Temperature 20°C

Sample 9/16/13	pH7	pH8	pH9	pH10
CR 64			++ Day 1	++ Day 1
CR 65			++ Day 1	++ Day 1
CR 66	+ Day 4	+ Day 4	+ Day 1	+ Day 1
CR 67			++ Day 1	++ Day 1
CR 68	+ Day 2		++ Day 1	+++ Day 1
CR 69			++ Day 1	+ Day 1
CR 70	+ Day 2	+ Day 2	+ Day 1	+ Day 1
CR 71	++ Day 2		++ Day 1	+++ Day 1
CR 72	+ Day 2	+ Day 7	+ Day 1	+ Day 1
CR 73			++ Day 1	++ Day 1
CR 74	+ Day 7	+ Day 4	+ Day 1	++ Day 1

Table 8: An example table showing how optimum temperature and pH was determined. The date beside 'Sample" describes the start of the experiment. Each set of pH 7, 8, 9, and 10 plates were grown at 20°C in this case and +, ++, +++, describes the start and density of microbial growth a number of days after inoculation

Experimental physiological data of each isolate were then compared to the environmental data of the CROMO well it was isolated from. Figure 14 depicts the relationship between the environmental pH of each CROMO well and the average experimental optimum pH of the isolates from each well. Each plot represents each serpentinizing well at the CROMO site. Based upon the data there seems to be a strong relationship between the environmental pH and the experimental optimum pH of the isolates. To test the correlation of the environmental and experimental pH a linear regression line was made on the plots and the value of $R^2 = 0.09772$ indicates there is a strong relationship between experimental and environmental pH. The data suggest that the isolated microorganisms, selecting specifically for alkaliphiles, are true alkaliphilic bacteria or microorganisms that have adapted to the alkalinity of the serpentinite environment. Microbial isolates from the wells may have several of the previously named mechanisms that allow them to adapt to the alkaline conditions, which may be the reason for the preference of alkaline conditions in a lab cultured setting.

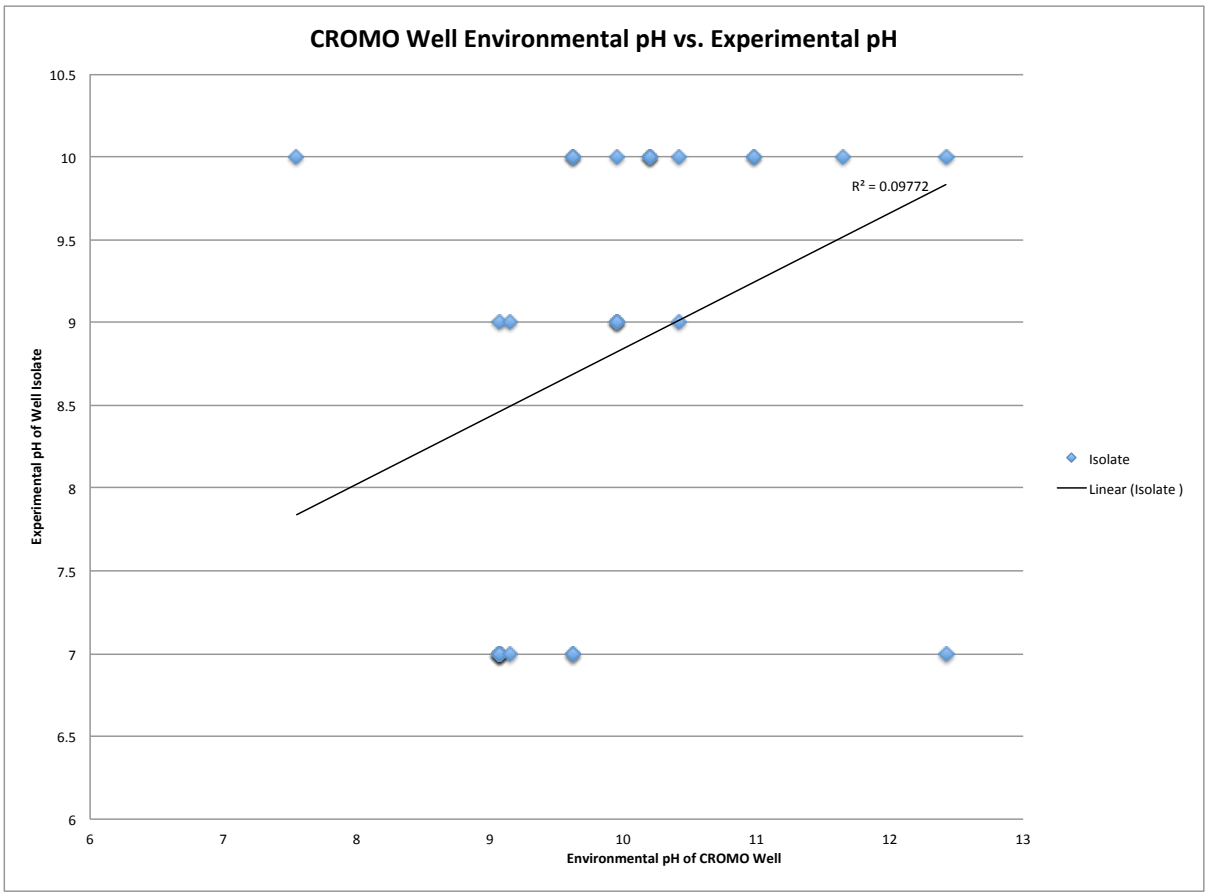

Figure 14: Scatterplot comparing the experimental optimum pH of isolates to the environmental pH of the CROMO well it was isolated from. The y-axis identifies the average experimental optimum pH of the isolates, while the x-axis states the environmental pH of the well. Different colored and shaped plots represent each of the CROMO wells. R^2: 0.09772

To test whether there was any relationship with the experimental optimum temperature and the environmental temperature of each well, isolates were incubated at three different temperatures as mentioned previously. The temperature between the wells did not vary much from on another, the average between all of the well sites were around 16°C. One might assume that because the relative temperature of the well was relatively low that the optimum growth temperature of the isolate would also follow closely to the well temperature. However, the data suggests that there is not a clear correlation between the temperature of the well and the experimental optimum temperature of the isolates. (Figure 15)

There was no correlation between the environmental temperature and the average experimental temperature of the isolates, $R^2 = 0.02635$. When grown in a lab setting isolates preferred warmer growing conditions. It can be noted that the samples were cultured and isolated using a selective filter of ~25°C, thus isolates should grow relatively well at this temperature. The optimum experimental growing temperatures of a majority of the isolates were typically higher than 25°C. There was no obvious correlation between the temperature effects on the growth of the isolates and their sample temperature. This suggests that temperature is not a major selecting factor for the microorganisms that inhabit the CROMO wells. It can also be said that because the experimental optimum temperature varied so differently from the well temperature, that these microorganisms may not be growing optimally in their natural environment.

