MOLECULAR AND PHENETIC CHARACTERIZATION OF THE BACTERIAL ASSEMBLAGE OF BASQUE LAKE, BC, AN ENVIRONMENT WITH HIGH CONCENTRATIONS OF MAGNESIUM SULPHATE, AND ITS RELEVANCE TO MARS

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science with a major in Biological Sciences.

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Success is not final, failure is not fatal: it is the courage to continue that counts.

-Winston Churchill

ABSTRACT

Halotolerant bacteria favor environments containing high concentrations of salts. While there are a multitude of hypersaline environments containing various salts on Earth, those heavily dominated with sodium chloride (NaCl) have been of academic rigor. This thesis pertains to environments with high concentrations of magnesium sulphate ($MgSO_4$), which presents ample opportunity for discovery. Basque Lake, BC is one such environment that is dominated by magnesium sulphate. Basque Lake is an ephemeral lake containing near-saturated levels of magnesium sulphate that precipitates as epsomite ($MgSO_4 \bullet 7H_2O$). Natural environments containing high concentrations of magnesium sulphate are rare and previous microbiological effort is limited. For a microbe to persist in Basque Lake it must withstand extreme conditions similar to those present on the Martian surface including salinity, aridity, and temperature. Any microbe isolated from Basque Lake could give astrobiologists key details on what traits a Martian life-form may have and to limit potential forward contamination. Approximately 65 bacterial isolates were obtained through repetitive streak-plating in high salt media. The bacterial isolates were characterized phenotypically and subjected to 16S rRNA sequencing and phylogenetic analyses. Gram-positive bacteria dominated the culture collection including members of *Virgibacillus*, *Marinococcus*, *and Staphylococcus*. Members of the Gram-negative genera *Halomonas* and *Salinivibrio* were represented in the culture collection as well. Results indicate that microbes isolated from epsom-rich environments such as Basque Lake present a potential risk of forward contamination.

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LIST OF ABBREVIATIONS

BC	British Columbia
BL	Basque Lake
BLAST	Basic Alignment Search Tool
bp	Base Pairs
COSPAR	Committee On Space Research
DNA	Deoxyribonucleic acid
GSP	Great Salt Plains
HL	Hot Lake
kb	Kilobases
MEGA	Molecular Evolutionary Genetic Analysis
MEPAG	Mars Exploration Program Analysis Group
MH	Moderate Halophiles Medium
MRO	Mars Reconnaissance Rover
NaPP	Sodium Pyrophosphate
NASA	National Aeronautics and Space Administration
OD	Optical Density
PCR	Polymerase Chain Reaction
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
SP	Salt Plains
UV	Ultraviolet Light
w/v	Weight to Volume

LIST OF SYMBOLS

c	Celsius
°	Degree
g	Gram
km	Kilometer
L	Liter
µ	Micro
µl	Microliter
µM	Micromolar
ml	Milliliter
M	Molar
%	Percent
α_w	Water Activity

CHAPTER 1

INTRODUCTION

"A bewildering assortment of (mostly microscopic) life-forms has been found thriving in what were once thought to be uninhabitable regions of our planet. These hardy creatures have turned up in deep, hot underground rocks, around scalding volcanic vents at the bottom of the ocean, in the desiccated, super-cold Dry Valleys of Antarctica, in places of high acid, alkaline, and salt content, and below many meters of polar ice. ... Some deep-dwelling, heat-loving microbes, genetic studies suggest, are among the oldest species known, hinting that not only can life thrive indefinitely in what appear to us totally alien environments, it may actually originate in such places." (David Darling- *Life Everywhere: The Maverick Science of Astrobiology*).

1.1 Extremophiles

Life exists in environments that prove otherwise difficult for most known organisms (Rothschild and Mancineli, 2001; Mesbah and Wiegel, 2008). An environment like this is classified as "extreme" according to Brock (1979), due to low species diversity and possible absence of one or more taxa. Numerous sites that represent a wide-variety of extremes exist on Earth. Environments such as the Atacama Desert and Yellowstone Hot Springs present a unique set of challenges to which an organism must adapt. Organisms existing in such environments possess distinct traits that ensure survival and viability in extreme conditions – thus earning the name "extremophiles" (MacElroy, 1974). Conditions that an extremophile must overcome include: excessive and low temperatures, increased acidity and alkalinity, desiccation,

and high salinity. Research involving extremophiles has changed phylogeny and taxonomy in profound ways. This was evident by the groundbreaking study by Woese, Kandler, and Wheelis (1990) that resulted in the recognition of a new domain of life. Additional research involving extremophiles has resulted in a paradigm shift from a focus on biochemistry to molecular biology in the life sciences, achieved by the isolation of Taq polymerase from *Thermus aquaticus* that made polymerase chain reaction possible (PCR) – subsequently resulting in Karry Mullis winning a Noble Prize in 1993 (Saiki et al., 1988). More recently, extremophiles have been of prominent interest in the search for life on celestial objects – primarily Mars (Horneck, 2000; Ramelotto, 2010).

1.2 Halotolerance

1.2.1 Hypersaline Environments

Environments containing high concentrations of salt are documented to harbor organisms known as “halophiles” that grow among a wide range of salinities-including saline soils, salt lakes, and food products salted for preservation (Kushner, 1985; Ventosa, 1998). Well-known sites such as the Dead Sea and Great Salt Lake have been extensively studied to gain insight into their microbial community structure(s). Such studies have resulted in the identification of novel elements of physiology, complex community structures, refinement of taxonomy and phylogeny, and numerous industrial applications (Nissenbaum, 1975; Post, 1977; Oren, 2002). Oddly, these discoveries came from what are considered inhospitable environments. Halophiles are frequently mentioned when the Atacama Desert is brought to the public’s attention. The reason for this attention is the similarity in environments between the Atacama Desert

and Mars; more specifically, the potential for astrobiologists to find novel microbes on Mars. Being possibly the driest desert on Earth with virtually no organics and radical daily temperature fluctuations, the Atacama Desert is considered an analogue to a Martian environment. A set of experiments to simulate the exact tests performed by the Viking landers were carried out by Navarro-González et al. (2003) and confirmed the absence of organics and reported oxidizing conditions – most likely due to perchlorates – as the Viking 1 and Viking 2 previously had (Biemann et al., 1977; Oyama and Berdahl, 1977). Another interesting characteristic of the Atacama Desert is its high salinity – mainly consisting of sulphates and nitrates from atmospheric sources (Bhölke et al., 1997; Michalski et al., 2004). It is estimated that the concentration of salt ranges from 5% to 28%, with the later occurring in the hyperarid Yungay region. Microbial community analyses at different locales within the Atacama mainly found *Gemmatimonadetes* and *Planctomycetes* bacteria, and the presence of *Actinobacteria* and *Proteobacteria* (Drees et al., 2006; Connon et al., 2007). There is variation among environments of high salinity. Different sites may have differing concentrations of salts, temperature, elevations, aridity, and salt composition.

One such place is the Great Salt Plains (GSP) in Cherokee, Oklahoma. GSP contains a barren salt flat that consists of evaporates from Permian brine that rises to the surface. Na^+ and Cl^- are the principal ionic make-up of the brine. Salt concentrations often change dramatically due to heavy rainfall causing both temporal and spatial fluctuations. Any halophiles present must adapt to diluted and saturated concentrations. Other obstacles that GSP presents are scorching surface temperatures, frigid winters, UV radiation, desiccation, and high alkalinity (Johnson, 1980; Canton et al., 2004).

Given the range of salinities that occur at GSP, any isolated microbe could be considered halophilic or halotolerant; halophilic meaning that the microbe requires hypersaline concentrations and halotolerant meaning that the microbe can grow in both hypersaline and oligosaline conditions. Isolated microbes could then further be classified or categorized according to optimal growth – various classifications have been defined, such as that of Schneegurt (2012) shown in Table 1, though it is widely accepted that microbes are halotolerant and not halophilic unless at extreme salinities. This premise led to a series of studies that identified community structures and diversity and involved the characterization of numerous isolates of both bacteria and archaea that were representative of numerous phylotypes (Caton et al., 2004; Litzner et al., 2006; Caton et al., 2009). In addition to the Atacama Desert and GSP, other hypersaline territories have been explored as well – representing both terrestrial and aquatic environments. Further investigation will increase understanding of microbial community structure and function, phylogeny, abiogenesis, industrial applications, and has astrobiological implications (Longo and Blaber, 2014).

1.3 Mars

1.3.1 $MgSO_4$

Salt tolerance may be required by an organism if it is to remain viable on Mars. Salt concentrations mainly consist of sulphates in the form Mg, Fe, and Ca (Clark and Van Hart, 1981); though chlorides and perchlorates do persist at a lower quantity. Sulphate-heavy brines may exist if underlying permafrost were to melt. These brines would be dense with $MgSO_4$ given the compound's stability at low pH. A sulphate-rich

brine is allowed since its eutectic point (lower freezing point due to mixture of compounds) is below that of some equatorial temperatures on Mars (McEwen et al., 2011). Such brines are known as "cryobrines" given their ability to exist at eutectic temperatures under 0 °C (Möhlmann and Thomsen, 2011). It has even been mentioned that $MgSO_4$ salts may be the greatest near-surface obstacle present on Mars (Tosca, et al., 2008).

1.3.2 Epsotolerance

If microbes are to exist on Mars than a tolerance to $MgSO_4$ is possibly a vital requirement. Halotolerance has the connotation of being tolerant to NaCl, while salinotolerance is a broader term for salt tolerance. To separate any confusion between NaCl and $MgSO_4$ tolerance it is advised to use the word "epsotolerance" when referring to growth at $MgSO_4$ concentrations (Crisler et al., 2012; Kilmer et al., 2014).

1.3.3 Challenges

In addition to displaying epsotolerance, microbes must confront a multitude of other life threating conditions on the Martian surface: low temperature, desiccation, a diurnal cycle that alternates between freezing and thawing, low water activity, an anoxic atmosphere, and radiation (Clark, 1998). Mars lacks an atmosphere which means no oxygen for aerobes and continuous bombardment by cosmic rays and solar radiation. Within the past decade bacteria have been detected and isolated from class 100K spacecraft-assembly clean rooms and spacecraft-assembly facilities. Bacteria detected were representative of multiple phylotypes (Satomi et al., 2006; La Duc et al., 2007; Vaishampayan et al., 2010; Vaishampayan et al., 2012). Interestingly, many bacteria

detected through pyrosequencing were non-spore producing. The ability to persist in UV-irradiated cleanrooms may allow a facultative anaerobic bacterium to persist without the protection of an atmosphere. Having an arid and barren landscape, any microbe faces the possibility of dehydrating or desiccating. To counteract this effect a cell needs to increase its internal osmolality. The first method is through accumulation of compatible solutes within the cytoplasm. Most solutes are amino acids, sugars, and betaines. The second method is mainly used by halophiles, and requires an influx of potassium ions into the cytoplasm also known as 'salting-in' (Brown, 1976; Galinski, 1995). Crisler et al. (2012) determined desiccation tolerances for epsotolerant bacteria isolated from GSP through alternate drying and rewetting (vacuum-drying and pellet rewetting) cycles as shown in Figure 1; although there is a noticeable decrease in cell numbers after 5 cycles, it is suspected that planktonic responses may differ when compared to organisms within soil particles. A diurnal cycle that alternates between freezing and thawing could potentially "shock" a cell to lyse its contents. If thawing is not an option then the temperature may be low enough to slow down any metabolic activity to a standstill (Jakosky et al., 2003). Crisler et al. (2012) determined freeze tolerances for epsotolerant bacteria isolated from GSP through alternate freeze-thaw cycles (-75 to 25°C) as shown in Figure 2; though no live organisms were detected after 15 cycles results could change dependent on soil quality influences on thermal change.

Water activity (α_w) is of concern since it is essentially a measure of the amount of water available in an environment. Based on a unitless scale that ranges from 0 (complete desiccation) to 1 (pure water), it is widely used in food science and pharmaceuticals as an indicator of shelf life. The Martian surface being an arid

environment has little to no water available except in the form of brines consisting of hygroscopic salts. Salt based media used to isolate and cultivate halotolerant and epsotolerant bacteria are displayed along with salinity and α_w in Table 2 (Crisler, et al., 2013). Amazingly, there are organisms that can survive through the extreme-conditions listed. Venkateswaran et al. (2003) describe a bacterial species isolated from a spacecraft-assembly facility that is facultatively anaerobic and is resistant to radiation and desiccation. Organisms that harbor these traits are not only a curiosity but of ethical concern. Foreword contamination of Mars is the concern with these types of microbes.

1.4 Basque Lake

Hypersaline waters can be classified based on their ionic composition as either thalassohaline or athalassohaline. Thalassohaline environments mainly exist as bodies of water of marine origin that are subject to extensive evaporation leaving chloride-heavy brines (NaCl) with an ionic composition similar to the sea (Oren, 2006). Athalassohaline environments tend to consist of cations other than Na^+ such as Ca^{+2}, Mg^{+2}, and K^+ and when evaporated result in heavily laden brines rich in salts other than NaCl (Litchfield and Gillevet, 2002). Known athalassohaline environments include the Dead Sea, Mono Lake, and Big Soda Lake. While most biotic research has focused on thalassohaline environments, less is known of athalassohaline environments, especially those concentrated with $MgSO_4$. Epsomitic sites are rare in nature and little microbiological experimentation has been performed (Schneegurt, 2012). One such epsomitic environment is Basque Lake (Figure 3 and 4). An athalassohaline epsomite concentrated lake; Basque Lake provides the unique opportunity to study epsotolerant and epsophilic microorganisms (no epsophilic microorganism is known to exist at this

time). Basque Lake is located in south-central British Columbia 100 km west of Kamloops. Considered to have the highest concentration of magnesium in North America, Basque Lake is ephemeral and has a low concentration of chloride and is heavily laden with sulfate (Goudge, 1926). The lack of chloride and high concentration of magnesium results in SO_4^{-2} as the major anion and Mg^{+2} as the major cation (Nesbitt, 1990). Standing water is present after spring runoff but evaporates by late summer resulting in the precipitation of epsomite that forms a crust over concentrated brine pools. Brine will remain below the crust during arid periods (Goudge, 1926; Nessbitt, 1990). Basque Lake is an ideal analog to Mars given its high concentration of magnesium sulfate and aridity-an average annual rainfall of 25 to 30 cm-and fluctuating temperatures that span from frigid winters to mild summers (Goudge, 1926). Any microbes isolated could provide key details to astrobiologists about certain traits a Martian life-form may have.