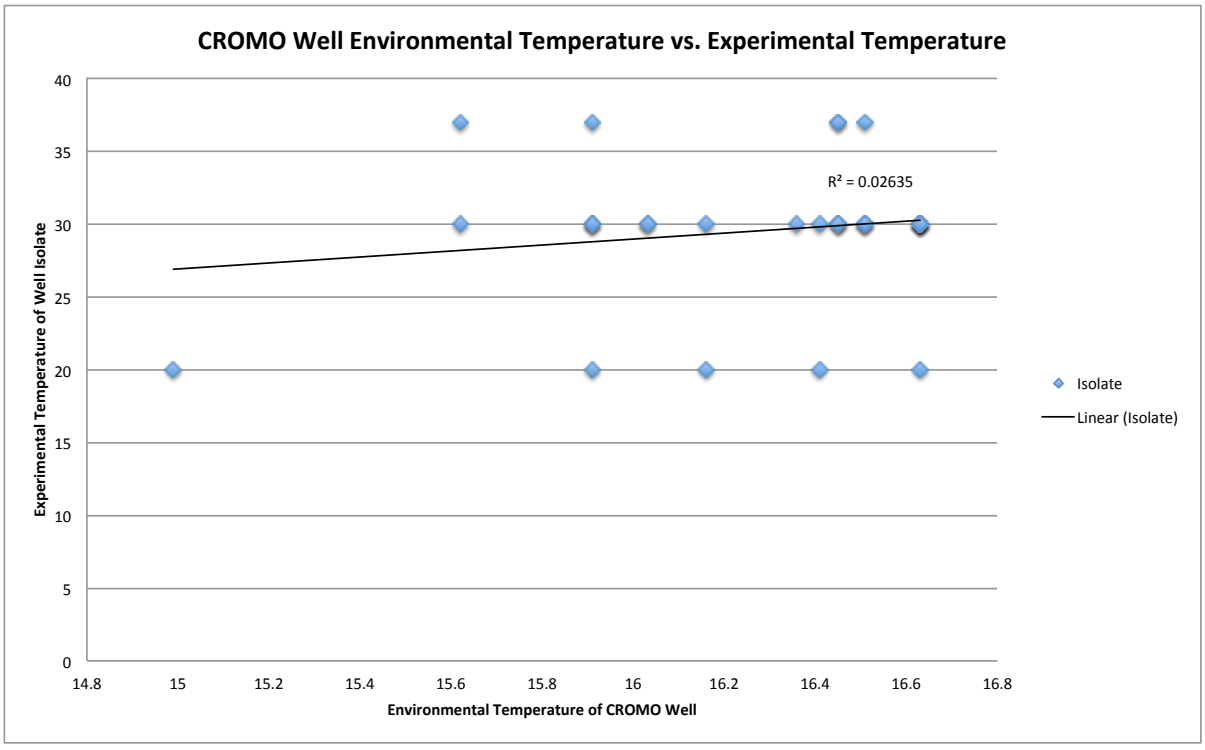

Figure 15: Scatterplot comparing the experimental optimum temperature of isolates to the environmental temperature of the CROMO well it was isolated from. The y-axis identifies the average experimental optimum temperature of the isolates, while the x-axis states the environmental temperature of the well. Different colored and shaped plots represent each of the CROMO wells. R^2-value: 0.02635

Oxidase Test Results

Microorganisms from the CROMO wells were isolated and cultured under aerobic conditions. The relative amounts of dissolved oxygen in each of the wells were relatively consistent with one another (Table 5). Most aerobic organisms use the cytochrome c oxidase enzyme to transfer electrons from the electron donors to the electron acceptor oxygen. It has been shown in previous studies that the levels of cytochromes are elevated in alkaliphiles. Microorganisms that are oxidase positive are typically aerobic, however that does not mean they are strict aerobes. The data from the oxidase test of the isolates show there was slightly more oxidase positive isolates 58.6 % that there was oxidase negative isolates 41.4% (Figure 16). These data may support the idea that cytochromes are more prominent in alkaliphiles. However, there seems to be no trend between the alkalinity and the amount of dissolved oxygen in relation to whether the isolate contained cytochrome c oxidase. The results suggest that some isolates have the ability to use oxygen as an electron acceptor in the electron transport chain or that they are using some other molecule as their electron acceptor or they use alterative oxygen detox mechanisms. *Hydrogenophaga* sp. is known to use oxygen as an electron acceptor while *Bacillus sp.* can also use nitrate and Actinobacteria can use nitrite (Suzuki et al., 2014; Nakano et al., 2007; Ventura et al., 2007). There are so few electron acceptors in the serpentinite environment it would be interesting to determine the range of alternative electron acceptors are being utilized by the isolates in this particular serpentinite environment. It could be possible based upon the NO_x (uM) levels (Table 5) of the CROMO wells that different nitrogen species (nitrate, nitrite, etc.) are being used as electron acceptors.

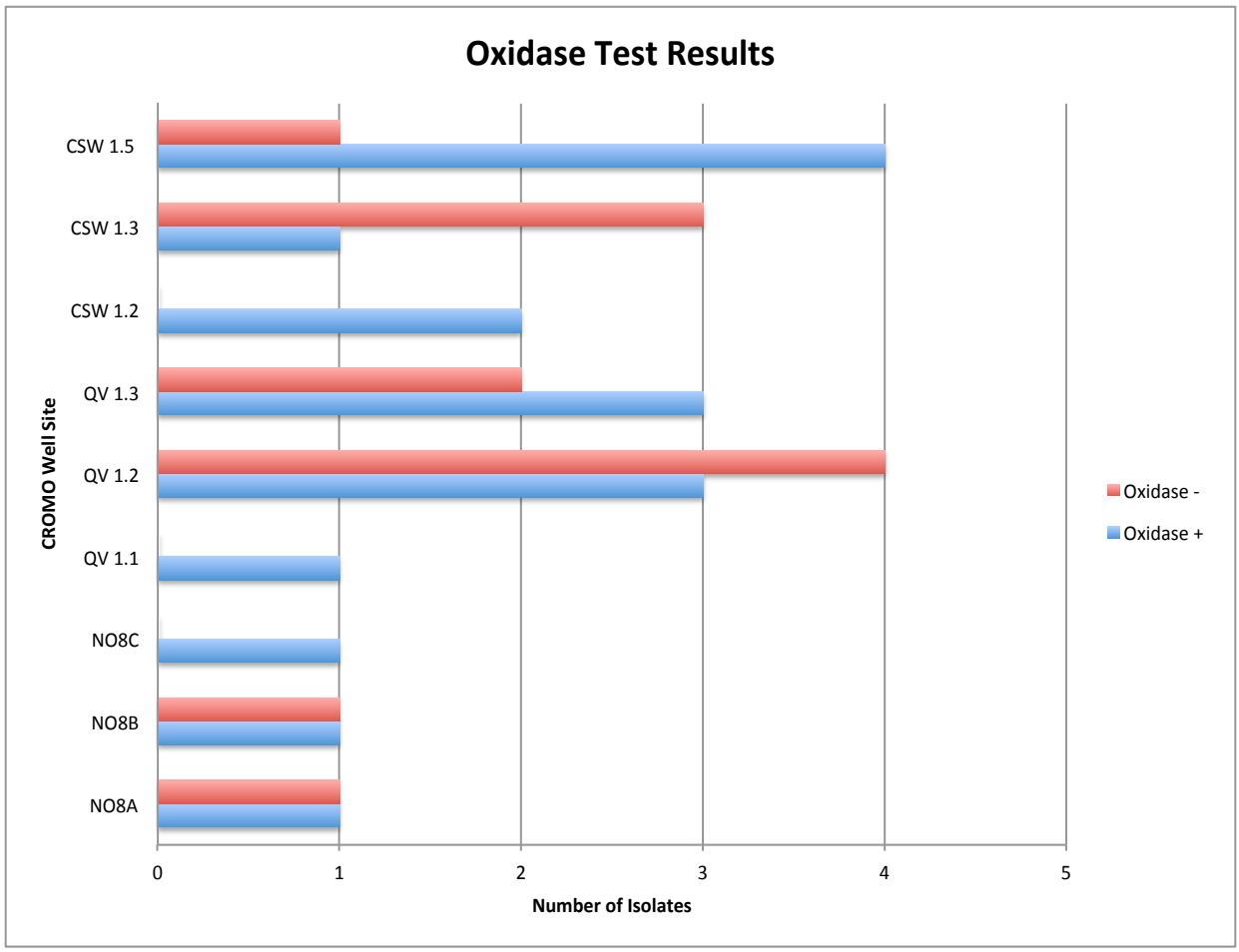

Figure 16: The number of oxidase +/- isolates for each CROMO well site. The light blue bar indicates the number of oxidase positive isolates for the particular well, while the dark blue bar indicates the number of oxidase negative organisms

Catalase Test

As a product of redox reactions happening inside aerobic microorganisms hydrogen peroxide is constantly accumulating. Hydrogen peroxide can result in higher mutation rates, growth defects, and possibly death. Hydrogen peroxide is toxic to these organisms, so microorganisms produce enzymes such as catalase to keep the toxin levels relatively low. The catalase enzyme is present in many aerobic bacteria that break down hydrogen peroxide into molecular oxygen and water (Mishra et al., 2012). Based upon the experimental results most of the organisms isolated were catalase positive 89.7% versus 10.3% of the catalase negative

isolates (Figure 17). There does not seem to be any relationship between the number of catalase positive organisms with the amount of dissolved oxygen in each well or the number of experimentally oxidase positive organisms. It is common to see catalase in most aerobic bacteria and since these microorganisms were isolated aerobically it is not surprising to see that most are catalase positive. Isolates were also grown in anaerobic conditions and it was found that a large majority was able to grow aerobically and anaerobically (Appendix A). This suggests that these isolates were not strict aerobes or anaerobes, but perhaps were facultative anaerobes that required oxygen detox mechanisms such as the enzyme catalase when grown under oxic conditions.