1.4.1 Martian Analog

As an athalassohaline epsomite concentrated lake; Basque Lake is known to precipitate and form small brine pools. Basque Lake's brine pools are analogous to various landmarks found on Mars; most notably the epsomite-rich outcrops of Meridiani Planum and Valles Marineris. This similarity can give clues into the history of Mars, in which liquid water was abundant and stable, as well as for the present day (Clark et al., 2005). Recent findings from the Curiosity rover have pointed towards a period of time in which Mars lacked sulfates, indicating a non-acidic environment more hospitable to life (Grotzinger et al., 2014). These findings coincide with observations from the Mars

Reconnaissance Orbiter (MRO) that indicate the flow of briny water on Mars (McEwen et al., 2013).

1.5. Salinotolerance

Currently, there have been little microbiological efforts to isolate and characterize any salinotolerant organism from Basque Lake; though Foster et al. (2010) quantified biomass and observed biosignatures. A more recent study by Fox-Powell et al. (2016) performed a community analysis using pyrosequencing on brine enrichments from Basque Lake, supporting a community dominated by Gram-positive bacteria consisting of *Firmicutes* and *Actinobacteria*. It is of interest to note the presence of an archaea community entirely comprised of the *Nitrososphaera* genus within the *Crenarchaeota*. Other efforts have focused upon Hot Lake, another athalassohaline epsomite rich lake (Lindemann et al., 2013; Kilmer et al., 2014). Isolation of viable microbes from both water and soil samples collected from various points around Basque Lake was performed as part of this thesis work. Isolation protocols are similar to those of Hot Lake as performed by Kilmer et al. (2014).

1.6 Growth in Varying $MgSO_4$ and NaCl Concentrations

Any microbe isolated from Basque Lake is expected to be tolerant of high concentrations of $MgSO_4$. Also of interest is the range of epsotolerance. Perhaps an isolate will grow through a wide range of salinities, while others are more selective. It is unknown to what extent these isolates will display halotolerance. Given the use of selective media used for isolation – which contains a high concentration of both $MgSO_4$ and NaCl – halotolerance is a defined trait. As with epsotolerance, the range of

tolerance is to be seen. Crisler et al. (2012) and Kilmer et al. (2014) measured various ranges of halotolerance and epsotolerance for bacteria isolated from GSP and Hot Lake using modified versions of GSP media as described in Caton et al. (2004). Organisms isolated from GSP (Table 3) grew better in media that was supplemented with 10 % w/v $MgSO_4$ than 2 M $MgSO_4$. Organisms isolated from Hot Lake (Table 4) grew better in media that was supplemented with 0.1% w/v and 1% w/v $MgSO_4$ than 50% w/v (roughly 2 M). For both set of isolates growth dropped when increased to 2 M, with GSP showing lower growth success. The success of Hot Lake may be due to the selective pressure presented by its high concentration of epsomite. Curiously, both Crisler et al. (2012) and Kilmer et al. (2014) noted isolates that were atypical in their range of growth, suggesting different mechanisms may be involved for tolerance.

1.7 Effects of Water Activity upon Growth

There is little accessible water on Mars, resulting in decreased α_w. Any existing water on Mars may be present as brine formed through melting permafrost or deliquescent salts. Water activity is of great interest as to be categorized as a defining characteristic by both the Committee on Space Research (COSPAR) and the NASA Mars Exploration Program Analysis Group (MEPAG) for “Special Regions” on Mars where potential growth of a microorganism could occur (Rummel et al., 2014). On Earth, the majority of organisms cannot grow $\alpha_w < 0.9$, and only a handful are known to endure $\alpha_w < 0.85$ (Grant, 2004). Halotolerant and epsotolerant microbes are promising candidates to grow under these conditions. In addition to salting-in and producing compatibles solutes, they possess increased acidic residues on protein surfaces to help compete for water and remain in a stable hydration shell (Karan et al., 2012). These

properties along with deliquescence are capable of supporting microbial activity as displayed in the Atacama Desert. Photosynthetic activity was detected through pulse amplitude modulated fluorometry in halite nodules located within the hyperarid core which is sustained entirely by deliquescence (Davilla et al., 2013). Cyanobacteria and accompanying heterotrophic bacteria dominated the endolithic community. This finding is not only noteworthy due to habitability through deliquescence, but deliquescence in a hypersaline environment. Basque Lake may be a more appropriate analog to Mars than the Atacama Desert in certain aspects – Basque Lake containing high concentrations of $MgSO_4$ instead of NaCl.

1.8 Chaotropicity, Kosmotropicity, and Ionic Strength

Epsomitic brines, like Basque Lake, are unique because they are limited in chloride and dominate in sulfates. This differs significantly from the dominant brine type found on Earth. Mg^{+2} and SO_4^{-2} are divalent ions which form different ionic ratios compared to monovalent ions, Na^+ and Cl^-, found in marine type brines. Ionic ratios are relevant to biological molecules because of the change in osmotic pressure due to the charge density of the dominant ions (Hallsworth et al., 2007). The effect of these ions on the structure of water is defined by chaotropicity and kosmotropicity (Marcus, 2009). Chaotropic ions can disrupt and denature macromolecules such as proteins and nucleic acids due to their low charge density and hydrophobic effects. Kosmotropic ions can stabilize macromolecules due to their large charge density and by strengthening hydrogen bonds between intramolecules (Marcus, 2009).

It has been suggested that the chaotropicity of hypersaline waters is the limiting factor for microbial growth (Hallsworth et al., 2007), Crisler et al. (2012) determined the chaotropicities of various salt solutions (magnesium sulfate, magnesium chloride, lithium sulfate, and sodium chloride) with the agar gel-point assay of Hallsworth et al. (2003) over a range of salinities as shown in Figure 5. It is worth noting that magnesium sulfate was kosmotropic at low concentrations and chaotropic at high concentrations. More discrepancies have also been noted with the relationship between sucretolerance and salinotolerance suggesting compatible solute effects as an alternative (Fredsgaard et al., 2016). Even though high charge density is associated with kosmotropicity, (Fox-Powell., 2016) demonstrated that high divalent:monovalent ionic ratios resulting in high charge densities in Martian brines may make Mars uninhabitable despite the presence of water. In the same study, it was determined that Basque Lake brines had an ionic ratio near that of Mars and supports life. This is of interest because out all the hypersaline waters sampled for brines Basque Lake not only had nearly the same ionic ratio as Mars, but also the same dominant ions except $Fe^{2/3+}$.

1.9 Phylogeny of Epsom Rich Environments

The known phylogeny of epsom-rich environments is minimal and presents the opportunity for novel taxa. An increase in effort is needed to clarify associated community structures. Currently, phylogeny representative of halite (NaCl) concentrated environments is better understood and more available. Organisms representing all three domains of life can be found in these environments.

Eukaryotic microorganisms are present in many forms that include fungi, algae, invertebrates and flora; yet they lack in abundance when compared to the other domains. Halotolerant plants known as 'halophytes' are well documented as a potential biofuel and for being invasive (Glenn et al., 1999; Shafroth et al., 2005). MacKay (1935) details the growth of *Ruppia maritime* in two $MgSO_4$ concentrated lakes: Epsom Lake and Spotted Lake. Fungi have a wide range of growth capabilities and have been noted to grow in near saturated salt conditions (Petrovič et al., 2002). Of the eukaryotes, the green alga *Dunnaliella* and brine shrimp *Artemia* are worthy of mention given their ubiquitous presence.

What could be considered true "halophiles", archaea have been extensively studied in hypersaline environments. Though similar to bacteria in appearance, they are considered a separate domain as a result of the groundbreaking work of George Fox and the late Carl Woese. Archaeal species such as *Halobacterium salinarum* and *Haloferax volcanii* have significance as model organisms for the study of DNA replication, translation, and transcription as well as their interactions with each other due to increased stability – interest was especially bolstered in *H. salinarum* after the discovery of bacteriorhodopsin in 1971 (Oesterhelt and Stockenius, 1971; Soppa, 2006).

Bacteria may be the most diverse of the three domains. They expand across many subgroups and most species are Gram-positive or Gram-negative aerobic or facultative anaerobic (Ventosa et al., 1998) genera associated with Gram-positive halotolerant bacteria include *Halobacillus*, *Marinococcus*, and *Salinicoccus*; actinomycetes, which are Gram-positive and resemble fungi, include *Nesterenkonia* and

Nocardiopsis. Halotolerant Gram-negative species have members in *Halomonas*, *Halovibrio,* and *Deleya*; these genera in addition to others are widely represented throughout the *Proteobacteria*. Both cyanobacteria and spirochaetes have halotolerant species, with spirochaetes containing the sole genus *Spirochaeta* (Ventosa et al., 2012). There are also halotolerant species that belong to genera such as *Bacillus* and *Clostridium* that contain nonhalotolerant members, which may be a result of horizontal gene transfer.

Classification can prove difficult and has changed over the years, one prominent example being that of the family *Halobacteriaceae*. Originally thought to belong to the Bacteria – this was before the advent of 16S rRNA sequencing – it was later determined as a member of Archaea. The difficulty was a result of unorganized nomenclature rules, lack of distinguishing characteristics in earlier versions of what is now *Bergey's Manual of Systematic Bacteriology* (then known as *Bergey's Manual of Determinative Bacteriology*), and unexpected findings such as some species having divergent copies of the 16S rRNA gene in a single cell made classifying some genera and species rather difficult (Oren, 2012). Through various methods such as polar lipid analysis and multi-locus sequence typing it became possible to adequately classify problematic taxa.

At this moment, only a few studies have rigorously surveyed the microbial make-up and community structure of an epsom concentrated lake – Hot Lake – Linderman et al. (2013), Babauta et al. (2014), and Kilmer et al. (2014) are three such studies. The Kilmer study focused on various water and soil samples; Linderman et al. concentrated on seasonal phototropic mats, while Babauta et al. investigated the physiochemical properties of these mats. Linderman and colleagues found an abundance of

cyanobacteria in addition to *Proteobacteria* (alpha, beta, delta, and gamma). The Kilmer study isolated approximately 100 aerobic heterotrophic bacteria and constructed a 16S rRNA gene clone library from direct DNA extracts of a Hot Lake margin soil. The bacterial isolate collection of Kilmer et al. was primarily associated with hypersaline environments and widely displayed both halotolerance and epsotolerance, while the clone library was split between bacteria primarily associated with hyperhaline environments and salinotolerant variants of common soil bacteria. No archaea were isolated in either study even with near-saturated brines. Basque Lake is similar to Hot Lake in that they are both epsom-rich and located within an endorheic basin that becomes nearly saturated with $MgSO_4$ due to the evaporation of accumulated precipitation. The fact that the Hot Lake collection mainly consists of halotolerant and epsotolerant isolates leads to an inference of the same outcome for Basque Lake; yet, this does not rule out the possibility of finding novel taxa.

CHAPTER 2

RATIONALE AND HYPOTHESIS

2.1 Rationale

Experimental and molecular microbiology of epsom-rich environments is not only valuable from a biodiversity perspective but also from an astrobiological perspective. In addition to being an extreme ionic environment, Basque Lake is an analog to Mars because it has near-saturated $MgSO_4$ brines similar to those that would form if underlying permafrost were to melt and come into contact with the Martian surface. Previous work at GSP and Hot Lake has revealed phylotypes that were halotolerant and epsotolerant. Many isolates from these collections have grown in media containing over 2 M $MgSO_4$ – the highest concentration to date; it is expected that isolates from Basque Lake will also be halotolerant and epsotolerant. Characterization of their morphology, physiology, and biochemistry will show trends throughout the Basque Lake collection – giving clues as to how they remain viable in a near-saturated environment. Some Isolates collected from GSP and Hot Lake were related to halotolerant isolates collected from spacecraft assembly facilities (Caton et al., 2004; Kilmer et al., 2012; Kilmer et al., 2014). Given that there are halotolerant and epsotolerant microbes that can survive extreme ionic environments and therefore may survive Mars-like conditions it is imperative to take further measures to limit potential forward contamination. Once characterized, epsotolerant microbes from Basque Lake can increase astrobiogists' understanding of how potential life-forms functions on Mars. On Earth, epsotolerant

microorganisms are no less important. Taxonomy and phylogeny has already been reevaluated at the highest levels because of extremophiles. The potential of finding novel phylotypes at Basque Lake is there; it is reasonable to think that some microbes might have adapted to this type of environment and become discernable.

2.2 Hypothesis

Given the results of similar microbial studies concerning hypersaline environments the following hypothesis were put forth:

1. Bacteria from Basque Lake will be highly epsotolerant and halotolerant.
    - Similar studies at similar environments resulted in the isolation of microbes that possess both characteristics.
2. Bacteria from Basque Lake will be tolerant to low temperatures
    - Epsotolerant and halotolerant bacteria have demonstrated growth at low temperatures.
3. Isolates from Basque Lake will be from taxa previously observed at Hot Lake.

- Basque Lake and Hot Lake are similar environments that present similar challenges for life to persist. Isolates previously obtained from Basque Lake will have characteristics and traits similar to those of Hot Lake and their respected taxa.

4. Basque Lake isolates will not belong to taxa normally associated with common soils
    - Epsotolerant and halotolerant bacterial isolates observed at Hot Lake are rare in common soils.