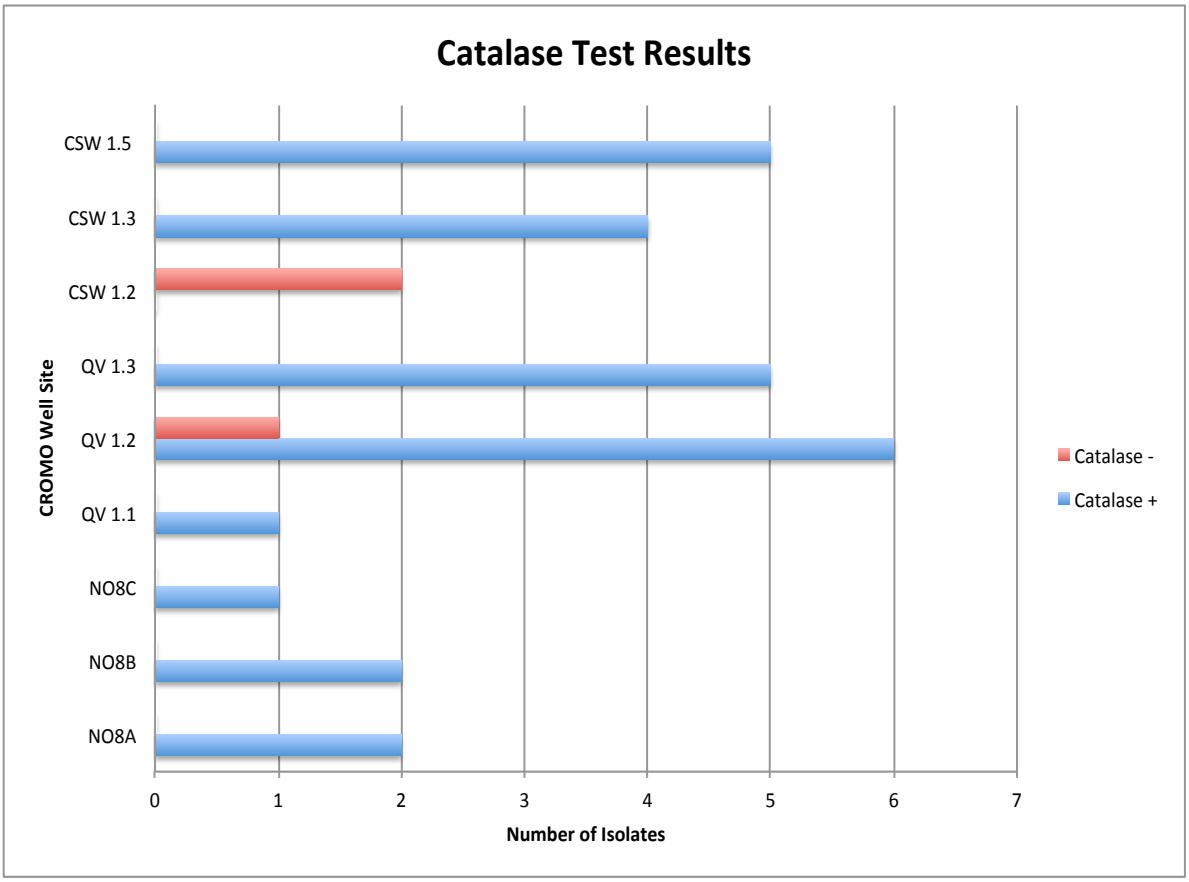

Figure 17: The number of catalase +/- isolates for each CROMO well site. The light blue bar indicates the number of catalase positive isolates for the particular well, while the dark blue bar indicates the number of catalase negative organisms.

CHAPTER 7: Discussion

Microbial diversity of the serpentinite subsurface has not been intensively studied and the number of cultured bacteria from these serpentinite sites is low. This study focuses on examining the biodiversity of alkaliphilic heterotrophic bacteria from several serpentinite sites in California, Canada, and Italy in comparison to previous studies. This study also evaluates the experimental microbial physiology of isolated alkaliphilic heterotrophic microbial cultures in relation to the characteristics of the environment from which they were isolated.

When examining microbial diversity at the phylum level we see three major phyla present at each of the three serpentinite sites, Firmicutes, Actinobacteria, and Proteobacteria. Similar studies on microbial diversity have also examined this phenomenon, where they find the same three phyla present at additional serpentinite habitats (Tiago et al., 2004), DeGrood et al., 2004, Daae et al., 2013, Blank et al., 2009, Tiago et al., 2013). Firmicutes not only made up a large portion of the total isolates from all the serpentinite sites, but also dominated in abundance at each individual site. This may indicate that the phylum Firmicutes are more adapted to being geographically dispersed and are better suited to this serpentinizing environment. Proteobacteria was the second most prevalent phylum when examining the isolates in their entirety, however for each individual serpentinite it was comparable in relative abundance with the phylum Actinobacteria. This predominance of Firmicutes and Proteobacteria from the total isolates could indicate that these phyla are more suited to the serpentinite environment; especially since their respective genera *Clostridia* and *Hydrogenophaga* have been previously found prominently in the deep subsurface and fluid systems of these serpentinite habitats (Schrenk et al., 2013).

Microbial diversity was then evaluated at the genus level of the microbial isolates. We find that microbial diversity is very low, indicated by the total number of genera, not only when

looking at the total number of isolates, but when examining the microbial diversity at each individual serpentinite site. Even when examining the microbial diversity of the sample plates, it was evident that the amount of diversity was very low despite the fact they were cultured on nutrient rich heterotrophic medium and concentrated 10-100 times. This indicates that the serpentinite habitat, because of its low nutrient availability, lack of electron acceptors, and extreme alkalinity, is selective on what types of microorganisms are able to survive its harsh habitat. It was found that there was overlap between genera in all of the serpentinite sites, in particular with the genus *Bacillus* that was present at every site including the background-sampling site. *Bacilli* are known spore formers and may be able to survive geographical dispersion better than microorganisms of another genera (Errington, 1993). It could also be possible that they reason we see *Bacillus* at every site is that they are very alkalitolerant genus of microorganism, as supported by previous data (Padan et al., 2005).

This supports the idea that microorganisms do not have any dispersal barriers, as most macroorganisms do. However, there is some variation between genera at each serpentinite site that were not present at other sites. Similar genera of each site were found to be more common between serpentinite habitats that were on the same continental plate. We see that these microorganisms may not have any dispersal barriers, since we see the same organisms at all serpentinizing habits. Isolates from geographically distant serpentinite sites were closely related to one another, with no distinct phylogenetic branching although they were from an entirely separate geographical location. This may be a consequence of just examining the highly conserved 16S rRNA gene of these isolates and it would be interesting to see if we find the same types of relationships with other genes. It could be possible that with further examination of the isolates genomes that we will see variation between core and variable genes between isolates of

the same species, as previously found in Reno's 2009 study. Overall, serpentinite isolates were phylogenetically more similar to one another than to the reference 16S rRNA sequences found in non-serpentinite habitats from the Silva database.

These data from both gene sequencing and physiological characterization suggest that serpentinite isolates have genetically evolved to become more adapted to survive in a serpentinite site and that they may also be genetically unique to the particular serpentinite site that they were isolated. This may be an indication that although we see the same organisms everywhere there are microbial adaptations to the environment through perhaps mutations, gene exchange, or even genetic drift. It would be interesting to sequence the genomes of all the isolates to make more thorough comparisons on the genetic content of the microorganisms and examine the genetic variations that occur between different serpentinite habitats. It is also imperative to get a more statistically representative number of isolates from more serpentinizing habitats.

Microbial communities were phylogenetically evaluated in the soil and water systems of the serpentinite sites to examine whether there were relationships between the isolates in the serpentinite microenvironment. There were no distinct genera that were more present in either the soil or fluid systems of the serpentinite habitats. There were also no instances of distinct phylogenetic differences between isolates from soil or fluid systems. Since there were no divergent microbial sub-communities in the soil or fluid systems it can be inferred that they have the similar microbial communities; perhaps due to microorganisms washing off of the soils in to the serpentinite fluids.

Previous studies have indicated that the genus *Hydrogenophaga* dominated the fluid systems of many serpentinite sites. All isolates of *Hydrogenophaga* were obtained from samples

of water were predominantly from the fluid systems of the CROMO site in California, with the exception of a single isolate from Canada. Since, *Hydrogenophaga* are so prominent in the mixing zones of these serpentinite habits, it is important to evaluate how they are phylogenetically related to other *Hydrogenophaga* isolated from a separate serpentinite habitat and to other non-serpentinite *Hydrogenophaga.* The isolated *Hydrogenophaga* from the CROMO site was determined to belong to two different *Hydrogenophaga* groups; one that was more adapted to the serpentinite environment and the other that was supposedly more "common" *Hydrogenophaga*. The group of *Hydrogenophaga* that seemed to be more suited to the serpentinite environment was closely related to previously characterized *Hydrogenophaga* found at the serpentinite site in Portugal. These serpentinite *Hydrogenophaga* were also found in greater abundance at various serpentinite habitats than their more common counterparts. These data may suggest that this group of serpentinite *Hydrogenophaga* may actually belong to a newly proposed genus *Serpentinomas* (Suzuki et al., 2014).

Serpentinomas are a group of microorganisms that are known to grow heterotrophically like *Hydrogenophaga* and also have the hydrogen oxidation capability. However, *Serpentinomas* can also utilize calcium carbonate, an abundant mineral in the highly alkaline serpentinite environment, and a carbon source in an otherwise carbon-limited environment. These new *Serpentinomas* strains are metabolically versatile, in the sense that they can utilize inorganic carbon as well as organic carbon sources. Utilization of calcium carbonate makes *Serpentinomas* well adapted to the serpentinizing habitat and it is possible that the serpentinite group of *Hydrogenophaga* is part of this proposed new genus (Suzuki et al., 2014).

To determine what physiological attributes of serpentinite isolates contribute to the microbial diversity present at these habitats, basic physiological testing of isolates from the

CROMO well sites in California was performed. To evaluate whether environmental pH played a role in determining microbial abundance, the number of colony forming units of alkaliphilic heterotrophs per milliliter of serpentinite water was determined for each well. Cells were concentrated for the enrichment of alkaliphiles by spread plating onto organic-rich pH 11 medium. It was surprising that the highest CFUs and microbial abundances were seen in serpentinite wells with a pH of 9. There was no relationship between the cell counts of the CROMO wells, which were relatively consistent throughout the wells, and the number of colonies that grew from sampled well water. The more neutral or extremely alkaline wells were found to have lower CFU counts. This suggests that perhaps the microbes existing in these wells neither are not strict alkaliphiles nor are they neutrophiles, but may be alkalitolerant. It would be interesting to also isolate microorganisms at a pH of 7 to analyze the colony forming units per milliliter and compare them to the microorganisms isolated at a pH of 11. These data would give insight to whether there is a predominance of alkaliphiles in these serpentinite wells or whether there were neutrophiles present that are dormant or growing suboptimally in these habitats.

The number of colony forming units from each well sample may actually be tied to the microbial diversity of each CROMO well. Identification of the genera present in each of the CROMO wells proved that the microbial diversity of each well site was relatively low and that the diversity between the wells also varied. Microbial diversity seemed to show similarities amongst each well site in NO8 and in CSW when comparing what genera are present in each well. However, there seems to be no similarities in microbial diversity amongst the QV well sites. This may indicate that each particular well site NO8, CSW, and QV has as specific microbial community specifically adapted to that particular environment. The microbial community of each well site may differ in the number of alkaliphiles or alkalitolerant

microorganisms, which may influence the number of colony forming units that formed on the alkaliphilic heterotrophic medium.

To determine which physiological characteristics played a role in the microbial abundance and diversity of the CROMO wells, the experimental optimum pH, temperature, oxidase and catalase test were determined for the isolates. Based upon the data there seems to be a strong correlation between the environmental pH of a particular well and the experimental optimum pH of the microorganism from which it was isolated. The fact that alkaliphiles were predominantly found in these wells, reinforces the idea that pH is a major selecting factor. The data suggest that the isolates are alkaliphilic or alkalitolerant and that the pH of the environment is a major selecting factor for the microbial diversity of the CROMO wells. It was also found that temperature was not a selecting factor for these serpentinite environments, since no correlation was found between the experiment optimum temperature and the temperature of the wells.

To determine the number of isolates that use oxygen as an electron acceptor by the use of cytochromes, the oxidase test was conducted. There were more oxidase positive isolates than oxidase negative, suggesting that some of the isolates are using oxygen as an electron acceptor. In a previous study it was discussed that alkaliphiles had elevated cytochrome levels to help maintain inter-membrane homeostasis and the presence of more oxidase positive isolates may be an indicator for the abundance of alkaliphiles present (Sorokin et al., 1999). As a by-product of redox reactions hydrogen peroxide is often produced. So it was unsurprising to see more catalase positive organisms than catalase negative organisms, since there were also more oxidase positive isolates. In fact there were very few catalase negative organisms, but this may expected

since the microorganisms were isolated aerobically. There was also no correlation between the amount of dissolved oxygen in the well and the number of catalase positive isolates.

Overall this study found that similar microorganisms were found at all serpentinizing sites and that they were adapted to the harsh habitat. Although, we see the same microorganisms globally at these serpentinizing habitats in most cases they are genetically most similar to other organisms from the same site, suggesting that microbial biogeography influenced by dispersal barriers does play a role. It was also determined that pH was the only selecting factor measured that was related to the physiology of microorganisms isolated from serpentinites habitats, although other physiological and phenotypic factors are also likely to play a role.