2.3 Objectives

The main objective of this study was the phenetic characterization and molecular analysis of cultivated bacterial isolates obtained from the water and lake margin soils of Basque Lake. The following objectives were pursued as follows:

1. Cultivation of isolates on selective media
2. Characterization of the isolate collection
    - Morphology
    - Physiology
    - Biochemistry
3. Measurement of epsotolerance and halotolerance, of the isolate collection
4. Measurement of temperature tolerance, of the isolate collection
5. Molecular analysis of isolates through sequencing and phylogenetic characterization

CHAPTER 3

METHODOLOGY

3.1 Sample Collection

Water and lake margin soil samples were obtained in November 2009. Arrangements were made with a collaborator who had access to Basque Lake to retrieve and send the samples. Jim Britton from the British Columbia Geological Survey (Vancouver, BC), a regional geologist based in Kamloops, BC-not far from Basque Lake agreed to collect water and lake margin soils from diverse locations around Basque Lake. Sterile lab equipment was gathered to assist in the collection of samples and included: 50 ml plastic centrifuge tubes, sterile spatulas, and a shipping container. Once gathered, the lab equipment was packaged in the Microbial Ecology Lab at Wichita State University and delivered to Mr. Britton for sterile collection of the samples and to lessen any cost associated with collecting the samples. Three random sites around Basque Lake were chosen S1 (50° 36’02.5” N 121°21’32.2 W), S2 (50°35’59.8” N 121° 21’27.6” W) and S3 (50°35’58.9” N 121°21’27.2” W) were sampled for both water and soil. Samples were collected using aseptic technique into sterile 50 ml plastic centrifuge tubes from the 3 sites around Basque Lake. An aerial view labeled with the sample sites can be seen in Figure 6. For each sampling site, two 50 ml sterile plastic centrifuge tubes were filled with either water or soil. After collection at an individual sampling site, one of the tubes was immediately frozen in the field and put on dry ice to maintain it for molecular analysis. The other set was kept at ambient temperature for cultivation work. Samples were shipped overnight via FedEx to the Microbial Ecology Lab at Wichita

State University. After arrival, the still-frozen set of samples were placed into a freezer at -80° C to preserve for molecular analysis and the “fresh” cultivation set of samples were immediately used to inoculate an assortment of media.

3.2 Enrichment and Isolation

Isolation of halotolerant and epsotolerant microbes involved direct plating, dilution plating and liquid enrichment of Basque Lake waters and margin soils. Kilmer et al. (2014) successfully isolated both halotolerant and epsotolerant microbes from Hot Lake using modified "SP" and “MH” mediums based on (Caton et al., 2004). SP and MH are selective mediums that are rich in nutrients and moderate to highly saline (NaCl). The SP medium contained per litre: NaCl, 100.0 g (10%); KCl, 2.0 g; $MgSO_4$●7H2O, 1.0 g; $CaCl_2$●$2H_2O$, 0.36 g; $NaHCO_3$ 0.06; NaBr, 0.23 g; $FeCl_3$●$6H_2O$, 1.0 mg; trace minerals, 0.5 ml; Bacto tryptone, 5.0 g; yeast extract, 10.0 g; glucose, 1.0 g; final pH 7.0. SP medium was prepared with either 10% (w/v) NaCl, or modified with 10% (w/v) $MgSO_4$ or 493.0 g (2 M) $MgSO_4$. These mediums were prepared as a liquid or solid with solid mediums being used as plates with 15 g/L Bacto-agar. Agar plates did not successfully gel at 2 M $MgSO_4$, so could only be made as a liquid. The MH medium contained per litre: NaCl, 220.0 g (20%); $MgSO_4$●$7H_2O$, 10.0 g; KCl, 5.0 g; sodium citrate, 3.0 g; KNO_3, 1.0 g; Bacto-tryptone, 5.0 g; yeast extract, 1.0 g; $CaCl_2$●$2H_2O$, 0.20 g; $FeCl_3$●6H2O, 1.0 mg; trace minerals, 0.5 ml; final pH 7.3. As with the SP medium, the MH medium was prepared as either a liquid or solid with solid plates containing 15 g/L Bacto-agar.

Upon arrival aliquots of “fresh” Basque Lake samples were immediately used to inoculate all mediums via the isolation methods previously stated. Direct plating was performed by smearing approximately 0.5 g of each soil sample or spreading 1 ml of each water sample onto the surface of 10% NaCl and 10% $MgSO_4$ solid media. Dilution plating only involved the use of soil samples and was diluted 10-fold using either 10 g or 10 ml of the sample with a suitable media. Prior to plating, samples were serial diluted at 1:10 dilution using a 0.1% sodium pyrophosphate (NaPP) solution. This involved vigorously agitating the slurry mixture for 30 minutes on a shaker allowing approximately 10 minutes for settlement. Once the mixture had settled with no particulates evident 1 ml of the dilution was spread plated onto 10% NaCl and 10% $MgSO_4$ solid media. Each plate was wrapped in parafilm and kept at an appropriate temperature in a moist container to help prevent drying of agar in the media.

All water and soil samples were individually used for inoculation in each type of liquid medium. Liquid enrichment cultures were obtained by inoculating 250 ml Erlenmeyer flasks containing approximately 100 ml with individual aliquots of 0.5 g soil, 1.0 ml of water, or 1 ml of soil dilutions. Both plating and liquid enrichment cultures were duplicated and incubated at 7, 25, or 37° C. Liquid enrichment cultures were incubated and maintained on a rotary shaker at 150 rpm. Aliquots of 100 µl from each liquid enrichment culture were spread on agar plates containing the same medium matching that of the liquid culture after 24 or 48 h. Although practical for liquid enrichments, agar plates with 2 M $MgSO_4$ would not gel, any isolates obtained from liquid cultures of this medium were transferred to 10% NaCl plates.

Colonies were collected from the plates within a couple days of inoculation and kept for several weeks in incubation to include new colony types representative of slower growing microorganisms. Selection of colonies for isolation was based on gross morphological and physiological features, including pigmentation, size, margin or rate of growth. Duplicate colonies originating from the same Basque Lake sample were excluded to manage the size of the culture collection and limit potential contamination. Isolates were secured by transferring colonies to fresh SP agar plates with 10% NaCl or 10% $MgSO_4$ using the streak-plate method. To ensure clonal purity each isolate underwent a minimum of five successive streak-platings. Pure cultures were transferred to duplicate agar slants, of an appropriate medium, and stored in a moist container at room temperature. Isolates were curated as 50% glycerol stocks at -80°C. Two isolate sets on agar slants were maintained to prevent contamination and ensure collection purity. One set of pure cultures is sealed and only used to make other pure duplicate sets. The other set is used as a "working set" and used for phenetic analysis.

3.3 Morphological, Physiological, and Biochemical Tests

All tests were incubated in duplicate at room temperature and results recorded over the span of one week unless otherwise noted. Differential media was supplemented with 10% (w/v) NaCl unless otherwise noted. All media was autoclave sterilized unless otherwise noted.

3.3.1 Gram Stain

Gram-stains were done using the PROTOCOL® Gram-staining kit (Fisher Diagnostics) following the manufacturer's instructions.

3.3.2 Endospore Stain

Endospore stains were performed in accordance with the procedures as listed in *Brief Microbiology Laboratory Theory and Application*, (2012). Leboffe and Pierce, Morton Publishing, 2nd Ed.

3.3.3 Acid Fast Stain

Acid fast stains were performed in accordance with the procedures as listed in *Brief Microbiology Laboratory Theory and Application*, (2012). Leboffe and Pierce, Morton Publishing, 2nd Ed.

3.3.4 SIM Test

Motility and hydrogen sulfide production isolates was determined by stab inoculation of sulfur-indole-motility medium deeps (SIM, BBL Fisher Scientific).

3.3.5 Catalase Test

Catalase detection was determined using a 3% hydrogen peroxide solution that was applied to individual smears of liquid broth culture isolates on individual microscope slides.

3.3.6 Oxidase Test

Oxidase testing was done using the BBL DrySlide® (Fisher Scientific) kit following the manufacturer's instructions.

3.3.7 Urease Test

Urease activity was determined at 5 days using urea broth medium which contained the following per litre: urea, 20.0 g; K_2HPO_4, 9.5 g; KH_2PO_4, 9.1 g; phenol red, 0.01 g; yeast extract, 0.1 g; final pH 7.0. Urea broth medium was filtered sterilized using a 0.22 μm filter.

3.3.8 Glucose Test

Production of acid and gas as a result of glucose fermentation was tested with 0.5% (w/v) glucose medium in culture tubes containing inverted Durham tubes. Glucose medium contained the following per litre: glucose, 5.0 g; NaCl, 100.0 g; Bacto tryptone, 10 g; phenol red, 0.018 g; final pH 7.3.

3.3.9 Lactose Test

Production of acid and gas as a result of lactose fermentation was tested with 0.5% (w/v) lactose medium in culture tubes containing inverted Durham tubes. Glucose medium contained the following per litre: glucose, 5.0 g; NaCl, 100.0 g; Bacto tryptone, 10 g; phenol red, 0.018 g; final pH 7.3.

3.3.10 Sucrose Test

Production of acid and gas as a result of sucrose fermentation was tested with 0.5% (w/v) sucrose medium in culture tubes containing inverted Durham tubes. Sucrose medium contained the following per litre: glucose, 5.0 g; NaCl, 100.0 g; Bacto tryptone, 10 g; phenol red, 0.018 g; final pH 7.3.

3.3.11 Starch Hydrolysis Test

Amylase production on Starch agar (Difco) was determined by flooding lawn plates with Gram's iodine after incubating for 5 days.

3.4 Epsotolerance and Halotolerance

Epsotolerance and halotolerance were measured over a wide range of salinities. Modified SP mediums containing limited to near saturated concentrations of either NaCl or $MgSO_4$ were used. A minimal concentration of 0.1% was used for both NaCl and $MgSO_4$ with a maximum concentration of 30% NaCl and near- saturated concentration of 67% $MgSO_4$. The following concentrations of SP media was used: 0.1% NaCl, 1.0% NaCl, 10%, NaCl 15%, NaCl, 20% NaCl, 30% $MgSO_4$, 0.1% $MgSO_4$, 1% $MgSO_4$, 10% $MgSO_4$, 20% $MgSO_4$, 30% $MgSO_4$, 40% $MgSO_4$, 50% $MgSO_4$, 60% $MgSO_4$, 67% $MgSO_4$. A liquid shake tube size of 13x100 mm containing 3 ml of media was used for all salinities. Growth was measured at 2-day intervals for a period of two weeks. Measurements were recorded as optical density (OD) at A_{600} by spectrophotometry (ThermoFisher Genesys 10S®). Shake-tube cultures were kept at room temperature in a moist sealed bin and placed on a rotary shaker set to 150 rpm.

3.5 Temperature Tolerance

Temperature tolerance was measured at varying temperatures. For each temperature, a 10% SP plate was inoculated with Basque Lake isolates (four isolates per plate) and left at the appropriate temperature for two weeks. Plates were checked for growth on a weekly basis. The following temperatures were used: 4 °C, 7 °C, 10 ° C, 20 °C, 37 °C.

3.6 pH Tolerance

Tolerance to a wide range of pH levels was measured across the pH scale using 10% SP mediums with an adjusted pH below or above pH 7. The following pH levels were used: pH 4, pH 5, pH 6, pH 7, pH 8, pH 9, pH 10, pH 11. A liquid shake tube size of 13x100 mm containing 3 ml of media was used for all salinities. Growth was measured at 2-day intervals for a period of two weeks. Measurements were recorded as optical density (OD) at A_{600} by spectrophotometry (ThermoFisher Genesys 10S®). Shake-tube cultures were kept at room temperature in a moist sealed bin and placed on a rotary shaker set to 150 rpm.

3.7 DNA Extraction

3.7.1 Isolate Extracts

DNA extracted from pure-culture isolates involved a freeze-thaw technique modified from Caton et al. (2004). This involved centrifugation to collect cells from 3 ml of dense culture for each isolate. The cells then were then resuspended in 0.5 ml of 10 mM Tris-HCl (pH 8.0) and subsequently underwent alternate freezing in liquid nitrogen (1 minute) and thawing in a -90 °C water bath (1 minute) for a total of six cycles. Suspensions were refined by centrifugation for 10 minutes. The supernatant (200 µl) was stored at -20 ^{0}C. Concerns over the lack of steps to remove humic acids and organics are minimal since pure-culture samples were used.

3.8 PCR Amplification

PCR amplification was done for each isolate. Genomic DNA in the supernatant from the DNA extraction was the target of amplification of 16S rRNA gene fragments with an approximate nucleotide length of 1.5 kb (kilobases) using the bacterial primers (EUBPA: 5'-AGAGTTTGATCCTGGCTCAG-3' and EUBPH: 5'-AAGGAGGTGATCCAGCCGCA-3') (Edwards et al., 1989). All PCR amplification was performed in a thermocycler (Eppendorf Mastercycler®) as 25 µl reactions. Each reaction was prepared in 0.2 ml PCR tubes (Eppendorf) which included 0.2 µl of each primer, 200 µM dNTP, 5 µl 10X PCR buffer, 1 U Takara Ex Taq® (Takara Bio, Shiga, Japan) and 5 µl of genomic DNA template. A negative and positive control reaction was included with each PCR run. The negative control lacked a DNA template and the positive control contained *Bacillus subtilis* DNA extract. The PCR reaction profile included the following thermal cycler conditions: an initial denaturation for 2 minutes at 95 °C, followed by 40 cycles of denaturation for 1 minute at 95 °C, annealing for 1 minute at 50 °C, and extension for 1 minute at 72 °C. This was followed by a final extension for 5 minutes at 72 °C. To determine amplification success, 5 µl from each reaction was run through a 1% agarose gel via electrophoresis and stained with ethidium bromide after which was exposed to UV light for visual confirmation. The resulting amplicons representing the Basque Lake isolate collection were purified using Wizard® SV Gel and PCR Clean-up System (Promega) and stored at -20 °C until shipped for sequencing.

3.9 Sequencing and Phylogenetic Analysis

PCR amplicons derived from the Basque Lake isolate collection were single-passed sequenced by Eurofin Genomics (Louisville, KY) using the EUBPA primer. A NanoDrop 2000® (Fisher Thermo Scientific, Bartlesville, OK) was used to measure the DNA concentration of each sample. All samples were diluted to a concentration of 50 ng/ µl before sequencing. The purified amplicons along with 10 µM of EUBPA primer were shipped overnight via UPS for sequencing the next day.