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Appendix A: Taxonomic & Physiological Data of Isolate

Canada Sample

Re-named	Accession Number	Max Identity	Site	Type	Year	BLAST Result
TL-1	NR_042286.1	99%	TLED	water	2009	Bacillus herbersteinensis
TL-2	JF505968.1	99%	TLED	water	2009	Microcella alkaliphila
TL-3	JF505968.1	99%	WHC2A	water	2009	Microcella alkaliphila
TL-4			WHC2A	water	2009	
TL-5	AJ717373.1	99%	WHC2A	water	2009	Dietzia natronolimnaea, isolate CV46
TL-6	NR_025634.1	96%	WHC2A	water	2009	Aquiflexum balticum strain BA160
TL-7	KF011690.1	99%	WHC2A	water	2009	Paenibacillus
TL-8	NR_025634.1	96%	WHC2	water	2009	Aquiflexum balticum strain BA160
TL-9	FJ263011.1	99%	WHC2	water	2011	Bacillus sp. RA5
TL-10	FJ263011.1	99%	WHC2	water	2011	Bacillus sp. RA51
TL-11	HM567001.1	99%	WHC2	water	2011	Bacillus sp. DU120(2010)
TL-12	FJ263011.1	99%	WHC2	water	2011	Bacillus sp. RA51
TL-13	CP001186.1	99%		water	2011	Bacillus cereus G9842
TL-14	DQ490379.1	100%		water	2011	Bacillaceae bacterium KVD-1700-03
TL-15	KC172059.1	99%	WHC2A	water	2010	Bacillus altitude strain SH164
TL-16	JX266376.1	99%	WHC2A	water	2010	Bacillus sp. B2066
TL-17	JX266376.1	99%	WHC2A	water	2010	Bacillus sp. B2066
TL-18	AB300163.1	97%	WHC2A	water	2010	Hydrogenophaga sp AH-24
TL-19	AB437410.1	98%	WHC2			Bacillus sp. JAEA
TL-20	AB043854.1	99%	WHC2			Bacillus sp. N6
TL-21	AB044748.1	99%	WHC2			Bacillus sp KSM-K38
KL3	AB298545.1	99%			2013	Dietzia natronolimnaea
KL4	KF040368.1	99%			2013	Geomicrobium
KL5	KF040368.1	99%			2013	Geomicrobium
KL6	KF040368.1	99%			2013	Geomicrobium
KL7			A4A-1			

Italy Samples

Re-named	Accession Number	Max Identity	Site	Type	Year	BLAST Result
LS-1	GQ352404.1	99%		water	2010	Kocuria sp. 104
LS-2				water	2010	
LS-3	DQ270677.1	99%		water	2010	Microbacterium sp. B-1001
LS-4	HM222680.1	99%		water	2010	Arthrobacter sp. 0713C4-1
LS-5	JN099789.1	99%		water	2010	Microbacterium arborescens strain BAB-264
LS-6	FJ666055.1	99%		water	2010	Agrobacterium tumefaciens
LS-7	HQ222345.1	99%	L43	water	2010	Bacillus sp. H6
LS-8	GU570653.1	99		water	2010	Planococcus
LS-9	GQ284392.1	99%	BR2	water	2010	Bacillus niabensis strain THWCSN9
LS-10	AB188222.1	99%		water	2010	Cellulosimicrobium sp. TUT1242
LS-11	CP000903.1	100%	LER20	water	2011	Bacillus weihenstephanensis KBAB4
LS-12	FJ957641.1	100%	LER20	water	2011	Uncultured Bacillus sp. clone JPL-S3_K17
LS-13	CP000903.1	100%	LER20	water	2011	Bacillus weihenstephanensis KBAB4
LS-14	GU385872.1	100%	LER20	water	2011	Bacillaceae bacterium QT26

California Samples

Re-named	Accession Number	Max Identity	Site	Type	Year	BLAST Result
CR-1	CP000813.1	100%	N08A	water	2011	Bacillus pumilus
CR-2	FJ607051.1	100%	N08B	water	2011	Bacillus sp. 42-7
CR-3	DQ448755.1	100%	N08B	water	2011	Bacillus sp. CNJ759
CR-4	GQ304778.1	99%	N08B	water	2011	Bacillus horikoshii
CR-5	FJ607051.1	100%	N08B	water	2011	Bacillus sp. 42-7
CR-6	FJ607051.1	100%	N08B	water	2011	Bacillus sp. 42-7
CR-7	AB362829.1	99%	N08B	water	2011	Bacilllus sp. YT0027
CR-8	JN644574.1	99%	OldCSW	water	2011	Exiguobacterium aurantiacum
CR-9	NR_043478.1	99%	OldCSW	water	2011	Exiguobacterium aurantiacum strain DSM 6208
CR-10	NR_042259.1	97%	CSW5	core	2011	Planomicrobium
CR-11	HM439456.1	97%	CSW5	core	2011	Paenibacillus lautus strain
CR-12	AJ717367.1	99%	CSW22	core	2011	Micrococcus luteus
CR-13	HQ697771.1	98%	QV41	core	2011	Unculutred bacterium clone Bms_CK242
CR-14	FR749913.1	99%	QV10	core	2011	Bacillus horneckia
CR-15	AB013919.2	99%	QV33	core	2011	Microbacterium kitamiense
CR-16	JF766689.1	99%	OldCSW	water	2011	Pseudomonas sp. BIHB
CR-17	AF439803.1	99%	OldCSW	water	2011	Pseudomonas anguilliseptica
CR-18	AB300163.1	97%	CSW9	core	2011	Hydrogenophaga sp. AH-24
CR-19	DQ986320.1	99%	OldCSW	water	2011	Hydrogenophaga sp. CL3
CR-20	AB334763.1	99%	CSW9	core	2011	Bacillus thuringiensis
CR-21	GU213502.1	99%	QV10	core	2011	Micrococcus
CR-22	JF970589.1	99%	CSW9	core	2011	Sporosarcina globispora
CR-23	X76438.1	99%	CSW9	core	2011	Bacillus sp. (DSM 8714)
CR-24	AP012157.1	100%	CSW9	core	2011	Solibacillus silvestris
CR-25	EF101552.1	99%	CSW9	core	2011	Bacillus psychrodurans strain KOPRI24934
CR-26	GU385871.1	99%	CSW9	core	2011	Bacillus psychrodurans strain QT25
CR-27	NR_040904.1	97%	CSW9	core	2011	Malikia spinosa strain IAM
CR-28	EU685820.1	97%	CSW9	core	2011	Bacillus sp. PK-1
CR-29	DQ986320.1	99%	OldCSW	water	2011	Hydrogenophaga sp. CL3
CR-30	EU373398.1	99%	OldCSW	water	2011	Dietzia natronolimnaea strain TPL19
CR-31	NR_028716.1	99%	OldCSW	water	2011	Hydrogenophaga taeniospiralis strain 2K1
CR-32	DQ986320.1	99%	OldCSW	water	2011	Hydrogenophaga sp. CL3
CR-33	EU558286.1	99%	OldCSW	water	2011	Paenibacillus sp. B17a
CR-34	AB300163.1	97%	CSW20	core	2011	Hydrogenophaga sp AH-24
CR-35	AB300163.1	97%	CSW20	core	2011	Hydrogenophaga sp AH-24
CR-36			CSW23	core	2011	
CR-37	JF970589.1	99%	CSW9	core	2011	Sporosarcina globispora
CR-38	EU558286.1	99%	CSW23	core	2011	Paenibacillus sp. B17a
CR-39	KF562253.1	99%	OldCSW	water	2011	Paenibacillus
CR-40	KC466132.1	100%	QV18	core	2011	Bacillus sp H69
CR-41	EU880532.1	99%	QV18	core	2011	Bacillus pumilus
CR-42	HM030747.1	98%	QV8	core	2011	Exiguobacterium aurantiacum strain M-4
CR-43	AM398212.1	99%	QV8	core	2011	Exiguobacterium sp. EP03
CR-44	KF475828.1	100%	QV 1,1	water	2012	Bacillus altitudinis
CR-45	AB300163.1	97%	QV 1,1	water	2012	Hydrogenophaga sp. AH-24 gene
CR-46	EU880532.1	99%	QV 1,1	water	2012	Bacillus pumilus
CR-47	NR_028717.1	97%	QV 1,1	water	2012	Hydrogenophaga pseudoflava strain GA3
CR-48	HM030754.1	97%	CSW2	core	2011	Pseudomonas stutzeri strain M16-9-4

California Samples

Re-named	Accession Number	Max Identity	Site	Type	Year	BLAST Result
CR-49	KF475828.1	99%	CSW 1,1	water	2012	Bacillus altitudinis
CR-50	AB300163.1	98%	CSW 1,1	water	2012	Hydrogenophaga sp. AH-24
CR-51	FR749913.1	99%	CSW 1,1	water	2012	Bacillus horneckiae partial
CR-52	EU880532.1	99%	CSW 1,1	water	2012	Bacillus pumilus strain PRE14
CR-53	AY289507.1	99%	CSW 1,1	water	2012	Paenibacillus sp. IDA5358
CR-54	EU880532.1	99%	CSW14	core	2011	Bacillus pumilus strain PRE14
CR55			QV1.1			
CR56	EU330348.1	99%	QV1.1			Micrococcus
CR57	KF040368.1	99%	QV1.1			Geomicrobium
CR58	AB298545.1	99%	CSW OLD			Dietzia sp. P27-19
CR59	FR749913.1	98%	CSW OLD			Bacillus horneckiae
CR60	AB298545.1	100%	CSW OLD			Dietzia natronolimnaea
CR61	AB298545.1	99%	CSW OLD			Dietzia natronolimnaea
CR62	AB298545.1	99%	CSW OLD			Dietzia natronolimnaea
CR63	AY741723.1	99%	CSW OLD			Brachybacterium
CR64	KF040368.1	99%	NO8A	water	2013	Geomicrobium
CR65	KF040368.1	99%	NO8C	water	2013	Geomicrobium
CR66	JQ661108.1	99%	CSW 1.1	water	2013	Bacillus sp G47
CR67	KF040368.1	99%	CSW 1.1	water	2013	Geomicrobium
CR68	KC166730.1	99%	CSW 1.5	water	2013	Uncultured Alishewanella
CR69	KC166730.1	99%	CSW 1.5	water	2013	Uncultured Alishewanella
CR70	AB298545.1	99%	CSW 1.5	water	2013	Dietzia natronolimnaea
CR71	KC166730.1	100%	CSW 1.5	water	2013	Uncultured Alishewanella
CR72	EF554919.1	97%	CSW 1.5	water	2013	Pseudomonas sp. G-R2A7
CR73	KF040368.1	99%	NO8B	water	2013	Geomicrobium
CR 74	NR_042275.1	99%	NO8B	water	2013	Microcella alkaliphila
CR 75	DQ234215.2	91%	QV 1.2	water	2013	Erysipelothrix
CR76			QV 1.2	water	2013	
CR77			QV 1.2	water	2013	
CR78	HQ132455.1	98%	QV 1.2	water	2013	Thauera
CR79	AB055907.1	90%	QV 1.2	water	2013	Erysipelothrix rhusiopathiae
CR80	NR_042275.1	99%	NO8A	water	2013	Microcella alkaliphila
CR81			QV 1.1	water	2013	
CR82	AB298545.1	99%	QV1.2	water	2013	Dietzia natronolimnaea
CR83			QV 1.2	water	2013	
CR84						
CR85						
CR86	KC166730.1	100%	QV 1.3	water	2013	Alishewanella
CR87	AB284047.1	99%	QV 1.3	water	2013	Pseudomonas
CR88	NR_042275.1	99%	CSW 1.3	water	2013	Microcella alkaliphila
CR89	KC166730.1	100%	CSW 1.3	water	2013	Alishewanella
CR90	KC166730.1	100%	QV 1.3	water	2013	Alishewanella
CR91	AB284047.1	99%	QV 1.3	water	2013	Pseudomonas
CR92	JQ323147.1	99%	QV 1.3	water	2013	Microcella
CR93	NR_042275.1	99%	CSW 1.3	water	2013	Microcella alkaliphila
CR94	KC166730.1	100%	CSW 1.3	water	2013	Alishewanella
CR95	JX945764.1	99%	CSW 1.2	water	2013	Pseudomonas sp. LARP66
CR96	EU817498.2	99%	CSW 1.2	water	2013	Alishewanella jeotgali strain