Sequences were automatically aligned using Clustal-W (Thompson et al., 1994) and then manually inspected and trimmed within MEGA 7 (Kumar, Stecher, and Tamura, 2015). Contextual 16S rRNA gene sequences were identified in GenBank using BLAST (BASIC ALIGNMENT SEARCH TOOL) (Altschul et al., 1998) or by comparison to relevant literature. MEGA 7 generated phylogenetic trees using distance analysis with Jukes-Cantor rules and the neighbor-joining algorithm. Sequences were trimmed to equal lengths, with sequences less than 500 bp (base pairs) removed, and positions with gaps and ambiguous bases ignored, giving 500-600 bases for analysis. Bootstrap analysis was used to assess the relative support for each branch with a total of 100 replicates conducted heuristically using the distance-based neighbor-joining algorithm and the nearest-neighbor interchange algorithm in MEGA. The trees were rooted using *Methanospirillum hungatei* as the functional outgroup.

CHAPTER 4

RESULTS

4.1 Assemblage and Identification of Bacterial Isolates

All three soil and water samples provided bacterial isolates, with the soil samples providing the bulk at approximately 75%- all 3 sites contributed roughly the same. Culturing efforts resulted in nearly 65 aerobic hetrotrophic bacterial isolates from Basque Lake by dilution plating and repetitive streaking. Most the bacterial isolates were acquired from enrichment cultures at room temperature in 10% NaCl SP medium. Approximately a tenth of the isolates were acquired at 30° or 37° C in 10% or 2 M SP medium (Table 5). Although precautions were taken to collect colonies based on unique colony morphology, representatives of the most common colony types from each of the water and soil samples were collected, increasing duplication and limiting diversity throughout the collection. No archaea were isolated despite Fox-Powell et al. (2016) detecting *Crenarchaeota* through culture-independent community analysis. Phylogenetic analysis was performed on all Basque Lake isolates using 16S rRNA gene sequences, A phylogenetic tree of the Basque isolates (designated BL) can be seen in Figures 7 and 8. *Virgibacillus* dominated the Basque Lake isolate collection with 36 representative isolates. Another abundant Gram-positive genus was the low G+C *Marinococcus* (12 isolates). Gram-negative genera also had a strong showing with *Halomonas* claiming 13 isolates. *Salinivibrio* and *Staphylococcus* made up the

remainder of the Basque Lake collection with each containing only one representative isolate (Figure 9). The genus for each Basque Lake isolate is given in Table 6.

4.2 Phenotypic Analysis of Bacterial Isolates

4.2.1 Stains

The Gram-positive bacteria dominated the collection at 49 isolates, while the Gram-negative bacteria only had 14 isolates in the Basque Lake collection. Thirty-six isolates produced endospores and all isolates in the collection tested negative when acid-stained. Total results for all stains are listed in Tables 7 and 8.

4.2.2 Morphological, Physiological, and Biochemical Tests

All Basque Lake isolates were catalase-positive and over three-quarters were oxidase-positive. Amylase activity was scarce in the culture collection. Almost all the isolates fermented (84%) glucose and (81%) sucrose, while over (76%) thee-quarter fermented lactose, however; only BL62 and BL90 produced gas. Urea hydrolysis occurred in less than a (17%) fifth of the isolates. Hydrogen sulfide production using SIM medium was limited to 13 isolates, with none of them positive for indole. Mobility was demonstrated by nearly the entire culture collection with BL90 being the exception. Total results for all physiological and biochemical tests are listed in Tables 9-11. The morphology and motility of each colony isolate are presented in Table 12.

4.3 Epsotolerance and Halotolerance

The Basque Lake isolate collection was screened through a wide range of salinities to determine the epsotolerance and halotolerance of individual isolates.

Modified SP medium containing limited to near saturated concentrations $MgSO_4$ or NaCl were used. A minimal concentration of 0.1% was used for both NaCl and $MgSO_4$ with near saturated concentrations of 30% NaCl and 67% $MgSO_4$. Basque Lake Isolates demonstrated epsotolerance by growing across the spectrum with nearly all growing between 0.1-40% $MgSO_4$ (94%), and (87%) at 50% $MgSO_4$ (~ 2 M). More than half grew (65%) at 60% $MgSO_4$. Isolate growth dropped dramatically (8%) at 67% $MgSO_4$, saturated concentration (Tables 13-15). Epsotolerance was expansive as expected at Basque Lake given its ephemeral nature. Although some isolates could grow at saturated concentration, no epsophilic microbe was isolated from the Basque Lake isolate collection.

Extraordinary tolerance to both high and low concentrations of NaCl was displayed by Basque Lake isolates. This was unexpected since Basque Lake is rich in divalent ions unlike environments rich in chlorides. All isolates grew at 10% NaCl and almost three-quarters (70%) grew at 20% NaCl (~ 3.5 M). A handful of isolates grew at 30 % NaCl (Tables 16 and 17). Epsotolerance and halotolerance do not complement each other as some isolates representing *Virgibacillus* grew at 60% $MgSO_4$ but did not at 20% NaCl (Figure 9). This was also noted by Kilmer et al. (2014) with isolates from the Hot Lake collection.

4.4 Temperature Tolerance

Growth tolerance to a variety of temperatures was screened for using the Basque Lake isolate collection to determine the temperature tolerance of individual isolates. Screened temperatures varied from a minimum temperature of 4° C (psychrophilic) to a

maximum of 37° C (mesophilic). Almost all the Basque isolates were “Psychrotolerant”- meaning they are tolerant to low temperatures- with nearly (81%) all of the Basque Lake isolates growing between 10-37° C. Many of the Basque isolates were exceptionally (75%) psychrotolerant showing growth at 4° C including *Halomonas* and *Marinococcus*. (Tables 18 and 19) Multiple isolates grew at every temperature screened, mainly those of *Virgibacillus* and *Marinococcus*. A possible explanation for the widespread psychrotolerance among Basque Lake isolates could be attributed to the average seasonal temperature at Basque Lake. The temperature fluctuates from an average of -10° C in January to an average of 25-30° C during July and August, well within the agreed upon psychrotolerant temperature range (Goudge,1926; Morita,1975).

4.5. pH Tolerance

Acidity and alkalinity growth tolerance was determined for the Basque Lake isolate collection. Basque Lake isolates were screened throughout a wide spectrum of the pH scale, ranging from pH 4 to pH 11. Given the expansive range of pH screened for tolerance, Basque Lake isolates displayed both acid and alkaline-tolerance. All the isolates grew at pH 8, and over half grew (79%) grew at pH 9. Growth dropped to a third of the culture collection at pH 10. Growth occurred in a few isolates at pH 11. Basque isolates were more vulnerable as acidity increased with only a little over a fifth (22%) of the collection showing growth at pH 5. There was no growth at pH4 (Tables 20 and 21). Some Basque isolates were both acid-tolerant and alkaline-tolerant by growing from pH 5 to pH 10 including *Virgibacillus*.

CHAPTER 5

DISCUSSION

5.1 Epsotolerance and Halotolerance

The Basque isolates were tolerant to a wide array of salinities. This growth tolerance spanned from growth in media with trace amounts of NaCl and $MgSO_4$ to near-saturated concentrations; a few of the Basque isolates could tolerate the point of saturation for magnesium sulfate, like the sulfate heavy brines that exist on Mars. Among the isolates, halotolerance is just as widespread as epsotolerance. This outcome was expected since previous studies with isolates from GSP and especially Hot Lake had nearly the same results regarding epsotolerance and halotolerance. Basque Lake isolates grew better than Hot Lake isolates at higher concentrations of $MgSO_4$, however; Basque Lake isolates had a similar tolerance to Hot Lake at higher concentrations of NaCl, particularly 30% NaCl (Kilmer et al., 2014). Basque Lake isolates high tolerance to $MgSO_4$ may be attributed to Basque Lake being more concentrated in epsomite and limited in chlorides which allowed for specialization. The lack of chlorides and inhabiting an environment predominate in divalent ions may have led to Basque Lake having a lower growth tolerance to high concentrations of NaCl than that of $MgSO_4$. High epsotolerance or halotolerance isn't a guarantee for success in the other. BL6a is one such isolate that could grow well at 60% $MgSO_4$ but not above 10% NaCl. It is currently unknown what physiochemical factors are involved, and they could be solicited by any number of influences including specific solute effects such as the

viscosity of the medium the microbe was grown on. Regardless, nearly all the isolates from Basque Lake were halotolerant and epsotolerant such as those isolated from Hot Lake.

5.2 Temperature Tolerance

Growth at psychotropic temperatures was widespread among the Basque Lake culture collection with temperatures ranging from 4-37 °C. Although not originally screened for growth throughout the isolate collection, growth was observed on a handful of plates below 4 ^{0}C. Psychrotolerant and Psychrophilic microorganisms have been closely associated with hypersaline environments (Bowman et al., 1997). In fact, the coldest temperatures reported for microbial growth and metabolic activity are set at -15^{0} C and -32^{0} C by bacteria isolated from subzero permafrost brines (Bakermans and Skidmore, 2011; Mykytczuk et al., 2013). Microbes are viable at low temperatures by having higher levels of unsaturated fatty acids in the cell membrane to increase fluidity, induce cold-shock proteins if there is a sudden drop in temperature, and accumulate compatible solutes to present freezing or decrease metabolism to a dormant state to later revive (Cary et al., 2010; De Maayer et al., 2014). It has even been proposed that microbes could survive cold temperatures such as those on Mars by going into metabolic stasis (Jakosky et al., 2003). In addition to dormancy, a microbe with a broad range of temperature tolerance like those isolated from Basque Lake could possibly survive bitter temperatures on the Martian surface.

5.3 Basque Lake Phylogeny

Overall, The Basque Lake bacterial isolate collection had less distinct taxa than the Hot Lake isolate collection. Out of the approximate 65 bacterial isolates from Basque Lake roughly half was *Virgibacillus salarius*, with the rest of the collection consisting of members from *Marinococcus*, *Staphylococcus*, *Halomonas* and *Salinivibrio*. All taxa in the Basque Lake isolate collection were present in the Hot Lake collection down to the family level minus that of *Vibrionaceae* which contained *S. costicola* as the lone representative (Kilmer et al., 2014). In addition to duplicate colony selection, the low number of taxa within the Basque Lake isolate collection may be attributed to *V. salarius* behaving as a “microbial weed” by dominating communities that have developed at an otherwise vacant Basque Lake (Cray et al., 2013; Oren and Hallsworth, 2014). This study did not utilize any culture-independent methods or analysis to determine community structures after numerous unsuccessful attempts to extract DNA from Basque Lake soil samples. Difficulty in extracting DNA can be attributed to Basque Lake soil samples having little biomass and primarily consisting of clay and chaotropic salts (Nesbitt, 1990; Schneegurt, Dore, and Kulpa, 2003; Cai et al., 2006).

The limited number of taxa obtained from Basque Lake water and margin soil could signal that any isolated microbe may have specifically adapted to endure the unique extremes exhibited by Basque Lake. In addition to extreme salinity, aridity, and fluctuating temperatures, a microbe must overcome extreme ionic ratios. There is currently no known place on Earth that has a higher ionic ratio than Basque Lake (Fox-Powell et al., 2016). Nearly all the Basque Lake isolates were primarily associated with

hypersaline environments. One notable exception is *Staphylococcus succinus* which was originally isolated from amber and more recently isolated from ripened cheese (Lambert et al., 1998; Place et al., 2002). As expected none of the Basque Lake isolates belonged to taxa normally associated with common soil, like the Hot Lake isolate collection. Even though common soil taxa were rare among both the Hot Lake and Basque Lake isolate collections, Kilmer et al. (2014) prepared a16S rRNA gene clone library from direct DNA extracts of Hot Lake margin soil which yielded numerous clones representing common soil bacteria which include actinomycetes, *Bacillus*, and *Legionella*. A culture-independent community analysis of direct DNA extracts of Basque Lake margin soil using a culture-independent clone library and 16S rRNA phylogeny might be similar in diversity and coverage. Any comparison between the clone libraries of Basque Lake and Hot Lake will match closely given the similarity in environments.

CHAPTER 6

CONCLUSION

The Basque Lake isolate collection has proved to be versatile to a whole host of extremes that include salinity, acidity and alkalinity, cold temperatures, and low water activity as found on the Martian surface. Although Mars lacks oxygen for an aerobic microbe to survive it may be possible for an anaerobic or facultative anaerobic microbe to remain viable on the Martian surface. A versatile microbe such as the facultative anaerobic bacterium *S. costicola* (BL62) is a prime candidate. Microbes isolated from Basque Lake are a potential threat to forward contamination of not only Mars but other planetary bodies and satellites including Titan and Enceladus. It is of importance that astrobiologists are aware of such risks. More effort to understand the bacterial assemblage of athalassohaline epsomite dominate bodies of water is needed to provide better insight into not only extreme organisms and their extreme niches on Earth, but that of life contaminating or originating on Mars or any other celestial object. Great care to limit such risks needs to be a top priority for future rover missions.

CHAPTER 7

FUTURE EFFORTS

A community analysis through culture-independent methods was unobtainable for this thesis work. Future efforts will seek to extract DNA from Basque Lake margin soils for deep sequencing or constructing a culture-independent clone library so that community analysis can be performed to help better understand the microbial community structures that make up Basque Lake. Upon completion, comparisons with Hot Lake and other hypersaline environments will be of focus. Other efforts will consist of additional physiological and biochemical testing of Basque Lake isolates which include: eutectic temperatures, increased pressure, deliquescence, fatty acid and polar lipid analysis. Once testing has concluded results will provide even more insight and guidance into resolving current and future concerns regarding the potential forward contamination of Mars and other planetary bodies from future rover and manned missions.

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APPENDICES

APPENDIX A

TABLES

Table 1. Growth requirements of microorganisms at specified concentrations of sodium chloride (NaCl) as adapted from Schneegurt (2012).

Category	Growth Requirement
Halotolerant	Trace to > 0.6 M (seawater)
Halophilic	>0.6 M

Table 2. Commonly used media to grow salinotolerant microbes (Fredsgaard et al., 2016).

Water Activities of Salt-Based Media	
Media Type	a_w
1% NaCl SP	0.95
10% NaCl SP	0.92
15% NaCl SP	0.88
20% NaCl SP	0.85
25% NaCl SP	0.8
30% NaCl SP	0.76
1% $MgSO_4$ SP	0.989
10% $MgSO_4$	0.983
20% $MgSO_4$ SP	0.977
30% $MgSO_4$ SP	0.966

TABLE 2 (continued)

40% $MgSO_4$ SP	0.954
2M $MgSO_4$ SP	0.936
60% $MgSO_4$ SP	0.907

Table 3. GSP isolate growth at high salinities (Crisler et al., 2012).

$MgSO_4$	NaCl	Growth/Total	Growth%
10%	10%	44/46	95.6%
10%	1%	44/52	84.6%
2 M	10%	5/52	11.5%
2 M	1%	18/52	34.6%

Table 4. Growth of Hot Lake isolates at various salinities (Kilmer et al., 2014).

Salinity	Growth/Total	Growth %
0.1% NaCl	66/100	66%
1%NaCl	99/100	99%
10% NaCl	73/100	73%
0.1% $MgSO_4$	90/100	90%
1% $MgSO_4$	89/100	89%
50% $MgSO_4$	82/100	82%
60% $MgSO_4$	58/100	58%

Table 5. Sampling site and conditions Basque Lake bacterial isolates were obtained. A (-) indicates that isolate was obtained from other salt-based SP media.

Basque Isolate	Sampling Site	Temperature	NaCl SP	MgSO4 SP
BL1	S3	25°	10%	-
BL2	S2	25°	10%	-
BL4c	S2	25°	10%	-
BL5	S1	37°	10%	-
BL6	S3	25°	10%	-
BL6a	S3	25°	10%	-
BL6B	S3	25°	10%	-
BL6c	S3	25°	10%	-
BL7	S2	25°	10%	-
BL7a	S2	25°	10%	-
BL7b	S2	25°	10%	-
BL10	S1	37°	10%	-
BL10a	S1	25°	10%	-
BL10c	S1	25°	10%	-
BL11	S3	25°	10%	-
BL14	S3	37°	-	2 M
BL15	S1	25°	10%	-
BL15A	S2	25°	10%	-
BL15B	S2	25°	10%	-
BL15C	S2	25°	10%	-
BL16	S1	37°	10%	-
BL17B	S1	25°	10%	-
BL21	S3	25°	10%	-
BL22	S1	30°	10%	-
BL25	S3	25°	-	2 M
BL27a	S3	25°	10%	-
BL30	S3	25°	10%	-
BL32	S2	25°	-	2 M

TABLE 5 (continued)

BL35	S2	25°	10%	-
BL41	S1	25°	10%	-
BL42	S1	30°	10%	-
BL43A	S1	25°	10%	-
BL43a	S1	25°	10%	-
BL43c	S1	25°	10%	-
BL44a	S3	25°	10%	-
BL46	S2	25°	-	2M
BL48	S2	25°	10%	-
BL49	S1	25°	-	2 M
BL50	S2	25°	10%	-
BL54	S3	25°	-	2 M
BL55	S1	25°	10%	-
BL56	S1	25°	10%	-
BL57	S3	25°	10%	-
BL59b	S2	25°	10%	-
BL60	S2	25°	10%	-
BL61	S2	25°	10%	-
BL62	S2	25°	10%	-
BL66A	S3	25°	10%	-
BL66a	S3	25°	10%	-
BL66b	S3	25°	10%	-
BL67	S1	25°	10%	-
BL69	S1	25°	10%	-
BL69a	S1	25°	10%	-
BL76A	S3	25°	10%	-
BL76a	S3	25°	10%	-
BL76B	S3	25°	10%	-
BL76b	S3	25°	10%	-

TABLE 5 (continued)

BL79b	S3	25°	10%	-
BL84	S2	37°	10%	-
BL84a	S2	25°	10%	-
BL85	S1	25°	-	2 M
BL90	S3	37°	10%	-
BL90A	S3	25°	10%	-

Table 6. Basque Lake bacterial isolates organized by genus.

Basque Isolate	Genus
BL1	*Halomonas*
BL5	*Halomonas*
BL11	*Halomonas*
BL30	*Halomonas*
BL35	*Halomonas*
BL41	*Halomonas*
BL42	*Halomonas*
BL43c	*Halomonas*
BL60	*Halomonas*
BL66a	*Halomonas*
BL67	*Halomonas*
BL84	*Halomonas*
BL84a	*Halomonas*
BL2	*Marinococcus*
BL7	*Marinococcus*
BL7a	*Marinococcus*

TABLE 6 (continued)

BL7b	*Marinococcus*
BL10	*Marinococcus*
BL15	*Marinococcus*
BL25	*Marinococcus*
BL48	*Marinococcus*
BL49	*Marinococcus*
BL54	*Marinococcus*
BL59b	*Marinococcus*
BL79b	*Marinococcus*
BL62	*Salinivibrio*
BL90	*Staphylococcus*
BL4c	*Virgibacillus*
BL6	*Virgibacillus*
BL6a	*Virgibacillus*
BL6B	*Virgibacillus*
BL6c	*Virgibacillus*

TABLE 6 (continued)

BL10a	*Virgibacillus*
BL10c	*Virgibacillus*
BL14	*Virgibacillus*
BL15A	*Virgibacillus*
BL15B	*Virgibacillus*
BL15C	*Virgibacillus*
BL16	*Virgibacillus*
BL17B	*Virgibacillus*
BL21	*Virgibacillus*
BL22	*Virgibacillus*
BL27a	*Virgibacillus*
BL32	*Virgibacillus*
BL43a	*Virgibacillus*
BL43A	*Virgibacillus*
BL44a	*Virgibacillus*
BL46	*Virgibacillus*

TABLE 6 (continued)

BL50	*Virgibacillus*
BL55	*Virgibacillus*
BL56	*Virgibacillus*
BL57	*Virgibacillus*
BL61	*Virgibacillus*
BL66A	*Virgibacillus*
BL66b	*Virgibacillus*
BL69	*Virgibacillus*
BL69a	*Virgibacillus*
BL76a	*Virgibacillus*
BL76A	*Virgibacillus*
BL76b	*Virgibacillus*
BL76B	*Virgibacillus*
BL85	*Virgibacillus*
BL90A	*Virgibacillus*

Table 7. Stain characterization totals for the Basque Lake isolate collection.

Stain	Total
Gram (G+/G-)	G+ 49/63
	G- 14/63
Endospore	36/63
Acid-Fast	0/63

Table 8. Stain characterization for individual Basque Lake bacterial isolates. A (+) indicates a positive result or if an isolate stained as Gram- positive (G+). A (-) indicates a negative result or if an isolate stained as Gram-negative (G-).

Basque Isolate	Gram Stain (G+/G-)	Endospore	Acid-Fast
BL1	-	-	-
BL2	+	-	-
BL4c	+	+	-
BL5	-	-	-
BL6	+	+	-
BL6a	+	+	-
BL6B	+	+	-
BL6c	+	+	-
BL7	+	-	-
BL7a	+	-	-
BL7b	+	-	-
BL10	+	-	-
BL10a	+	+	-
BL10c	+	+	-
BL11	-	-	-
BL14	+	+	-
BL15	+	-	-
BL15A	+	+	-
BL15B	+	+	-
BL15C	+	+	-
BL16	+	+	-
BL17B	+	+	-
BL21	+	+	-
BL22	+	+	-

TABLE 8 (continued)

BL25	+	-	-
BL27a	+	+	-
BL30	-	-	-
BL32	+	+	-
BL35	-	-	-
BL41	-	-	-
BL42	-	-	-
BL43A	+	+	-
BL43a	+	+	-
BL43c	-	-	-
BL44a	+	+	-
BL46	+	+	-
BL48	+	-	-
BL49	+	-	-
BL50	+	+	-
BL54	+	-	-
BL55	+	+	-
BL56	+	+	-
BL57	+	+	-
BL59b	+	-	-
BL60	-	-	-
BL61	+	+	-
BL62	-	-	-
BL66A	+	+	-
BL66a	-	-	-

TABLE 8 (continued)

BL66b	+	+	-
BL67	-	-	-
BL69	+	+	-
BL69a	+	+	-
BL76A	+	+	-
BL76a	+	+	-
BL76B	+	+	-
BL76b	+	+	-
BL79b	+	-	-
BL84	-	-	-
BL84a	-	-	-
BL85	+	+	-
BL90	+	-	-
BL90A	+	+	-

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Table 9. Physiological and biochemical characterization totals for the Basque Lake isolate collection.

Test	Total
Amylase	3/63
Catalase	63/63
Glucose	53/63
Indole	0/63
Lactose	48/63
Motility	62/63
Oxidase	53/63
Sucrose	51/63
Sulfur	13/63

Table 10. Physiological and biochemical characterization of individual Basque Lake bacterial isolates. A positive result for a biochemical test is (+) and (-) is a negative result for a biochemical test.

Isolate Number	Amylase	Catalase	Indole	Oxidase	Sulfur	Urease
BL1	+	+	-	+	-	-
BL2	-	+	-	+	-	+
BL4c	-	+	-	+	-	-
BL5	-	+	-	-	+	-
BL6	-	+	-	+	-	-
BL6a	-	+	-	+	-	-
BL6B	-	+	-	+	-	-
BL6c	-	+	-	+	-	-
BL7	-	+	-	+	-	+
BL7a	-	+	-	+	-	+
BL7b	-	+	-	+	-	+
BL10	-	+	-	+	-	-
BL10a	-	+	-	+	-	-
BL10c	-	+	-	+	-	-
BL11	-	+	-	-	+	-
BL14	-	+	-	+	-	-
BL15	-	+	-	+	-	+
BL15A	-	+	-	+	-	-

TABLE 10 (continued)

BL15B	-	+	-	+	-	-
BL15C	-	+	-	+	-	-
BL16	-	+	-	+	-	-
BL17B	-	+	-	+	-	-
BL21	-	+	-	+	-	-
BL22	-	+	-	+	-	-
BL25	-	+	-	+	-	+
BL27a	-	+	-	+	-	-
BL30	-	+	-	-	+	-
BL32	-	+	-	+	-	-
BL35	-	+	-	-	+	-
BL41	-	+	-	-	+	-
BL42	-	+	-	-	+	-
BL43A	-	+	-	+	-	-
BL43a	-	+	-	+	-	-
BL43c	-	+	-	-	+	-
BL44a	-	+	-	+	-	-
BL46	-	+	-	+	-	-
BL48	-	+	-	+	-	+

TABLE 10 (continued)

BL49	-	+	-	+	-	+
BL50	-	+	-	+	-	-
BL54	-	+	-	+	-	+
BL55	-	+	-	+	-	-
BL56	-	+	-	+	-	-
BL57	-	+	-	+	-	-
BL59b	-	+	-	+	-	-
BL60	-	+	-	-	+	-
BL61	-	+	-	+	-	-
BL62	+	+	-	+	+	-
BL66A	-	+	-	+	-	-
BL66a	-	+	-	+	+	-
BL66b	-	+	-	+	-	-
BL67	-	+	-	-	-	-
BL69	-	+	-	+	-	-
BL69a	-	+	-	+	-	-
BL76A	-	+	-	+	-	-
BL76a	-	+	-	+	-	-
BL76B	-	+	-	+	-	-

TABLE 10 (continued)

BL76b	-	+	-	+	-	-
BL79b	-	+	-	+	-	+
BL84	-	+	-	+	+	-
BL84a	-	+	-	+	+	-
BL85	-	+	-	+	-	-
BL90	+	+	-	-	+	+
BL90A	-	+	-	+	-	-

Table11. Fermentation of sugars by individual Basque Lake bacterial isolates. A positive result for acid fermentation is (+) and a positive result for both acid fermentation and gas production is (++). A negative result for both acid and gas is (-).

Basque Isolate	Glucose	Lactose	Sucrose
BL1	+	+	+
BL2	-	-	-
BL4c	+	+	+
BL5	+	+	+
BL6	+	+	+
BL6a	+	+	+
BL6B	+	+	+
BL6c	+	+	+
BL7	-	-	-
BL7a	-	-	-
BL7b	-	-	-
BL10	+	+	+
BL10a	+	+	+
BL10c	+	+	+
BL11	+	+	+
BL14	+	+	+

TABLE 11 (continued)

BL15	-	-	-
BL15A	+	+	+
BL15B	+	+	+
BL15C	+	+	+
BL16	+	+	+
BL17B	+	+	+
BL21	+	+	+
BL22	+	+	+
BL25	-	-	-
BL27a	+	+	+
BL30	+	+	+
BL32	+	+	+
BL35	+	+	+
BL41	+	-	-
BL42	+	+	+
BL43A	+	+	+
BL43a	+	+	+
BL43c	+	+	+
BL44a	+	+	+

TABLE 11 (continued)

BL46	+	+	+
BL48	-	-	-
BL49	-	-	-
BL50	+	+	+
BL54	-	-	-
BL55	+	+	+
BL56	+	+	+
BL57	+	+	+
BL59b	+	+	+
BL60	+	+	+
BL61	+	+	+
BL62	++	+	-
BL66A	+	+	+
BL66a	+	-	+
BL66b	+	+	+
BL67	+	-	+
BL69	+	+	+
BL69a	+	+	+
BL76A	+	+	+

TABLE 11 (continued)

BL76a	+	+	+
BL76B	+	+	+
BL76b	+	+	+
BL79b	-	-	-
BL84	+	-	+
BL84a	+	-	+
BL85	+	+	+
BL90	+	++	+
BL90A	+	+	+

Table 12. Cell and colony morphology of individual Basque Lake bacterial isolates. Cell Morphology: SR-Small Rod (less than 1 µm), SC-Small Cocci (less than 1 µm), R-Rod, C- Cocci. Colony Morphology: Colony Shape (first field): C-Circular. Colony Margin (second field): E-Entire (smooth), ER-Erose (serrated), L-Lobate, U-Undulate. Colony Elevation (third field): C-Convex, R-Raised. Colony Texture (fourth field): S-Smooth. Colony Color (fifth field): B-Brown, C-Cream, O-Orange, W-White, Y-Yellow. An isolate is positive for motility if labeled (+) and nonmotile if (-).