Canada Sample

Re-named	Color	Form	Catalase Test	Oxidase Test	Optimum pH	Optimum Temperature
TL-1	White	Circular	-	-		
TL-2	Yellow	Circular	-	-		
TL-3	Yellow	Circular	+	-		
TL-4	Yellow					
TL-5	Orange	Circular	+	-	7	37°C
TL-6	Red					
TL-7	White	Circular	+	+	7	20°C
TL-8	Red					
TL-9	White	Rhizoid	+	-	7	20°C
TL-10	White	Circular	+	-	9	30°C
TL-11	White	Circular	+	-	7	20°C
TL-12	White	Circular	+	-	7	20°C
TL-13	White	Circular	-	+	7	30°C
TL-14	White	Circular	+	-	7	20°C
TL-15	White		+	+	7	30°C
TL-16	White		+	+	7	30°C
TL-17	White		+	+	7	30°C
TL-18	Clear		+	-	8	30°C
TL-19						
TL-20						
TL-21						
KL3						
KL4						
KL5						
KL6						
KL7	White					

Italy Sample

Re-named	Color	Form	Catalase Test	Oxidase Test	Optimum pH	Optimum Temperature
LS-1	Yellow		+	+	7	20°C
LS-2						
LS-3	Yellow	Circular	+	-	9	20°C
LS-4	Yellow	Circular	-	-		
LS-5	Yellow	Circular	+	-	7	20°C
LS-6	White/Nude	Circular	+	-	7	20°C
LS-7	White	Circular	+	+	8	30°C
LS-8	Orange/Nude	Circular	+	-	9	20°C
LS-9	White	Circular	+	+	8	37°C
LS-10	White	Circular	+		8	30°C
LS-11	White		-	+	8	27°C
LS-12	White		+	+	8	30°C
LS-13	White		+	-		
LS-14	White		-	+		

California Samples

Re-named	Color	Form	Catalase Test	Oxidase Test	Optimum pH	Optimum Temperature	Aerobic	Anaerobic
CR-1	Yellow	Circular	+	-	7	37°C		
CR-2	White	Circular	+	-	10	30°C		
CR-3	White	Circular	+	-	9	30°C		
CR-4	White		+	-	9	20°C		
CR-5	White	Circular	+	-	9	37°C		
CR-6	White	Circular	+	-	7	37°C		
CR-7	White		+	+	7	30°C		
CR-8	Orange	Circular	+	-	9	30°C		
CR-9	Orange	Circular	+	-	9	37°C		
CR-10	Orange	Circular	+	-	9	30°C		
CR-11	White	Circular	-	+	7	30°C		
CR-12	Yellow	Circular	+	-	7	37°C		
CR-13	White	Circular	-	-	10	30°C		
CR-14	White	Irregular	+	+				
CR-15	Orange/Red	Circular	+	-				
CR-16	White		+					
CR-17	White		+	+				
CR-18	Yellow		+	-	7	30°C		
CR-19	White		-	+	7	20°C		
CR-20	White		+	-	7	37°C		
CR-21	Black				7	20°C		
CR-22	White		+	+				
CR-23	White		-	+	9	20°C		
CR-24	White		+	+	7	20°C		
CR-25	White		+	-	7	37°C		
CR-26	White		+	+	7	20°C		
CR-27	Yellow		+	-	10	30°C		
CR-28	White		+	+	7	20°C		
CR-29	Orange/White		+	-	10	30°C		
CR-30	Orange		+	-	7	37°C		
CR-31	Orange/White		+	-	7	30°C		
CR-32	Orange/White		+	-	7	30°C		
CR-33	White		-	+	2	20°C		
CR-34	White		+	-				
CR-35	White							
CR-36	White		-	+	7	20°C		
CR-37	White		+	+	8	20°C		
CR-38	White		-	+	7	20°C		
CR-39	White		-	+	7	20°C		
CR-40	White		+	+	8	30°C		
CR-41	White		+	+	8	30°C		
CR-42	Orange		+	-	7	20°C		
CR-43	Orange		+	+	7	37°C		
CR-44	White		+	+	7	37°C		
CR-45	Clear		-	-	9	37°C		
CR-46	White		+	+	7	37°C		
CR-47	Clear		-	+	9	37°C		
CR-48	Orange		+	+	10	20°C		

California Samples

Re-named	Color	Form	Catalase Test	Oxidase Test	Optimum pH	Optimum Temperature	Aerobic	Anaerobic
CR-49	White		+		7	37°C		
CR-50	Clear		-	+	9	20°C		
CR-51	White		+	+	9	30°C		
CR-52	White		+	+	10	37°C		
CR-53	White		-	+	7	30°C		
CR-54	White		+	+	10	37°C		
CR55	yellow							
CR56	yellow							
CR57	white							
CR58	orange							
CR59	yellow							
CR60	nude							
CR61	nude							
CR62	orange							
CR63	yellow							
CR64	white		+	+	10	20°C	+	+
CR65	white		+	+	10	20°C	+	+
CR66	white		+	-	7	30°C	+	+
CR67	white		+	+	10	20°C	+	+
CR68	off white		+	+	9	37°C	+	+
CR69	white		+	+	10	20°C	+	+
CR70	orange		+	-	9	30°C	+	+
CR71	off white		+	+	9	30°C	+	+
CR72	white		+	+	9	30°C	+	+
CR73	white		+	+	10	30°C	+	+
CR 74	yellow		+	-	10	30°C	+	+
CR 75	pink		+	-	7	30°C	+	+
CR76	orange		+	-	7	30°C	+	+
CR77	nude		+	+	7	30°C	+	-
CR78	pink		+	+	7	30°C	+	+
CR79	clear		-	-	9	20°C	+	-
CR80	yellow		+	-	9	30°C	+	+
CR81	nude		+	+	10	30°C	+	+
CR82	orange		+	-	7	30°C	+	+
CR83	yellow		+	+	7	30°C	+	+
CR84								
CR85								
CR86	nude		+	+	10	30°C	+	+
CR87	nude		+	+	7	30°C	+	+
CR88	yellow		+	-	10	30°C	+	+
CR89	nude		+	+	10	37°C	+	+
CR90	nude		+	+	10	37°C	+	+
CR91	yellow		+	-	7	30°C	+	-
CR92	yellow		+	-	10	37°C	+	+
CR93	yellow		+	-	10	30°C	+	+
CR94	nude		+	-	10	30°C	+	+
CR95	nude				7	30°C	+	+
CR96	nude				9	37°C	+	+