Basque Isolate	16S	Cell Morphology	Colony Morphology	Motility
BL1	*Halomonas aidingensis*	SR	C/E/R/S/B	+
BL2	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL4c	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL5	*Halomonas arcis*	R	C/E/R/S/C	+
BL6	*Virgibacillus salarius*	R	C/U/C/S/C	+
BL6a	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL6B	*Virgibacillus salarius*	R	C/ER/C/S/W	+
BL6c	*Virgibacillus salarius*	R	C/U/C/S/W	+
BL7	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL7a	*Marinococcus luteus*	SC	C/U/C/S/O	+
BL7b	*Marinococcus luteus*	SC	C/L/C/S/O	+
BL10	*Marinococcus tarijensis*	C	C/E/C/S/O	+
BL10a	*Virgibacillus salarius*	R	C/E/C/S/C	+
BL10c	*Virgibacillus salarius*	R	C/ER/C/S/C	+

TABLE 12 (continued)

BL11	*Halomonas arcis*	R	C/E/R/S/C	+
BL14	*Virgibacillus salarius*	R	C/U/C/S/C	+
BL15	*Marinococcus luteus*	SC	C/E/C/S/O	-
BL15A	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL15B	*Virgibacillus salarius*	R	C/L/C/S/C	+
BL15C	*Virgibacillus salarius*	R	C/ER/C/S/W	+
BL16	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL17B	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL21	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL22	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL25	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL27a	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL30	*Halomonas arcis*	R	C/E/R/S/C	+
BL32	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL35	*Halomonas arcis*	R	C/E/R/S/C	+
BL41	*Halomonas subterranea*	R	C/E/R/S/C	+
BL42	*Halomonas arcis*	R	C/E/R/S/C	+
BL43A	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL43a	*Virgibacillus salarius*	R	C/ER/C/S/W	+

TABLE 12 (continued)

BL43c	*Halomonas arcis*	R	C/E/R/S/C	+
BL44a	*Virgibacillus salarius*	R	C/U/C/S/C	+
BL46	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL48	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL49	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL50	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL54	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL55	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL56	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL57	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL59b	*Marinococcus tarijensis*	C	C/E/C/S/O	+
BL60	*Halomonas arcis*	R	C/E/R/S/C	+
BL61	*Virgibacillus salarius*	R	C/U/C/S/C	+
BL62	*Salinivibrio costicola*	R	C/E/C/S/W	+
BL66A	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL66a	*Halomonas zhaodongensis*	R	C/E/R/S/Y	+
BL66b	*Virgibacillus salarius*	R	C/E/C/S/W	+
BL67	*Halomonas gomseomensis*	R	C/E/R/S/C	+
BL69	*Virgibacillus salarius*	R	C/ER/C/S/C	+

TABLE 12 (continued)

BL69a	*Virgibacillus salarius*	R	C/U/C/S/C	+
BL76A	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL76a	*Virgibacillus salarius*	R	C/E/C/S/C	+
BL76B	*Virgibacillus salarius*	R	C/U/C/S/C	+
BL76b	*Virgibacillus salarius*	R	C/ER/C/S/W	+
BL79b	*Marinococcus luteus*	SC	C/E/C/S/O	+
BL84	*Halomonas zhaodongensis*	R	C/E/R/S/Y	+
BL84a	*Halomonas zhaodongensis*	R	C/U/R/S/Y	+
BL85	*Virgibacillus salarius*	R	C/ER/C/S/C	+
BL90	*Staphylococcus succinus*	C	C/E/R/S/W	-
BL90A	*Virgibacillus salaries*	R	C/ER/C/S/C	+

Table 13. Epsotolerance totals of the Basque Lake isolate collection.

Media Type	Growth/Total	Growth %
0.1% $MgSO_4$ SP	60/63	95%
1%$MgSO_4$ SP	62/63	98%
10% $MgSO_4$ SP	63/63	100%
20% $MgSO_4$ SP	63/63	100%
30% $MgSO_4$ SP	63/63	100%
40% $MgSO_4$ SP	62/63	98%
50% $MgSO_4$ SP	58/63	87%
60% $MgSO_4$ SP	41/63	65%
67% $MgSO_4$ SP	5/63	8%

Table 14. Epsotolerance of individual Basque Lake isolates at limited to high concentrations of $MgSO_4$ (w/v). Growth is indicated by (+) and little to no growth is indicated by (-).

Basque Isolate	0.1%	1%	10%	20%	30%
BL1	+	+	+	+	+
BL2	+	+	+	+	+
BL4c	+	+	+	+	+
BL5	+	+	+	+	+
BL6	+	+	+	+	+
BL6a	+	+	+	+	+
BL6B	+	+	+	+	+
BL6c	+	+	+	+	+
BL7	+	+	+	+	+
BL7a	+	+	+	+	+
BL7b	+	+	+	+	+
BL10	-	+	+	+	+
BL10a	+	+	+	+	+
BL10c	+	+	+	+	+
BL11	+	+	+	+	+
BL14	+	+	+	+	+
BL15	+	+	+	+	+
BL15A	+	+	+	+	+
BL15B	+	+	+	+	+
BL15C	+	+	+	+	+

TABLE 14 (continued)

BL16	+	+	+	+	+
BL17B	+	+	+	+	+
BL21	+	+	+	+	+
BL22	+	+	+	+	+
BL25	+	+	+	+	+
BL27a	+	+	+	+	+
BL30	+	+	+	+	+
BL32	+	+	+	+	+
BL35	+	+	+	+	+
BL41	+	+	+	+	+
BL42	+	+	+	+	+
BL43A	+	+	+	+	+
BL43a	+	+	+	+	+
BL43c	+	+	+	+	+
BL44a	+	+	+	+	+
BL46	+	+	+	+	+
BL48	+	+	+	+	+
BL49	+	+	+	+	+
BL50	+	+	+	+	+
BL54	+	+	+	+	+

TABLE 14 (continued)

BL55	+	+	+	+	+
BL56	+	+	+	+	+
BL57	+	+	+	+	+
BL59b	-	-	+	+	+
BL60	+	+	+	+	+
BL61	+	+	+	+	+
BL62	+	+	+	+	+
BL66A	+	+	+	+	+
BL66a	+	+	+	+	+
BL66b	+	+	+	+	+
BL67	-	+	+	+	+
BL69	+	+	+	+	+
BL69a	+	+	+	+	+
BL76A	+	+	+	+	+
BL76a	+	+	+	+	+
BL76B	+	+	+	+	+
BL76b	+	+	+	+	+
BL79b	+	+	+	+	+
BL84	+	+	+	+	+
BL84a	+	+	+	+	+

TABLE 14 (continued)

BL85	+	+	+	+	+
BL90	+	+	+	+	+
BL90A	+	+	+	+	+

Table 15. Epsotolerance of individual Basque Lake isolates at near-saturated concentrations of $MgSO_4$ (w/v). Growth is indicated by (+) and little to no growth is indicated by (-).

Basque Isolate	40%	50%	60%	67%
BL1	+	+	+	-
BL2	+	+	+	-
BL4c	+	+	+	-
BL5	+	+	+	-
BL6	+	+	-	-
BL6a	+	+	+	-
BL6B	+	+	-	-
BL6c	+	-	-	-
BL7	+	+	+	-
BL7a	+	+	+	+
BL7b	+	+	+	-
BL10	+	+	+	-
BL10a	+	+	+	+
BL10c	+	+	+	-
BL11	+	+	-	-
BL14	+	+	+	-
BL15	+	+	+	-
BL15A	+	+	+	-
BL15B	+	+	+	-
BL15C	+	+	+	-
BL16	+	+	-	-
BL17B	+	+	+	-
BL21	+	-	-	-
BL22	+	+	-	-
BL25	+	+	+	-
BL27a	+	+	+	-
BL30	+	+	+	-

TABLE 15 (continued)

BL32	+	+	+	-
BL35	+	+	+	-
BL41	+	+	-	-
BL42	+	+	-	-
BL43A	+	+	+	-
BL43a	+	+	+	-
BL43c	+	+	+	-
BL44a	+	+	+	-
BL46	+	+	+	-
BL48	+	+	+	+
BL49	+	+	+	+
BL50	+	+	-	-
BL54	+	+	+	-
BL55	+	-	-	-
BL56	+	+	+	-
BL57	-	-	-	-
BL59b	+	+	+	-
BL60	+	+	+	-
BL61	+	-	-	-
BL62	+	+	-	-
BL66A	+	+	+	-
BL66a	+	+	-	-
BL66b	+	-	-	-
BL67	+	+	+	-
BL69	+	+	-	-
BL69a	+	+	+	-
BL76A	+	+	+	-
BL76a	+	+	+	-
BL76B	+	-	-	-

TABLE 15 (continued)

BL76b	+	-	-	-
BL79b	+	+	+	+
BL84	+	+	-	-
BL84a	+	+	-	-
BL85	+	+	+	-
BL90	+	+	-	-
BL90A	+	+	+	-

Table 16. Halotolerance totals of the Basque Lake isolate collection.

Media Type	Growth/Total	Growth %
0.1% NaCl SP	60/63	95%
1%NaCl SP	61/63	97%
10% NaCl SP	63/63	100%
20% NaCl SP	42/63	67%
30% NaCl SP	12/63	19%

Table 17. Halotolerance of individual Basque Lake bacterial isolates from limited to near-saturated NaCl (w/v). Growth is indicated by (+) and little to no growth is indicated by (-).

Basque Isolate	0.1%	1%	10%	20%	30%
BL1	+	+	+	+	+
BL2	+	+	+	+	+
BL4c	+	+	+	+	-
BL5	+	+	+	-	-
BL6	+	+	+	+	-
BL6a	+	+	+	-	-
BL6B	+	+	+	+	-
BL6c	+	+	+	-	-
BL7	+	+	+	+	-
BL7a	+	+	+	+	+
BL7b	+	+	+	+	-
BL10	-	-	+	+	-
BL10a	+	+	+	+	+
BL10c	+	+	+	+	-
BL11	+	+	+	-	-
BL14	+	+	+	+	-
BL15	+	+	+	+	+
BL15A	+	+	+	+	+
BL15B	+	+	+	+	-
BL15C	+	+	+	+	-
BL16	+	+	+	-	-
BL17B	+	+	+	+	-
BL21	+	+	+	-	-
BL22	+	+	+	+	-
BL25	+	+	+	+	+
BL27a	+	+	+	-	-
BL30	+	+	+	+	-
BL32	+	+	+	+	-

TABLE 17 (continued)

BL35	+	+	+	-	-
BL41	+	+	+	-	-
BL42	+	+	+	-	-
BL43A	+	+	+	+	-
BL43a	+	+	+	+	-
BL43c	+	+	+	-	-
BL44a	+	+	+	+	
BL46	+	+	+	+	-
BL48	+	+	+	+	+
BL49	+	+	+	+	+
BL50	+	+	+	+	-
BL54	+	+	+	+	+
BL55	+	+	+	-	-
BL56	+	+	+	+	-
BL57	+	+	+	-	-
BL59b	-	-	+	+	-
BL60	+	+	+	+	-
BL61	+	+	+	+	-
BL62	+	+	+	+	-
BL66A	+	+	+	+	-
BL66a	+	+	+	+	-
BL66b	+	+	+	+	-
BL67	-	+	+	-	-
BL69	+	+	+	+	-
BL69a	+	+	+	+	-
BL76A	+	+	+	+	+
BL76a	+	+	+	+	-
BL76B	+	+	+	-	-

TABLE 17 (continued)

BL76b	+	+	+	-	-
BL79b	+	+	+	+	+
BL84	+	+	+	-	-
BL84a	+	+	+	-	-
BL85	+	+	+	+	-
BL90	+	+	+	-	-
BL90A	+	+	+	+	-

Table 18. Temperature tolerance totals of the Basque Lake isolate collection.

Temperature	Growth/Total	Growth %
4° C	47/63	75%
7° C	55/63	87%
10° C	61/63	97%
20° C	63/63	100%
37° C	63/63	100%

Table 19. Temperature tolerance of individual Basque Lake bacterial isolates. Growth is indicated by (+) and limited growth or no growth is indicated by (-).

Basque Isolate	4°	7°	10°	20°	37°
BL1	+	+	+	+	+
BL2	+	+	+	+	+
BL4c	+	+	+	+	+
BL5	+	+	+	+	+
BL6	-	+	+	+	+
BL6a	+	+	+	+	+
BL6B	-	+	+	+	+
BL6c	+	+	+	+	+
BL7	+	+	+	+	+
BL7a	+	+	+	+	+
BL7b	+	+	+	+	+
BL10	+	+	+	+	+
BL10a	-	-	+	+	+
BL10c	-	+	+	+	+
BL11	+	+	+	+	+
BL14	-	+	+	+	+
BL15	+	+	+	+	+
BL15A	+	+	+	+	+
BL15B	+	+	+	+	+
BL15C	+	+	+	+	+
BL16	-	-	+	+	+
BL17B	-	+	+	+	+
BL21	+	-	+	+	+
BL22	+	+	+	+	+
BL25	+	+	+	+	+
BL27a	+	+	+	+	+
BL30	+	+	+	+	+
BL32	-	+	+	+	+

TABLE 19 (continued)

BL35	+	+	+	+	+
BL41	+	+	+	+	+
BL42	+	+	+	+	+
BL43A	+	+	+	+	+
BL43a	+	+	+	+	+
BL43c	+	+	+	+	+
BL44a	+	+	+	+	+
BL46	+	+	+	+	+
BL48	+	+	+	+	+
BL49	+	+	+	+	+
BL50	-	-	+	+	+
BL54	+	+	+	+	+
BL55	-	-	+	+	+
BL56	+	+	+	+	+
BL57	-	-	+	+	+
BL59b	+	+	+	+	+
BL60	-	+	+	+	+
BL61	+	+	+	+	+
BL62	-	-	-	+	+
BL66A	-	+	+	+	+
BL66a	+	+	+	+	+
BL66b	-	+	+	+	+
BL67	+	+	+	+	+
BL69	+	+	+	+	+
BL69a	+	+	+	+	+
BL76A	-	+	+	+	+
BL76a	+	+	+	+	+
BL76B	-	-	+	+	+
BL76b	-	-	+	+	+

TABLE 19 (continued)

BL79b	+	+	+	+	+
BL84	+	+	+	+	+
BL84a	+	+	+	+	+
BL85	+	+	+	+	+
BL90	-	-	-	+	+
BL90A	+	+	+	+	+

Table 20. pH tolerance totals for the Basque Lake isolate collection.

pH	Growth/Total	Growth %
4	0/63	0%
5	14/63	22%
6	59/63	94%
7	63/63	100%
8	63/63	100%
9	50/63	79%
10	19/63	30%
11	3/63	5%

Table 21. pH tolerance of individual Basque Lake bacterial isolates. Growth is indicated by (+) and limited growth or no growth is indicated by (-).

Basque Isolate	pH 4	pH5	pH6	pH7	pH8	pH9	pH10	pH11
BL1	-	-	+	+	+	+	+	-
BL2	-	+	+	+	+	-	-	-
BL4c	-	-	+	+	+	+	+	-
BL5	-	-	+	+	+	+	+	-
BL6	-	-	+	+	+	+	-	-
BL6a	-	-	+	+	+	+	+	-
BL6B	-	-	+	+	+	+	-	-
BL6c	-	-	+	+	+	+	+	-
BL7	-	-	+	+	+	-	-	-
BL7a	-	+	+	+	+	-	-	-
BL7b	-	-	+	+	+	-	-	-
BL10	-	-	+	+	+	-	-	-
BL10a	-	-	+	+	+	+	-	-
BL10c	-	-	+	+	+	+	+	-
BL11	-	-	+	+	+	+	+	-
BL14	-	-	+	+	+	+	-	-
BL15	-	-	+	+	+	-	-	-
BL15A	-	-	+	+	+	+	-	-
BL15B	-	-	+	+	+	+	-	-

TABLE 21 (continued)

BL15C	-	-	+	+	+	+	-	-
BL16	-	-	+	+	+	+	-	-
BL17B	-	+	+	+	+	+	-	-
BL21	-	-	+	+	+	-	-	-
BL22	-	-	+	+	+	+	-	-
BL25	-	+	+	+	+	-	-	-
BL27a	-	-	+	+	+	+	-	-
BL30	-	-	+	+	+	+	+	-
BL32	-	-	+	+	+	+	-	-
BL35	-	-	+	+	+	+	+	-
BL41	-	-	+	+	+	+	+	-
BL42	-	-	-	+	+	+	+	-
BL43A	-	-	+	+	+	+	-	-
BL43a	-	-	+	+	+	+	-	-
BL43c	-	-	+	+	+	+	+	-
BL44a	-	-	+	+	+	+	-	-
BL46	-	-	+	+	+	+	-	-
BL48	-	-	+	+	+	-	-	-
BL49	-	-	+	+	+	-	-	-
BL54	-	+	+	+	+	-	-	-

TABLE 21 (continued)

BL55	-	-	+	+	+	+	-	-
BL56	-	-	+	+	+	+	-	-
BL57	-	-	+	+	+	+	-	-
BL59b	-	-	+	+	+	-	-	-
BL60	-	-	+	+	+	+	+	-
BL61	-	-	+	+	+	+	-	-
BL62	-	+	+	+	+	+	-	-
BL66A	-	-	+	+	+	+	-	
BL66a	-	-	-	+	+	+	+	+
BL66b	-	+	+	+	+	+	-	-
BL67	-	-	+	+	+	+	+	-
BL69	-	+	+	+	+	+	-	-
BL69a	-	+	+	+	+	+	-	-
BL76A	-	-	+	+	+	+	+	-
BL76a	-	+	+	+	+	+	-	-
BL76B	-	+	+	+	+	+	-	-
BL76b	-	-	+	+	+	+	-	-
BL79b	-	+	+	+	+	-	-	-
BL84	-	-	-	+	+	+	+	+
BL84a	-	-	-	+	+	+	+	+

TABLE 21 (continued)

BL85	-	-	+	+	+	+	-	-
BL90	-	+	+	+	+	+	-	-
BL90A	-	+	+	+	+	+	+	-

APPENDIX B

FIGURES

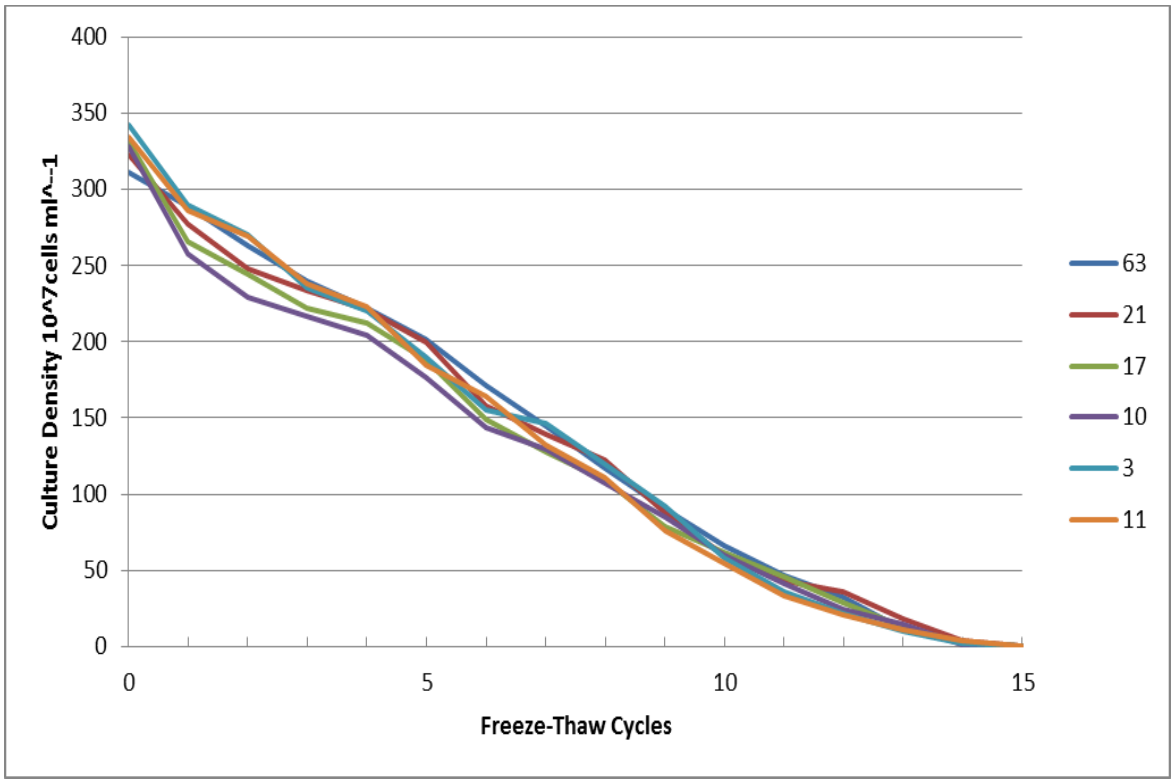

Figure 1. GSP Isolate Tolerance to Freeze-Thaw Cycles as Measured by Standard Plate Count (Crisler et al., 2012).

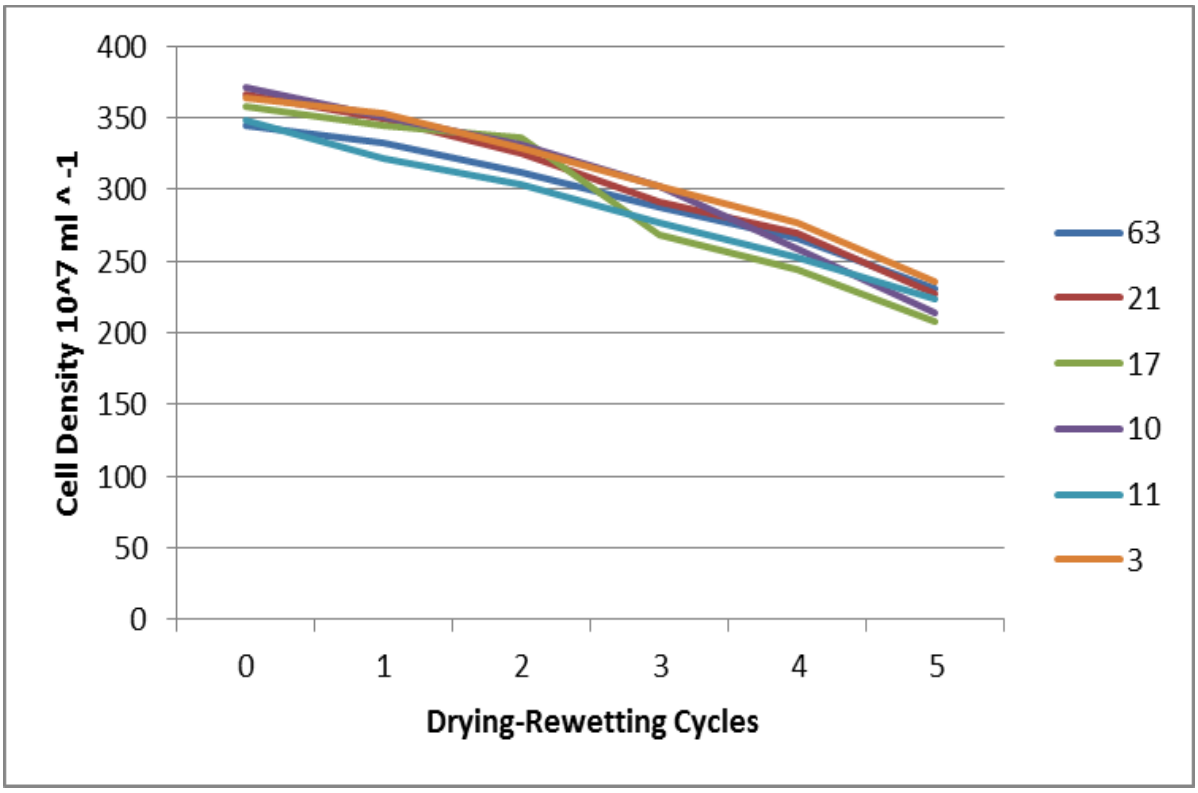

Figure 2. GSP Isolate Tolerance to Drying-Rewetting Cycles as Measured by Standard Plate Count (Crisler et al., 2012).

Figure 3. Basque Lake located near Kamloops, BC.

Figure 4. Near-saturated concentrations of $MgSO_4$ at Basque Lake

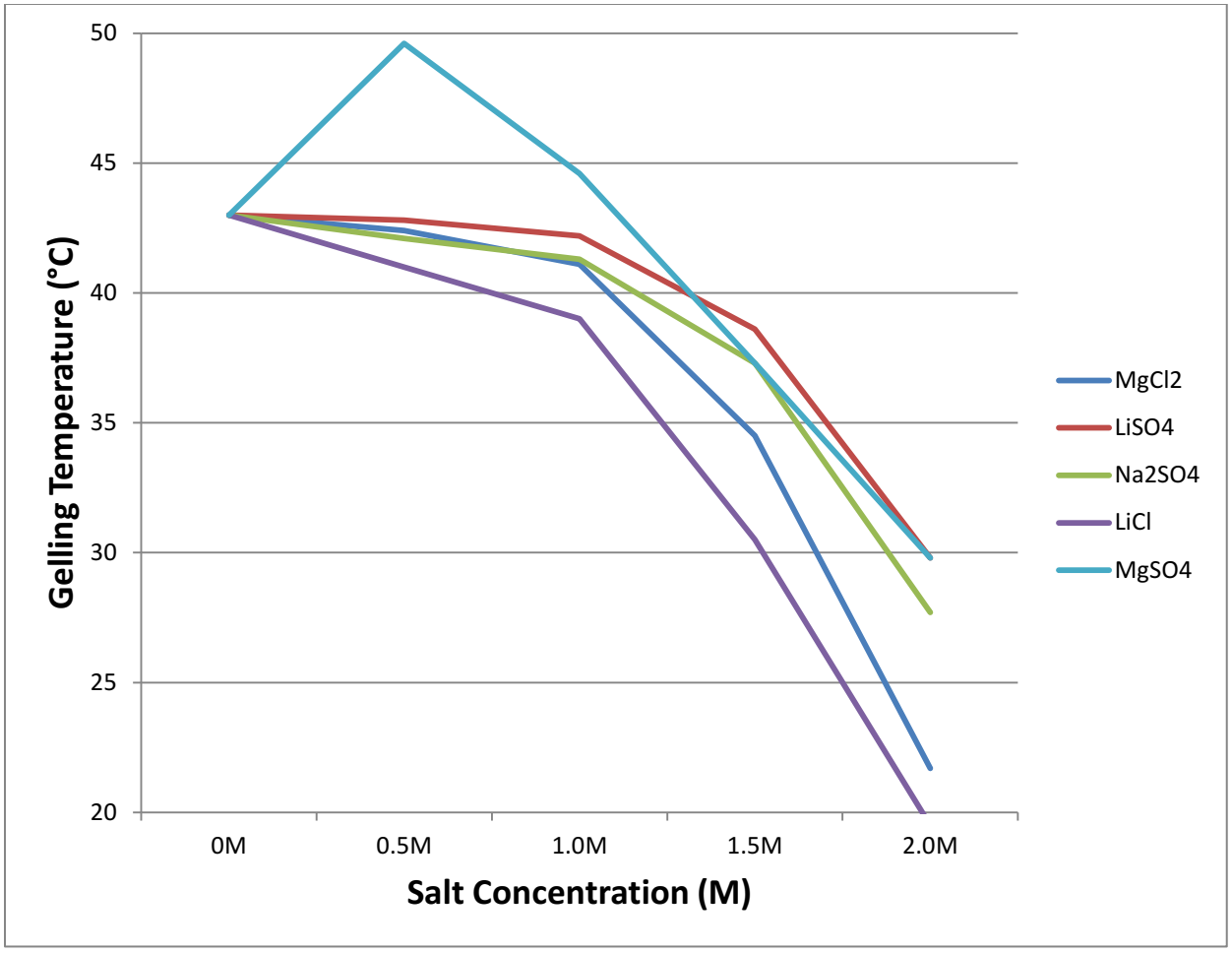

Figure 5. The chaotropicities of different salt solutions were determined by their effects on the gelation temperature of agar (Crisler et al., 2012).

Figure 6. Basque Lake Sampling Locations (Courtesy Google Maps).

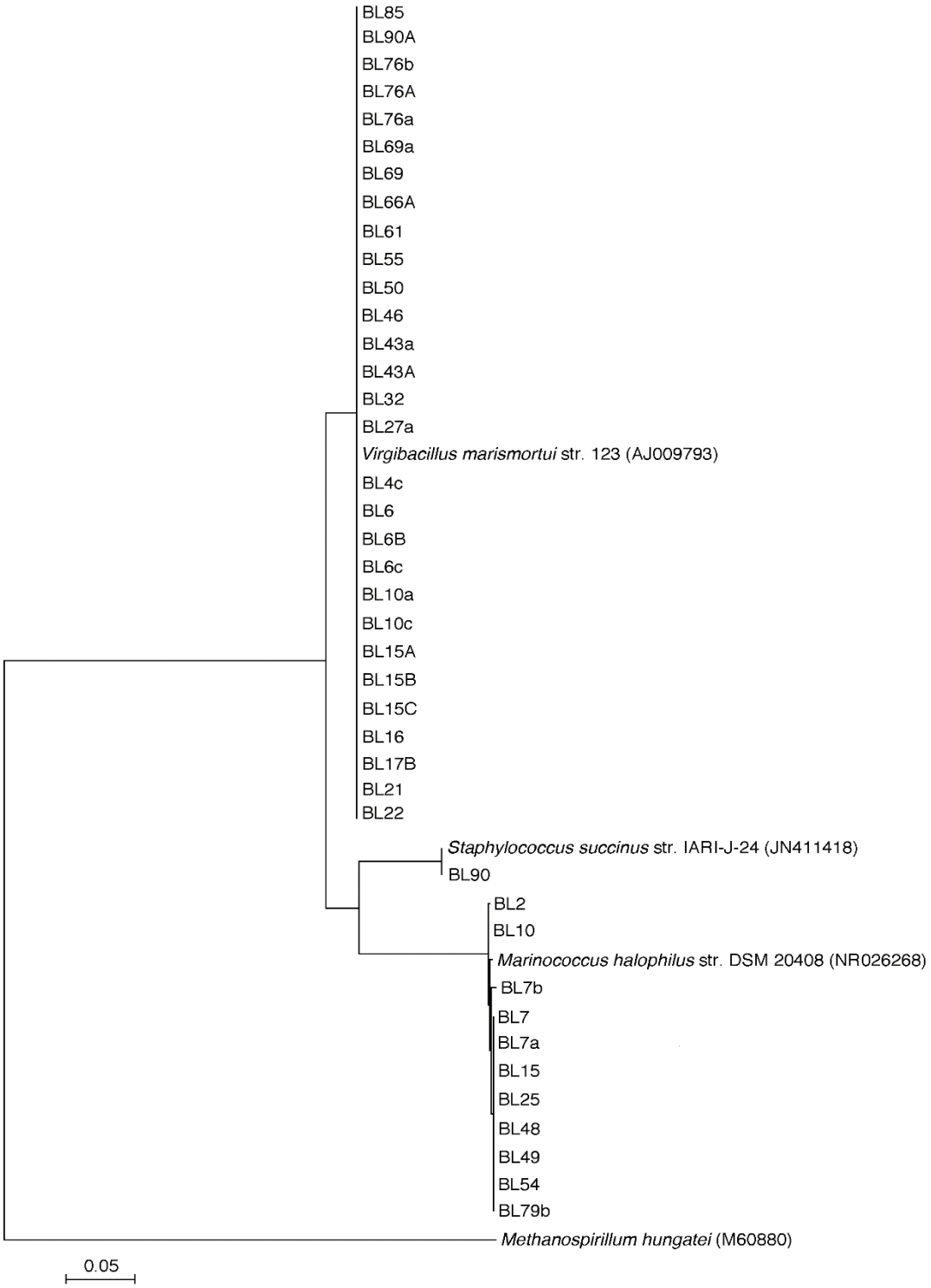

Figure 7. Phylogenetic Tree for Gram-positive Basque Lake Isolates.

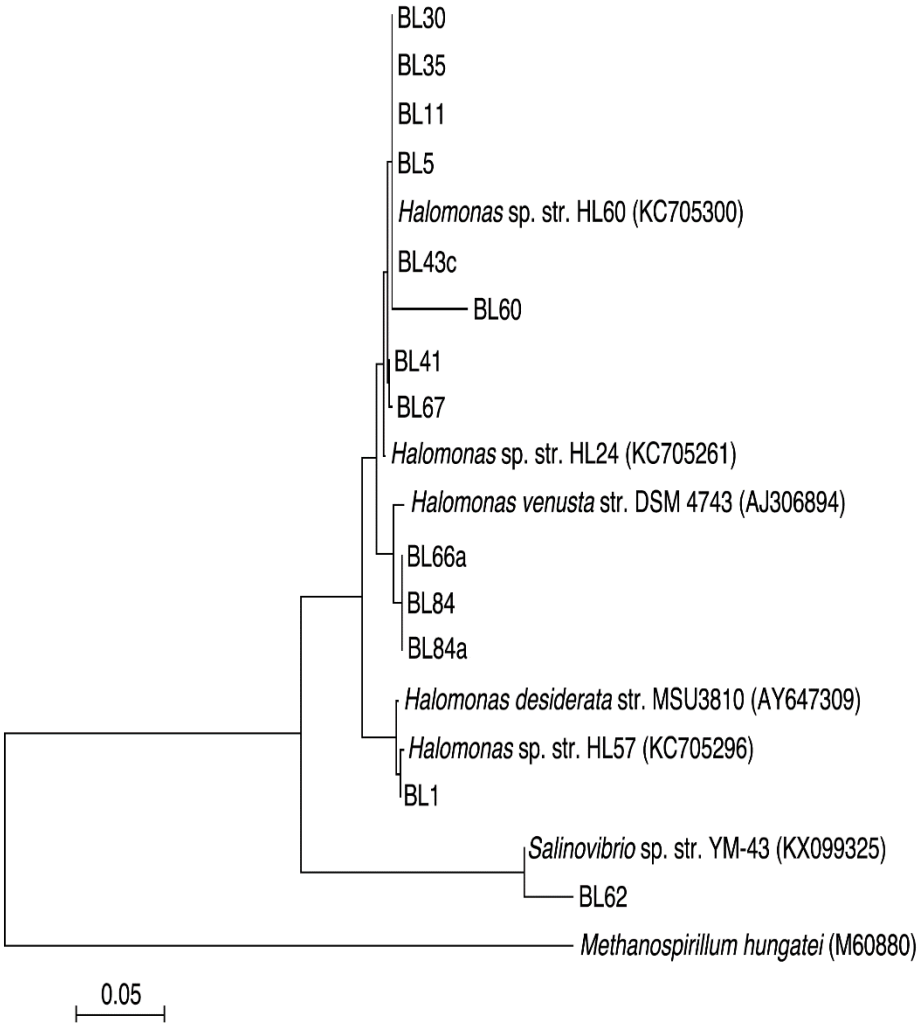

Figure 8. Phylogenetic Tree for Gram-negative Basque Lake Isolates.

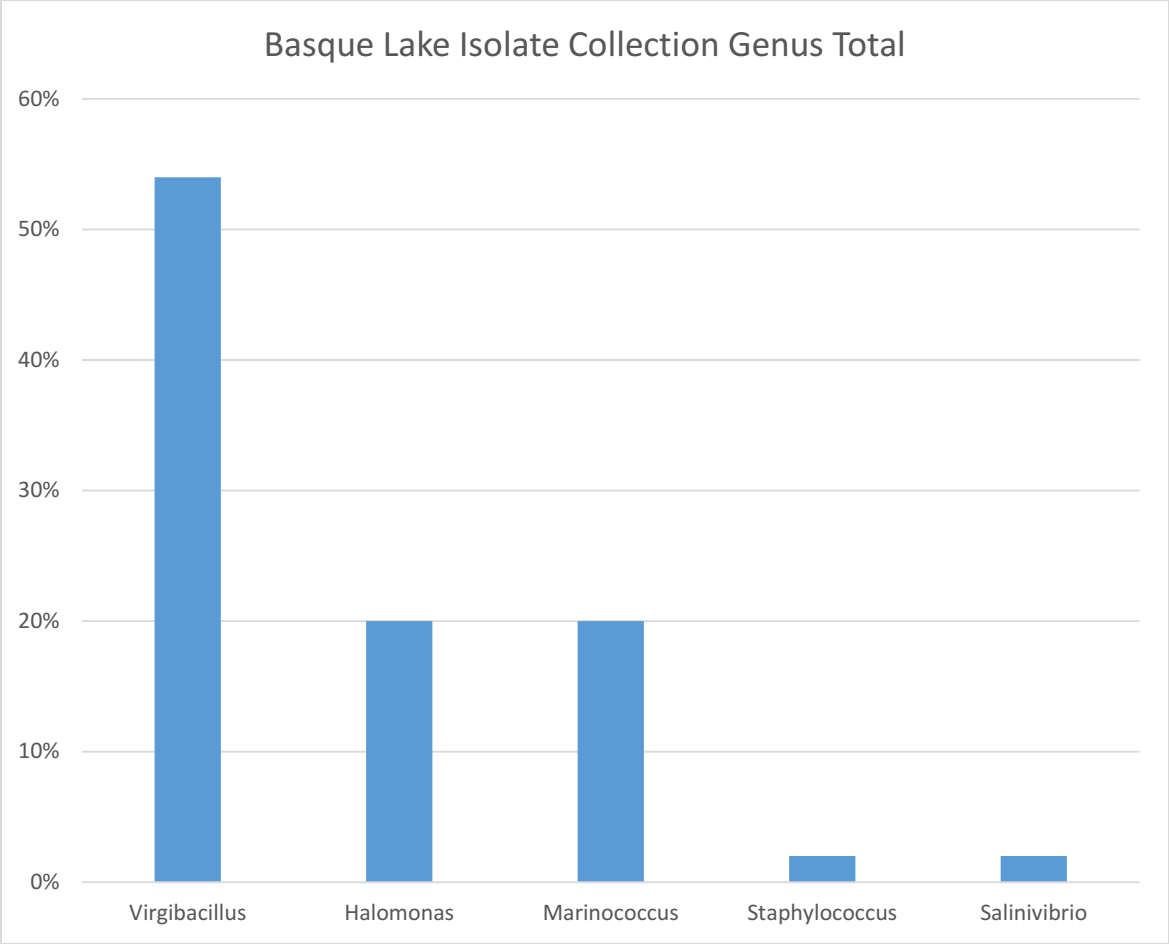

Figure 9. Genus Total of the Basque Lake Isolate Collection.

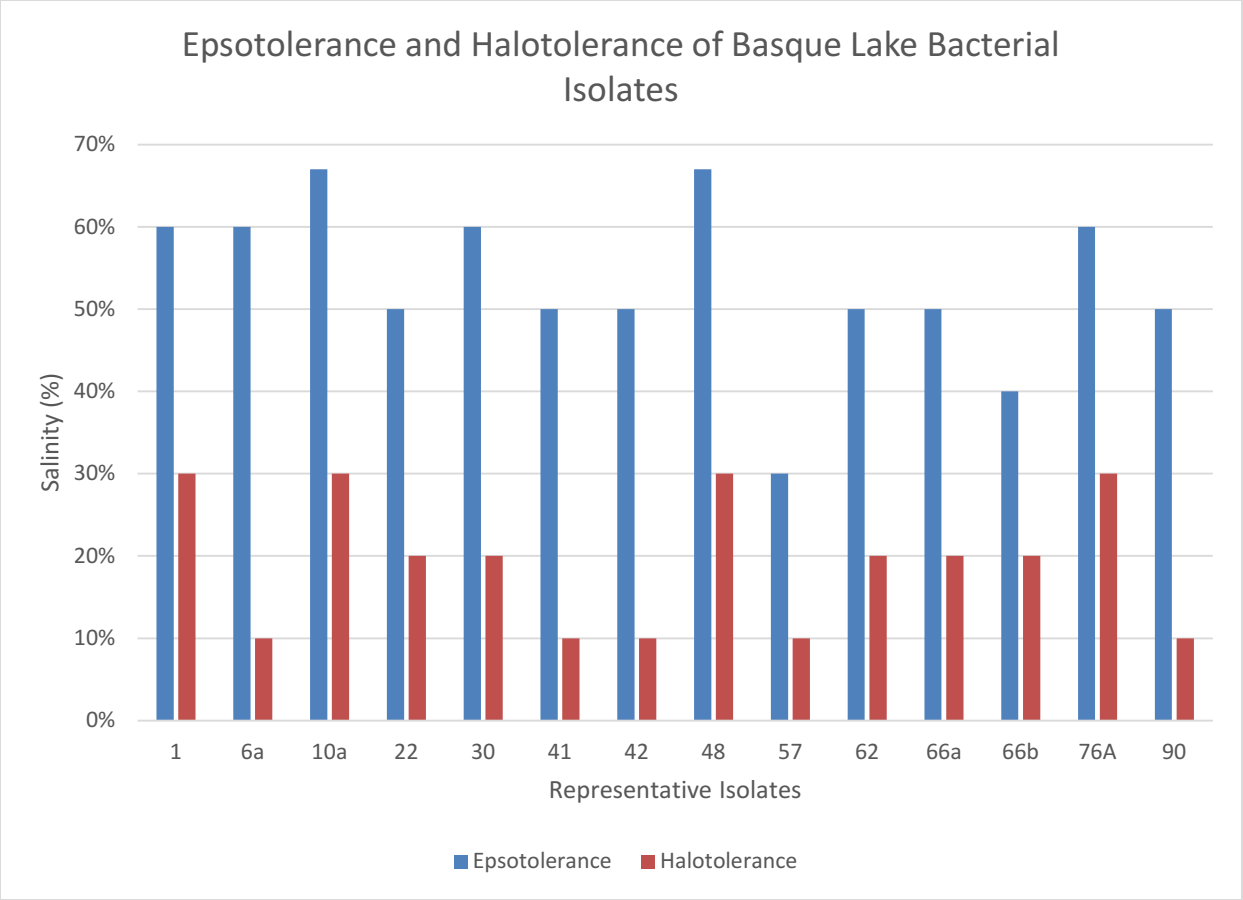

Figure 10. Epsotolerance and Halotolerance of Basque Lake Bacterial Isolates